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**TITOLO TESI DI DOTTORATO DI RICERCA**

**Avian viral disease prevention and control with plant-derived vaccines**

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# Summary of the thesis

The emergence and spread of viral infectious diseases in poultry remains a major challenge to the productivity of the global poultry market but also poses a threat to public health in the event of zoonotic diseases such as the H5N1 subtype of the influenza A virus. Infectious Bursal disease virus (IBDV) and Newcastle disease virus (NDV) are two of the most virulent avian viruses in the world and are, especially in the Mediterranean basin, the cause of economically important diseases. They are responsible for severe losses to the poultry industry due to high mortality rates, high degree of contagiousness, persistence in the environment and absence of etiological treatment. Therefore, vaccines are regarded as the most beneficial interventions to prevent this kind of infections and to control spread of these viruses. Immunization schedules are commonly based on conventional inactivated or live-attenuated vaccines, however, they possess several drawbacks such as insufficient protection, induction of post-vaccination symptoms and the impossibility to differentiate infected from vaccinated animals (DIVA). In consequence, many novel vaccine candidates are currently under development. Unlike conventional vaccines, they are based only on pathogen components (*i.e.* antigens) that are critical to initiate protective immune responses. The challenge for these new formulations is to mimic infection by stimulating a complete immune response (innate and adaptive, humoral- and cell-mediated), similarly to the conventional vaccines, while maintaining low production costs.

This PhD thesis project has been developed as part of the AVIAMED project (funded by the ERANET ARIMNet2 2015) that involves laboratories in Italy, Morocco, and Egypt in order to produce low-cost innovative vaccines against IBDV and NDV using plants as biofactories.

Indeed, plants are considered as a valid alternative to traditional expression systems for the production of recombinant heterologous proteins including biopharmaceutics, such as antibodies or antigens. Plant expression systems offer numerous advantages such as easy scale-

up, intrinsic biosafety and competitive production costs that are particularly suitable for the veterinary market. Plants can be used to massively produce the antigens or also for their delivery, if expression is induced in edible species.

NDV and IBDV antigens will be expressed transiently or stably as:

- i) fusion to the fragment crystallizable region of chicken IgY immunoglobulins (IgY-Fc or FcY);
- ii) fusion to the coat protein of the plant virus Potato virus X (PVX);
- iii) complex with plant Heat Shock Proteins 70 (HSP70).

This approach has for objective to result in:

- i) ease and rapidity of production scale-up at low costs due to the use of plants as expression systems;
- ii) simplification of the purification procedures;
- iii) improvement of the immunogenic properties of the antigens obtained by innovative fusion strategies;
- iv) production of vaccines able to differentiate infected from vaccinated animals (DIVA).

The antigens selected for each virus as well as the strategies used for expression are schematically presented in Figure 1.

The **Chapter 1** describes the state of the art in the field of: molecular farming, veterinary vaccines, proposed delivery platforms (IgY-Fc/ PVX/ HSP70) and selected viral targets (IBDV and NDV).



**Chapter 2:**

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.

**Chapter 3:**

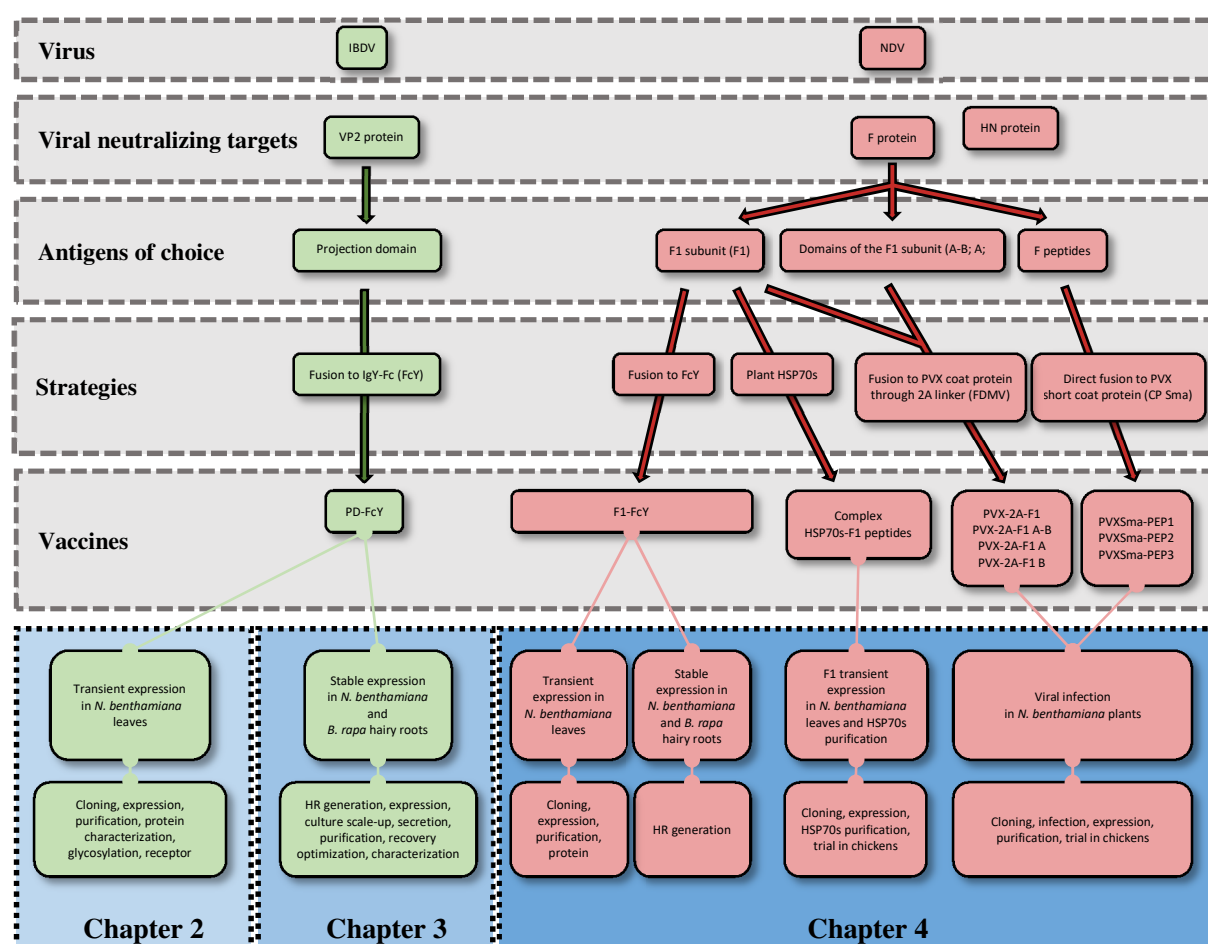
Hairy roots (HR) are a promising plant tissue-based platform for the production of biopharmaceuticals based on the possibility to induce the secretion of recombinant proteins in the culture medium, thus simplifying the purification and recovery steps. In this study, *Nicotiana benthamiana* and *Brassica rapa* subsp. *rapa* (turnip) HR cultures were generated to produce the secretory version of an infectious bursal disease virus chimeric antigen (PD-FcY) of interest for the formulation of poultry vaccines, with the aim of optimizing protein production and purification. Recombinant protein accumulation kinetics in the medium after induction with

the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), whose effects were still poorly characterized, was firstly investigated using a HR clone expressing the red fluorescent protein. The results demonstrated that HR cultures induced once with 2,4-D and grown 21 days efficiently secrete the protein and, even after replacement of the growth medium with a hormone-free counterpart, maintain the same secretion rate for several days. This protocol applied to HR cultures expressing PD-FcY resulted, for *N. benthamiana*, in similar accumulation kinetics and allowed to obtain protein recovery concentrations of ~4 mg/l. Conversely, no accumulation was observed for *B. rapa* HR cultures that were discontinued. A downstream processing procedure consisting in recombinant product concentration through ultrafiltration followed by two sequential chromatographic steps allowed to obtain final yields of 0.8 mg/l of purified PD-FcY. The HR-derived product showed a similar glycosylation pattern compared to the counterpart obtained from agroinfiltrated *N. benthamiana*, but a higher quality in terms of purity and protein degradation. In conclusion, the proposed expression and purification strategy holds promises for the development of an innovative platform to produce low-cost subunit vaccines.

#### **Chapter 4:**

Newcastle Disease Virus (NDV) 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 different formulations of innovative subunit vaccines constructions based on the fusion protein (F, the membrane glycoprotein involved in the virulence of the virus and target of the immune responses) of NDV. In particular, the F1 subunit of the F protein was transiently expressed in *Nicotiana benthamiana* plant platforms: i) as fusion to the constant region of avian immunoglobulin Y (FcY), ii) as fusion to the coat protein (CP) of Potato virus X (PVX) in order to obtain chimeric virus particles (CVPs);

iii) as single protein with the aim of purifying from plant tissues Heat Shock Proteins 70 (HSP70) chaperoning its peptides. In general, the expression of the protein in plants was difficult to achieve for all the strategies employed, nevertheless, purification of low antigen amounts for some of the formulations was performed. Preliminary immunization studies were set up, but no activation of anti-NDV immune responses in chickens was observed. However, this could be ascribed to the fact that the delivered doses were too low as the antigen content in the preparations could not be determined due to the lack of appropriate reagents. Further experiments will be necessary for production scale-up and immunogen quantification in order to test the efficacy of the proposed formulations in NDV prevention.



**Figure 1:** Schematic summary of the thesis

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# Chapter 1:

## **State of the art**



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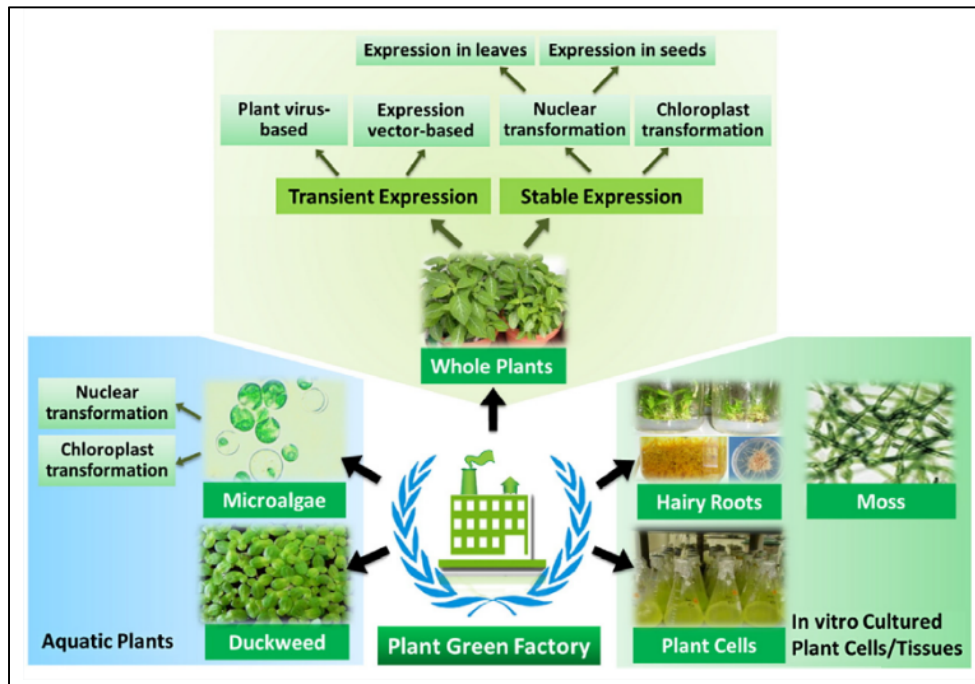
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## Molecular farming

The first successful expression in 1989 of a recombinant therapeutic protein in plants demonstrated that their use as "biofactories" (molecular farming) could be promising for the development and production of valuable recombinant products in agriculture, industry and medicine (Paul et al. 2011). Since then, molecular farming has proved to be an effective method to produce a wide variety of recombinant proteins: therapeutic proteins such as monoclonal antibodies, antigens, enzymes, growth factors or hormones, proteins of interest for industrial applications such as glycosidases, cellulases or proteases, and biopolymers such as collagen or spider silk protein (Xu et al. 2012).

Plant-based platforms are classified according to the transformation method (stable or transient) and the plant tissue used to target the expression (Figure 1). Stable transformation consists in inserting the gene encoding the protein of interest into the genome (nuclear or plastid) of plant cells that are cultured and used to regenerate an entire plant. By this approach, all the cells of the regenerated organism are genetically transformed, including germ cells that can transmit the heterologous information to the progeny (Gelvin 2017). The whole plant is transformed and can be exploited even if protein expression and accumulation can be targeted in leaves, seeds or any other storage organ (Lau and Sun 2009). For example, the nuclear transformation of the plant will result in the production in seeds or leaves, while chloroplastic transformation will result in the accumulation of proteins only in the leaves (Boyhan and Daniell 2011). The yields of the recombinant product that are obtained through stable transformation are generally low, mainly for nuclear transformation, because the gene is being inserted randomly into the plant genome and it also generally requires the screening of a large number of plants. In addition, all the process (transformation, regeneration and growth) is generally long and time consuming also because several generations are needed to obtain plants that are homozygous for the transgene (Yao et al. 2015). Aquatic plants, such as *Lemna minor*, can also be transformed and

will accumulate large amounts of biomass and therefore of the protein of interest thanks to their rapid and easy multiplication (Rival et al. 2008). *In vitro* plant culture systems under controlled and sterile conditions also exist for the expression of recombinant proteins with features close to existing conventional production platforms (mammalian cells, bacteria). The most common *in vitro* plant expression platforms currently in use are hairy roots, plant cell suspensions or moss and all have the potential of obtaining the secretion of the protein of interest in the culture medium (Xu et al. 2012; Specht and Mayfield 2014). From a technical point of view, the selection of the transformed cells/tissues by micropropagation and the first steps of scale-up are generally performed on a sterile semi-solid medium, and then in a liquid medium. Bioreactors are used for the *in vitro* culture of suspensions of plant cells or tissues and are diverse, including stirring tanks, bubble columns and airlift. Disposable bioreactors that look like plastic bags, such as the horizontal wave reactor, are also available. The advantages of this type of platform are that the transgenic plant material is contained with a reduced biological risk to the environment and that the growing conditions are strictly controlled to ensure the homogeneity of the product (Doran 2013).



**Figure 1:** Plant platforms for the expression of recombinant proteins (from Xu et al. 2012)

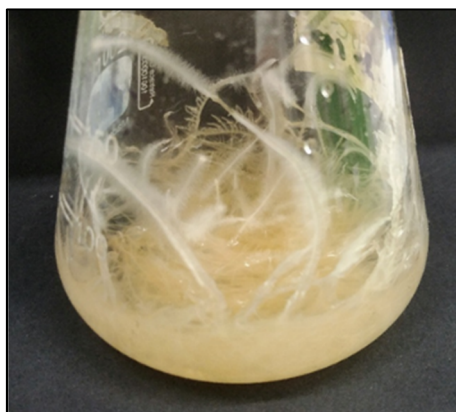
### **Stable expression: Hairy roots**

Hairy roots (HR) result from the infection of tissues of monocot and eudicot plants with *Rhizobium rhizogenes* (also known as *Agrobacterium rhizogenes*). This gram-negative soil bacterium of the *Rhizobiaceae* family infects plant cells similarly to *A. tumefaciens* but hosts the root-inducing plasmid Ri instead of the plasmid Ti. In nature, the pathogen generally enters the plant organism through a wound and introduces its genetic information into the plant cell. The generation of HR is induced by the integration of the T-DNA segment of the Ri plasmid into the plant genome (White et al. 1985). The T-DNA carries a set of oncogenes that modify the hormonal balance of the cell by disrupting the control of auxin production. Therefore, the transformation of the plant cell determines its differentiation and growth resulting in emerging neoplastic HR (Stiles and Liu 2013). The generation of HR expressing a heterologous recombinant protein can be obtained by two different methods. A first method consists in infecting tissue explants of transgenic plants (such as foliar discs) expressing the protein of interest with wild type (w.t.) *R. rhizogenes*. With this approach, the infected plant tissue at the

wound site will produce HR expressing the recombinant protein. This method is time-consuming because it is necessary to obtain the transgenic plant first. A second method consists of infecting w.t. plant tissues with a strain of *R. rhizogenes* carrying a T-DNA plant based binary vector with the gene of interest. Although this approach produces a lower percentage of HR clones expressing the protein of interest than those obtained with the first method, it is much faster and suitable for expressing complex multimeric proteins such as antibodies (Donini and Marusic 2018). Indeed, it is possible to simultaneously infect plant tissue with strains of *R. rhizogenes* carrying different genes of interest and finally obtain HR clones that co-produce the proteins. By adopting this strategy, it was possible to obtain HR clones of *N. benthamiana* producing a functional antibody by co-expressing and assembling the two heavy and light chains (Lonoce et al. 2016).

Once new HR clones are generated from the infected plant tissue, the clones are detached from the wound site and cultured on a semi-solid medium formulated to maximize proliferation. The composition of the medium depends on the plant species used but is generally based on Murashige and Skoog (MS) or Gamborg B5 medium enriched with sucrose. The random insertion of the transgene into the plant genome strongly influences the expression. Therefore, each generated HR clone will behave differently, either in terms of growth or protein yield. The selection of transformed clones is done by adding antibiotics to the medium. Subsequently, fast-growing clones are analysed by Polymerase Chain Reaction (PCR) and Reverse Transcriptase (RT)-PCR to verify DNA insertion and transcription, and Western Blot (WB) to verify protein production either inside the HR tissues or in culture medium if secretion is expected (Huet et al. 2014). All these steps are essential to select the clones producing the highest amount of recombinant protein. The selected clones are finally transferred in liquid cultures that can be gradually scaled up to increase biomass and production levels (Figure 2). Several plant species have been successfully engineered to obtain HR including *Nicotiana*

*tabacum*, *Nicotiana benthamiana*, *Brassica rapa* subsp *rapa* (turnip) and *Solanum lycopersicum* (Häkkinen et al. 2014; Ele Ekouna et al. 2017; Lonoce et al. 2019). It is also important to point out that recent genome editing methods such as the clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 system allows the induction of site-directed genomic modifications. Interestingly, these methods have been described in several studies for genome editing of newly generated HR, principally to knockout a gene of interest by site-directed insertion or deletion (Kirchner et al. 2017; Nakayasu et al. 2018). However, although HR generation using these techniques have not been yet studied for the expression of a recombinant protein, it is a promising perspective in which insertion of the transgene could be controlled and directed in order to improve its expression and avoid negative interference with the HR metabolism.



**Figure 2:** HR from *N. benthamiana* liquid culture in 250 ml-flask

HR are a particularly attractive platform for industrial-scale production because the root tissue can grow rapidly and indefinitely *in vitro*, in a manner similar to undifferentiated cells and in a simple and inexpensive medium without adding hormones. In addition, HR are genetically stable for years without significant loss of their metabolic activity (Häkkinen et al. 2016; Sun et al. 2017) and their growth in liquid medium can be scaled up using bioreactors (Mehrotra et al. 2015) making the platform convenient for industrial scales applications. Initially, this



platform was dedicated to the production of valuable secondary metabolites (Tian 2015), but in the last decade, many studies have been made on the use of HR for the production of biopharmaceuticals (Table 1). An important feature of HR cultures compared to other plant-based expression platforms is the ability to induce the accumulation of the heterologous protein of interest in the culture medium. This can be obtained by directing the protein to the apoplast through the addition of an appropriate secretory signal peptide. Secretion in the culture medium greatly facilitates the recovery and purification of the protein of interest by reducing the cost of downstream processing (Doran 2013). In addition to these positive aspects, this platform is not exempt from certain limitations. One of them is related to the difficulties of increasing HR production in very large bioreactors due to their particular entangled and filamentous morphology. A second challenge is the low yields generally obtained compared to those achieved with other plant platforms (*e.g.* transient expression systems), although recent studies have shown the possibility of increasing production by stimulating the secretion of recombinant proteins in the culture medium by adding auxins to the culture medium (Häkkinen et al. 2014). In particular, it has been shown that supplementation of the culture medium with a very small amount (0.1 mg/l) of the synthetic auxin-2,4-dichlorophenoxyacetic acid (2,4-D) induces a 28 fold increase in antibody accumulation (Lonoce et al. 2019). Several other strategies have been studied to increase the final recovery of the heterologous (secreted) protein such as the addition of a nitrogen source ( $\text{KNO}_3$ ) to the culture medium that can double the production of an antibody in tobacco HR (Häkkinen et al. 2014). Because of the possible degradation of the protein of by specific peptidases in the culture medium, it may be essential to stabilize the secreted protein by adding for example, polyvinylpyrrolidone (PVP) and gelatine (Sharp and Doran 2001). HR cultures have been successfully used to produce a large variety of recombinant proteins such as antibodies, enzymes, antigens or reporter proteins using different plant species (Donini and Marusic 2018).

|                                                                                                                     |
|---------------------------------------------------------------------------------------------------------------------|
| <b>Advantages</b>                                                                                                   |
| The final product can be secreted in the medium and is more homogeneous than a product extracted from plant tissues |
| Efficient downstream processing when the protein is secreted in the culture medium                                  |
| Absence of human pathogens                                                                                          |
| Production in sterile contained bioreactors                                                                         |
| Long-term genetic stability compared to cell suspension cultures                                                    |
| Fast growth and large biomass accumulation                                                                          |
| <b>Challenges</b>                                                                                                   |
| Low protein yield in the culture medium                                                                             |
| Culture scale-up                                                                                                    |
| Plant-type glycosylation of proteins                                                                                |

**Table 1:** Advantages/challenges of the production of heterologous proteins in HR (from Donini and Marusic 2018)

### **Transient expression: agroinfiltration and viral vectors**

With the aim to improve heterologous protein expression in plants overcoming the limitations imposed by stable expression strategies, technologies based on transient expression systems have been developed based on the extra-chromosomal expression of the transgene exploiting the plant cell machinery for a limited period of time, increasing production yields and drastically reducing manufacturing cycle time. Transient transformation, commonly used in research to evaluate gene expression in plants, offers several advantages, mainly because it is a method that allows large quantities of the recombinant protein to be obtained in a short time (days/few weeks). The main characteristic of transient expression, differently from stable transgenic plants, is that the transgene is not integrated into the plant genome and is therefore not transferred to the progeny.

The DNA sequence encoding the protein of interest is transferred into plant cells by plant pathogens: either bacteria carrying plant binary vectors or viruses *via* recombinant viral vectors (Komarova et al. 2010). The insertion of the foreign genetic information is generally mediated

by vacuum infiltration of a modified culture of *A. tumefaciens* (Figure 3) which is a gram-negative soil bacterium responsible in nature of the crown gall disease and is known to transfer part of its genetic material from the tumour-induced plasmid (Ti) to the plant genome. Molecular farming exploits this capability of *A. tumefaciens* to deliver the foreign gene inside the plant cells and to this aim specific binary vectors have been designed (Krensek et al. 2015). These vectors consist of a first component that is the T-DNA, delimited by right (RB) and left (LB) border sequence, which contains multiple cloning sites used to clone the gene of interest and marker genes for the selection of transformed plants. An advantage of T-DNA is the possibility of inserting a very long DNA fragment of interest (several kilobase pair, kbp) to produce proteins up to 100 kDa (kiloDalton) (Gelvin 2017). The second component is the vector backbone, which is mainly composed of sequences for plasmid replication in *Escherichia coli* and *A. tumefaciens* and antibiotic resistance genes for bacterial selection. The most commonly used binary vectors are the pBI and pEAQ (Peyret and Lomonosoff 2013). The agroinfection transient expression system is based on the infiltration of the bacteria carrying the gene of interest by applying the solution in the leaf tissue under pressure, by either syringe or vacuum. *A. tumefaciens* moves into the apoplastic spaces of the leaves through the stomata or wound and is then forced to enter the mesophyll of the leaf for infection. This system provides production of the protein of interest in a short period from 2 to 7 days after infiltration with a maximum production generally reached at 3-4 days with variability depending on the characteristics of the transgene. Vacuum agro-infiltration allows a simple and direct scaling up by increasing the volume of the culture of *A. tumefaciens* to treat a large number of plants and can be adapted at an industrial scale (Leuzinger et al. 2013). Many recombinant proteins have been produced with *A. tumefaciens* mediated non-viral transient expression systems, including life-size antibodies, viral antigens or enzymes (Xu and Zhang 2014).

The improved expression levels obtained by transient expression using agroinfection compared to stable transformation are influenced by several parameters that can be modified to further increase recovery (Egelkrout et al. 2012). In the T-DNA cassette, the promoter activity and transcription enhancer can be selected to improve expression. The most common combination in agroinfection to improve expression is the use of a strong promoter such as 35S cauliflower mosaic virus (CaMV), which can be repeated upstream of the DNA sequence of interest, and the use of the Tobacco Mosaic virus (TMV)  $\Omega$  translation enhancer (Peyret and Lomonossoff 2015). The selection of a signal peptide is also important to target the protein to the ideal subcellular component in order to optimize protein folding, stability and accumulation. For example, the KDEL retention peptide prevents the transit of the protein of interest outside the endoplasmic reticulum (ER) influencing the protein glycan composition by obtaining an N-glycosylation profile with high mannose sugars (Petrucelli et al. 2006). On the contrary, a secretion peptide can also be used to direct and accumulate the protein outside the plant cell into the apoplast (Thomas and Walmsley 2014). Engineering the DNA sequence of the transgene is also a way to increase transcription levels and improve protein translation. The codon composition of the heterologous sequence can, for example, be adapted to the host codon usage. Modification of the heterologous sequence to reduce the formation of detrimental messenger RNA (mRNA) secondary structures is also a method to improve the protein translation (Webster et al. 2017). Overexpression of the transgene results in a high number of transcripts that may cause the plant to activate a natural defence response called post-transcriptional gene silencing (PTGS) that takes place in the infiltrated tissue and can enormously reduce the expression efficiency of genes of interest. This response has been evolutionary developed by plants in order to defend themselves against RNA viruses infection by preventing the translation of heterologous viral transcripts into proteins (Pumplin and Voinnet 2013). A strategy has been developed to overcome this unwanted plant defence

response that is based on the co-expression, together with the gene of interest, of PTGS inhibitors derived from plant viruses (*e.g.* Tomato Bushy Stunt virus-derived p19 protein). By this approach, it is possible to restore high accumulation levels of the recombinant protein in agroinfiltrated plant tissues.

The most efficient transient expression systems are based on viral expression vectors instead of plant binary vectors. Plant viral vectors can generally use the same agroinfiltration system to introduce DNA into the plant cell and thus achieve the same simplicity and scalability process (Hefferon 2014). Viral vectors can be classified into two categories; the first generation is known as the full-virus strategy and involves the cloning of the gene of interest as an additional open reading frame (ORF) in the whole viral genome. Second generation vectors are obtained by deconstructing the viral genome and by using only those viral sequences essential to the production of the target protein such as strong promoters, translation enhancers, terminators and silencing suppressor. Unnecessary viral genes, such as the those encoding the envelope protein, are therefore removed from the vector resulting in the so called deconstructed viral vector strategy (Gleba et al. 2004).

The first generation of viral vectors was based on viruses still capable of infecting plant cells, the majority being RNA viruses due to their greater stability and plasticity, among which the most used ones were TMV, such as in the Kentucky BioProcessing's Geneware® system (Moore et al. 2016), or Potato virus X (PVX) (Lico et al. 2015). These vectors consist of the viral complementary DNA (cDNA) hosting the gene of interest either fused to the coat protein (*cp*) gene for the presentation of an antigen epitope in the case of a vaccine (obtaining Chimeric Virus Particles, CVPs), or inserted under the control of an additional subgenomic promoter. Viruses are inoculated into the leaf as: infectious RNA created from the vector through *in vitro* transcription or by agroinfiltration followed by the *in planta* transcription of the vector to obtain an infectious viral RNA. The protein of interest is then expressed along with the systemic spread

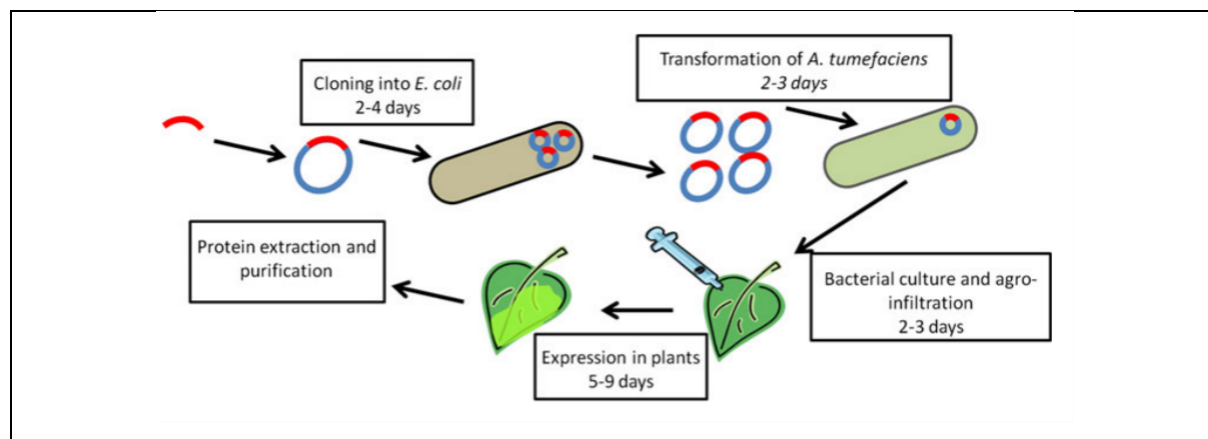
of the virus typically reaching the maximum levels of production after 1-2 weeks. One of the disadvantages of this technology is the limited size of the gene that can be inserted into the vector, indeed a DNA sequence greater than 1.5 kbp could cause problems to virus assembly and spreading in the plant (Matoba et al. 2011). The use of infectious virus particles can also pose a biosecurity problem and, in order to prevent the risk of environmental contamination, an appropriate level of greenhouse containment is required causing high costs. Several molecules have been successfully produced in plants using this strategy among which PVX CVPs exposing an immunogenic peptide of the influenza A virus nucleoprotein (Lico et al. 2009).

The deconstructed viral vector strategy is an improvement of the full-virus strategy and was developed in order to address the limitations of first-generation viral vectors. Since these type of vectors are not inducing the formation of infectious viral particles able to spread through the infected plants, they drastically reduce the safety issues correlated with biocontainment. On the other hand, the possibility of using automated systems of agroinfiltration through the use of vacuum for the delivery of these vectors in cells compensates for the defective viral movement and allows the rapid, homogeneous, and high-level expression even of complex proteins in different plant species (Hefferon 2014). The most well-known deconstructed viral vector is the TMV-based magnICON system developed by ICON Genetics GmbH (Marillonnet et al. 2004). This system has improved flexibility and efficiency and is widely used to produce a wide range of different recombinant proteins such as antibodies enzymes and vaccines based on Virus-Like Particles (VLPs) (Ibrahim et al. 2019). In the case of multimeric proteins the expression is obtained by co-infiltrating plants with several *A. tumefaciens* strains transformed with different constructs (Giritch et al. 2006). In addition to vacuum-agroinfiltration, another method has been developed with this viral strategy to maximize the scale-up of the magnification and is called the agrospray. It consists in spraying the solution of *A. tumefaciens* directly on the leaves together with surfactants allowing the modified bacteria to enter directly into the leaf tissue

through the created abrasions. This results in a reduction of the cost of agroinfiltration combined with acceptable levels of recombinant protein expression (Gleba et al. 2013).

Due to the speed of the production, easy scalability and high recombinant protein yield, transient expression systems have proved to be the most efficient technology for molecular farming applications. Plants are generally grown in greenhouses, in a confined environment and under controlled conditions facilitating regulatory acceptance even if genetically modified plants for specific glycan modifications of the recombinant proteins are used (Yao et al. 2015). Several plants have been tested for transient expression but tobacco species, in particular *N. tabacum* and *N. benthamiana*, are the most exploited for molecular farming in research laboratories. They are considered as model plants by the scientific community and are often studied because of the very deep and global knowledge acquired on their biology and genome. Moreover, they quickly produce a large amount of leaf biomass that make them economically more interesting than species such as *A. thaliana* (Tremblay et al. 2010). Moreover, they generally allow high expression levels of the heterologous protein and are very suitable for agroinfiltration. Their susceptibility to a wide variety of viruses make them an ideal host also of transient expression systems based on first generation viral vectors. In addition, being non-fodder and non-food crops they reduce the risk of the transformed plants entering the animal/human food chain (Matoba et al. 2011). However, due to the presence of alkaloids especially in *N. tabacum*, processing and purification of the recombinant proteins is mandatory before their possible administration. In the case of *N. benthamiana* its lower levels of alkaloids and nicotine allows the leaf extract to be administered directly, for example in animals (Sheludko et al. 2007; Liew and Hair-Bejo 2015).

Nevertheless, several other species have been tested for molecular farming applications such as lettuce, maize, rice, turnip, potato or carrot (Yao et al. 2015).



**Figure 3:** Schematic representation of the production process of a protein of interest in plants using pEAQ vector (from Peyret and Lomonossoff 2013)

### **Molecular farming characteristics**

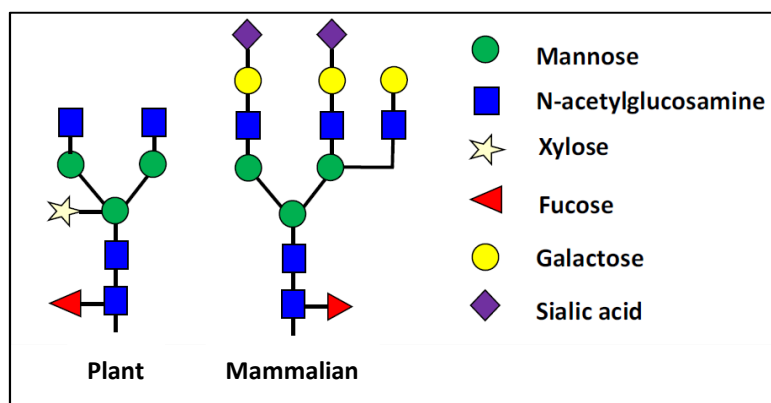
Plants have several advantages that make them competitive compared to other existing platforms, such as bacteria, yeasts, insects and mammalian cells, to produce recombinant proteins of interest (Schillberg et al. 2019).

First, they offer a better biosecurity guarantee because they do not support the replication of human and animal pathogens (Schillberg et al. 2013). Second, the simplicity and scalability of many plant-based platforms combined to low production costs make them economically very attractive for the production of valuable products. A typical example is the field cultivation of whole plants, which would allow a considerable amount of biomass to be obtained at very low cost, with the resulting economies of scale. However, regulatory laws and public perception of genetically modified organisms (GMOs) in open-field are important concerns in some countries that will need to be addressed in the future (Buyel et al. 2017). Upstream costs can also be reduced due to the exceptional biosynthetic capacities of plants as energy is delivered by light and then transformed by photosynthesis. The plant also requires only a simple and inexpensive substrate to obtain a significant accumulation of biomass and protein (Xu et al. 2012), while bacterial and mammalian systems require high mandatory costs such as culture medium and



equipment (*i.e.* bioreactors) (Ecker et al. 2015). Another advantage of some plant platforms is that, with the transient expression strategy, they can produce recombinant proteins in a few weeks, while "traditional" platforms can hardly meet demand in critical situations, such as influenza epidemics, when rapid production of a new vaccine is needed (Zhang et al. 2014). Molecular farming is also a viable option for the production of seasonal influenza vaccines that require a rapid manufacturing process. The Canadian company Medicago demonstrated that a flu vaccine, made with the circulating strain can be produced in less than 2 months without the risk of mutation during the process, whereas the platforms currently in use require more than 6 months and sometimes provide a vaccine with sub-optimal efficacy (Pillet et al. 2019).

In addition to these aspects, it is important to mention that plants can also be used to produce functional complex recombinant proteins, such as antibodies, which are impossible to obtain in bacterial systems that are unable to perform the majority of post-translational modifications required for the bioactivity of a majority of therapeutic proteins. The modifications that plant cells are able to perform include the formation of disulfide bonds, the assembly of subunits and N-glycosylation, although there is a certain difference with mammalian cells with regard to the latter (Gomord et al. 2010). In particular, in the plant cell, the specific addition of  $\beta(1,2)$ -xylose and  $\alpha(1,3)$ -fucose is observed as an alternative to that of sialic acid and  $\beta(1,4)$ -galactose, whose synthesis does not occur (Figure 4). These differences can affect the bioactivity of the product and cause allergic reactions in animals. To overcome this limitation, "glyco-humanized" plant lines have been developed in which the genes encoding the fucosyl- and xylosyl-transferases have been knocked-down and the genes encoding the galactosyltransferase and sialyltransferase pathways added (Fischer et al. 2018). However, difference in glycosylation can also be considered as an opportunity to produce a bio-better product with improved function and efficiency compared to a biosimilar (Schiermeyer 2020).



**Figure 4:** Comparison of typical N-glycan structures from glycoproteins produced in plants (left) and mammalian expression hosts (right) (from Margolin et al. 2018)

Molecular farming has proven its effectiveness in producing a wide variety of recombinant proteins, the challenge now is to achieve economic feasibility to move from proof-of-concept projects to commercialization. A first example in this direction is the commercialized therapeutic protein produced in plants, the human glucocerebrosidase expressed in carrot cells by Protalix Therapeutics, Inc (Mor 2015). A second example, developed in Japan by Hokusan co. Ltd, is the InterBerry  $\alpha$ , a canine interferon- $\alpha$  produced in the fruit of transgenic strawberries and used for the treatment of gingivitis in dogs by oral delivery. Improving the protein yield obtained in plants combined with the development of appropriate purification systems is necessary to reduce downstream costs and bring it closer to the pharmaceutical market demand. However, for some products such as veterinary vaccines, the plant production system is already a promising technology adapted to market demand with affordable products and attractive delivery methods such as oral vaccination (Kolotilin et al. 2014).

A wide variety of plant species can be adopted to set-up plant-based production strategies. It results in a considerable number of potential plant platforms compared to the limited number of mammalian cell lines or yeast and bacterial strains available. Plant bio-factory system has therefore a remarkable flexibility to adjust a production process to cost, yield, safety and

regulatory issues in order to find a high-performance and adapted platform, for example, in the case of proteins that are difficult to express in conventional systems (Donini and Marusic 2019).

## **Veterinary vaccines**

### **Vaccination and immunity**

There are available a wide variety of interventions to control bacterial and viral infections in animals but the most common therapeutic treatment relies on the use of antibiotics. Unfortunately, the inappropriate and excessive use of this type of therapeutics is resulting in a significant increase of antibiotic-resistant bacterial strains and, also because of the growing consumer demand for antibiotic-free foods, new solutions are urgently needed. Vaccination is generally considered as the safest and most cost-effective method to preserve health and control the spread of infectious diseases (Jansen and Anderson 2018). In the event of an outbreak associated to high risk of contamination, vaccination avoids also to slaughter animals to prevent infection spreading. Vaccination triggers immune responses specific to the target pathogen that will protect the organism in case of future infection. Immunity is the ability of multicellular organisms to develop sequential biological responses that give protection from harmful pathogens (Moser and Leo 2010).

Innate immunity is the first set of biological responses activated when a pathogen penetrates into the body and represent the first line of defence. Innate immune responses are quickly activated by a limited set of recognized structures, the pathogen-associated molecular patterns (PAMPs), which are shared by a large number of pathogens and include lipopolysaccharides, double- and single-stranded RNA (ds-RNA, ss-RNA) and unmethylated CpG-DNA motifs. These structures are recognized by pattern-recognition receptors (PRRs) among which Toll-like receptors (TLRs) are a pivotal component and are expressed on immune cells such as leukocytes (*e.g.* natural killer (NK) cells, mast cells) and phagocytic cells (*e.g.* macrophages, neutrophils and dendritic cells) that contribute to the effective elimination of pathogens through cytotoxic or phagocytic activities. Production of soluble factors, cytokines and chemokines in particular, also contribute to the activation of the inflammatory response and to the recruitment to the site

of infection of immune cells circulating in the blood. The activation of innate immunity is fundamental for the stimulation of antigen-specific adaptive immune responses through antigen presentation processes (Riera Romo et al. 2016).

Adaptive immune responses occur slower but are antigen-specific, *i.e.* capable of recognizing and eliminating virtually any known antigen/pathogen. The mediators of adaptive immune responses are B and T lymphocytes and their activation is induced through antigen-specific receptors expressed on the cell membrane. B lymphocytes specific receptors are immunoglobulins, or antibodies, characterized by almost infinite diversity, associated with B cell membranes or circulating in secreted forms and capable of recognizing and interacting very specifically with defined portions (epitopes) of the antigens. The exposure to an antigen activates B lymphocytes and induce their proliferation. The activated B lymphocytes then differentiate into plasma cells, synthesizing and secreting large quantities of immunoglobulins with the same specificity of those expressed on the membrane, or into memory B cells that remain in the blood and lymphoid organs for a long time and are able to secrete antibodies faster than naive lymphocytes in the event of subsequent contact with an antigen. Two main types of T lymphocytes have been described and are classified according to the expression of the CD4 or CD8 marker on the cell membrane. CD8-expressing T cells, also known as cytotoxic T-cells or killer cells, detect peptides of cytoplasmic origin complexed with major histocompatibility complex (MHC) class I molecules on infected or tumour cells and induce their death. CD4-expressing T cells, also known as helper, interact with peptide/MHC class II complexes, mainly present on the surface of immune cells, and secrete a large collection of cytokines that support the activity of other immune cells (Buck et al. 2015). The T-cell antigen receptor (TCR) has structural characteristics very similar to those of immunoglobulins but exists only in the form associated with the membrane. Unlike immunoglobulins, the TCR only recognizes the antigen

directly onto cells infected with the pathogen or after its processing by antigen-presenting cells (APCs).

After the pathogen has been eliminated, “memory” B and T lymphocytes provide effective protection against re-infection with the same infectious agent over time. Memory is a specificity of adaptive immunity as opposed to innate immunity characterized by the absence of memory-mediated immune responses and can be acquired by both infection and vaccination (Eibel et al. 2014).

### **Vaccine types**

The aim of vaccination is to elicit an immune response and to this purpose, a pathogen is inoculated in a harmless form to mimic the infection and stimulate the organism. For decades, the inoculated material consisted of viruses in an inactivated or live attenuated form (Bachmann and Jennings 2010). Inactivated vaccines are formulated with high titers of a cultivated pathogen that is inactivated by physical (radiation) or chemical methods based on binary ethylenimine or formaldehyde. Inactivation renders the virus non-infectious and unable to revert to virulence, to replicate and to spread among animals but can induce immunity. For optimal vaccine protection, it is important that during the inactivation process immunogenic epitopes of the protective antigens be not destroyed. On the other side, it is important that the exposure to the inactivating agent is sufficient to do not have residual infectious particles. A disadvantage of this immunization strategy, in which the virus cannot actively penetrate host cells and replicate, is that it activates mainly antibody-mediated immune responses being unable to stimulate mucosal and cell-mediated immunity. For this reason, this type of vaccines must be injected several times with adjuvants contributing to increase labour intensity and costs (Mak et al. 2014). In birds, inactivated vaccines are usually administered to long-living animals in a single injection with adjuvants as boost after priming with a live attenuated vaccine.

Attenuation is the alteration of an infectious agent that reduces its virulence while maintaining its ability to infect the vaccinated host and to effectively trigger the immune system. It is accomplished by performing several infective passages of the virus in a foreign host so that the virus loses its pathogenicity. In avian vaccines, in particular for the infectious bursal disease virus (IBDV), it can for example be obtained by multiplying and culturing the virus in chicken embryo fibroblast (CEF) cell cultures or in chicken embryos (Wang et al. 2004). Live attenuated vaccines are generally the method of choice to vaccinate flocks because they stimulate both mucosal and systemic immune responses inducing a very efficient protection also on the field, they can be used in a mass application and administered by drinking water or spray, making them well suited to agricultural labour with a very low administration time and cost. However, live attenuated viruses can revert to virulence and may spread infectious viruses in the environment. They can also cause complications in animals by weakening the immune system and inducing post-vaccination symptoms, such as respiratory reactions, that could affect herd performance. Post-vaccination reactions can also make animals more susceptible to secondary bacterial infections, which can be a significant problem due to restrictions on the use of antibiotics, particularly in intensive poultry production. These disadvantages make live attenuated vaccines less safe than inactivated vaccines (Zepp 2010).

However, new approaches to vaccine design are emerging, based only on the main immunogenic pathogen components (antigens) that possess the neutralizing epitopes. DNA vaccines are based on genetically engineered viral DNA that is transferred into the host cells, encoding the antigen directly produced intracellularly that trigger immune responses. On the other hand, subunit vaccines are developed by directly purifying the pathogen's antigen or by producing it in the recombinant form. Recombinant subunits offer several advantages such as the cost-effectiveness of production strategies, safety and the ability to serologically differentiate infected from vaccinated animals (DIVA) (Karch and Burkhard 2016). Moreover,

the expressed viral proteins can also, in some cases, further self-assemble into a structure that closely resemble the virus without the viral genetic material and called virus-like particles (VLPs) that trigger high immune responses due to the repetitive and high-density display of the antigens. However, due to the high purity of the final product (*e.g.* absence of viral RNA), the immunogenicity of these vaccines is limited mainly because they do not elicit efficiently the host innate immune system and like inactivated vaccines, they require adjuvant co-delivery to stimulate the immune system (Shi et al. 2019). Innovative approaches are currently investigated to improve subunit vaccines immunogenicity for example by specifically targeting their delivery to APCs, dendritic cells in particular, ensuring a more efficient stimulation of the immune system.

### **Vaccine delivery**

Vaccines are usually administered by intramuscular or subcutaneous injection, but other delivery approaches exist and their use depend on the vaccine formulation, the available infrastructure and the animal. Injection is the best way to adapt the vaccine dose to each animal and the method certifies the correct and complete administration of the necessary dose to the animal. However, some risks and disadvantages are inherent to this invasive method as the needle can cause contamination from the environment if used inappropriately and can break during vaccine distribution. Inactivated vaccines injection can also induce tissue damages due to the use of strong adjuvants (Bachmann and Jennings 2010). In addition, injectable vaccines are generally temperature sensitive and lose their effectiveness at room temperature, which can be problematic in developing countries. Injection is effective when the number of animals to be vaccinated is limited, but in the case of large herds, the time and cost of the technique become economically incompatible. Other non-invasive methods such as intranasal and oral vaccination are also available and effective. In this case, live attenuated vaccines are generally used as they



can be administered by drinking water, spraying or intranasal route. These methods are inexpensive, safe and can be used to rapidly immunize large animal populations (Abdul-Cader et al. 2018). In addition, they induce the activation of both mucosal and systemic immunity. Mucosal immunity is the first line of defence involved in preventing the access of many pathogens to the animal body and is not activated in the event of parenteral immunization. It is associated to oral and nasal mucous membranes that form an anatomical barrier to pathogens access. The barrier is further reinforced by the underlying mucosal lymphoid tissue composed in particular of gut-associated lymphoid tissue (GALT) and leads to the generation of secretory IgA and IgG. Thus, to mimic the infection as much as possible, it would be decisive to choose a mucosal vaccination approach (Mowat 2003).

Edible vaccines also exist and are of great interest to the veterinary industry with several advantages over the traditionally injected formulations. Moreover, it has been extensively demonstrated that oral immunization can stimulate both humoral and cellular immune responses both at a systemic and mucosal level representing an ideal line of defence against viral infections that generally occur through the mucosae (Müller et al. 1979b). These advantages are represented by the facilitated administration and by the simplification of the production processes that do not require expensive downstream purification steps. In the case of vaccines accumulated in plant seeds such as rice or maize, it has been demonstrated that the biomolecules are very stable over-time without the need of cold storage (Fischer et al. 2004).

### **Specificities of animal vaccination**

The vaccination mechanism is similar between humans and animals, but the objectives and strategies to reach those objectives can be very different. In the case of humans, the health and well-being of the vaccinated person are the main concerns. The same criteria of success often apply also to pets. The situation is strongly different when the vaccination concerns livestock

species; here the main goal is to improve the overall animal production with a cost-benefit ratio and to reduce the risk of disease propagation, also to consumers (Meeusen et al. 2007). Another important aspect of veterinary vaccination is the difficulty of monitoring the health status of each animal, particularly when there is high number of animals and in developing countries. In case of contagion, it would be therefore important to have develop methods allowing to differentiate between vaccinated and infected animals (DIVA) (Hasan et al. 2016) in order to avoid infection spreading and to ensure the safe international trade of animals and animal-derived products. Changes in legislation and consumer demand can also influence the use of veterinary vaccines and guide their development. For example, the restriction of the use of antibiotics in animal production in the European Union and the growing consumer demand for better animal welfare are contributing to change the current situation. Thus, the market of veterinary vaccines is constantly evolving to combine the existing situation with new constraints while adapting to very different situations depending on the animal species and production systems (Hoelzer et al. 2018). The need to reduce the use of antibiotics, the evolution and emergence of new zoonoses and the lack of effective solutions for the treatment of certain animal diseases are leading to high demand for new veterinary vaccines.

### **Avian vaccination**

Compared to other animal productions such as sheep or cows, poultry production has specific characteristics and the economic aspect of vaccination is one of the major concerns due to the very low value of each animal. A veterinary vaccine for the poultry market will only have economic value if its production cost is not excessive and if the inoculation method does not take time. The two main strategies adopted to vaccinate chickens are those based on inactivated vaccines and live attenuated vaccines. For this second approach, the main advantage is that it can be easily adopted to vaccinate a large number of animals because it can be administered by

drinking water or by aerial spraying to flocks (Abdul-Cader et al. 2018). Poultry vaccination with live attenuated vaccines is therefore usually carried out on a large scale, however injection is also used preferably and almost exclusively for "special" categories of animals such as laying hens, mainly with inactivated vaccines, that allows to follow more accurately the vaccination status of each animal. Another administration strategy, specific to birds, consist in immunizing the embryo *in ovo* but it is only available for some diseases. This approach allows to vaccinate a very large quantity of eggs at an early stage and at high speed using automated robots, but the equipment is very expensive and therefore limits its use to rich and developed countries (Peebles 2018). A final important aspect of poultry vaccination is passive immunization at an early stage of life through maternal antibodies. The transfer of maternal antibodies to the offspring takes place during embryo formation with direct transport of immunoglobulins inside the egg. Young chickens, compared to non-immunized chickens, can thus be partially or fully protected against diseases and pathogens present in the environment at the time of laying. The importance of passive immunization is maximal at the early growth stage of the chick and decreases with time ending after 3 weeks of age when the antibodies produced by the chick take over. Passive immunization should be therefore considered to avoid failure of early vaccination in chicks because maternal antibodies can interfere and reduce the effectiveness of vaccination, resulting in low protection (Vrdoljak et al. 2017).

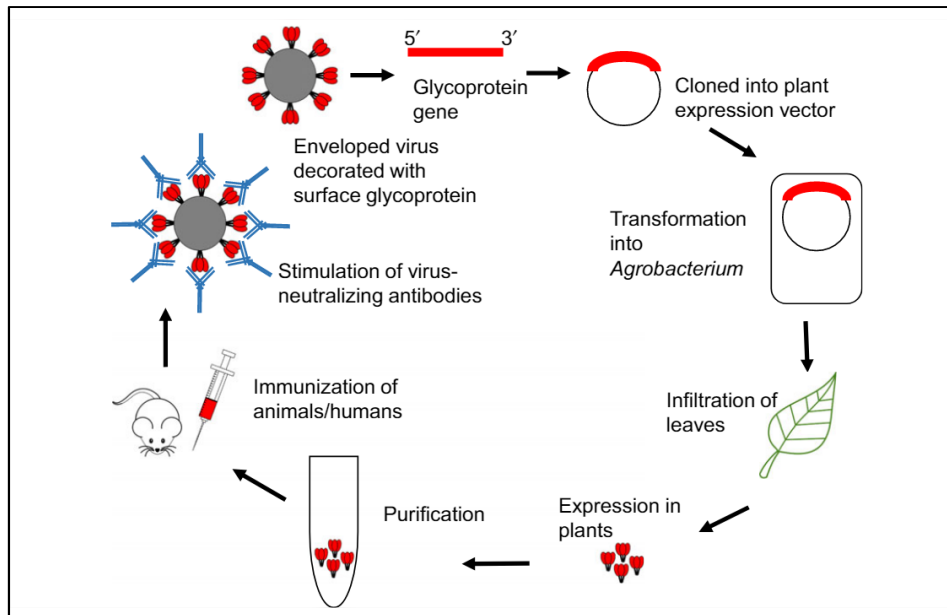
In conclusion, even if a considerable variety of vaccines is available, the main concerns in the field of new vaccines development for the poultry market are to catch up with the rapid evolution of diseases with increased pathogenicity while maintaining low production costs (including the use of adjuvants) and to provide effective DIVA strategies.

**Veterinary vaccines in plants**

The variety of platforms based on plants available for the production of recombinant antigens, both in terms of species and transformation methods render their use particularly attractive for the production of veterinary vaccines because the most suitable platform can be selected and studied to meet the needs of farmers. The choice is influenced by several parameters such as production time, antigen delivery route, final product characteristic (*i.e.* purified or not) and stability. As described above, the economic aspects of veterinary vaccination are crucial: effective vaccines may already be available on the market but, if they are too expensive for large-scale use, a low-cost crop-based production platform could be targeted to produce an economically competitive product (Figure 5) (Topp et al. 2016). In addition, cold conditions (4°C or less) are often required to transport and store vaccines, but in developing countries, in addition to the lack of infrastructures, this cost is generally unaffordable for farmers. Vaccination is therefore limited and plant-based vaccines offer promising solutions to bypass the cold chain because antigens can be very stable when stored in plant tissues. The plant matrix, such as in dried or lyophilized leaf tissues, protects the recombinant protein and allows long-term storage and distribution of the vaccine at room temperature without loss of quality (Kolotilin et al. 2014). Some other plant tissues can be selected for antigen accumulation to obtain the best storage conditions, such as seeds (Lau and Sun 2009). Plant extracts may have also inherent adjuvant activities that reduce the need for adjuvant in the preparation (Di Bonito et al. 2009). Plants can also be used to produce subunit vaccines designed to improve antigen uptake by APCs or to cross epithelial barriers, and can also be used to produce VLPs that are more immunogenic compared to soluble antigens (Kolotilin et al. 2014).

The definition of a strategy to produce an edible vaccine can be a promising approach to obtain a vaccine that is low-cost, easy to administer and offers improved therapeutic qualities. Indeed, the plant tissues used to produce the recombinant antigen can be consumed directly or with

minimal processing, by animals, simplifying vaccine delivery and reducing the costs associated to inoculation. This needle-free approach reduces risks associated to injection, reduces the time spent to vaccinate the animals, especially on large-scale, allowing to exploit to this aim food distribution infrastructures (Merlin et al. 2017). In addition, the edible vaccine may be designed to be effective also in activating mucosal immunity. The antigens expressed in plant are naturally “encapsulated” by the cell wall of cells and could thus be protected, depending from the plant species and tissue, from degradation by severe acid and enzymatic environment typical of the gastrointestinal tract (Topp et al. 2016) even if sometimes protection may be unsuccessful (Bertini et al. 2018). The seeds are particularly suitable for this “bioencapsulation” as the antigen may be accumulated in specific storage organelles such as protein storage vacuoles, starch granules and the surface of oil bodies (Khan et al. 2012). However, oral vaccination with plant-produced antigens requires the delivery of very high doses of plant material as compared to the parenteral delivery of a purified antigen, because the expression levels obtained by stable transformation methods are usually rather low and it is challenging to achieve a sufficient level of expression in plant organs (leaves, roots or seeds) (Mason et al. 2015).



**Figure 5:** Schematic representation of the recombinant production process in plant of a viral glycoprotein to be used as recombinant subunit vaccine (from Margolin et al. 2018)

Some plant species, such as soybean, maize and alfalfa, have been characterized with the specific aim of producing veterinary vaccines as they are among the most economically efficient crop production systems. A vaccine based on the expression of the LT-B protein of *E. coli* was produced in soybean (Moravec et al. 2007) and one against the porcine transmissible gastroenteritis virus was produced in maize (Lamphear et al. 2004). On the other hand, alfalfa was used to produce a vaccine against enterotoxigenic *E. coli* (ETEC) for pigs based on the main F4 fimbriae protein (Joensuu et al. 2006) and one against bovine viral diarrhoea virus based on the antigen protein E2 (Aguirreburualde et al. 2013). *Arabidopsis thaliana* was also used as production platform for a vaccine against the zoonotic Rift Valley fever virus that demonstrated to be immunogenic when orally delivered to mice (Kalbina et al. 2016) and for a vaccine against the porcine reproductive and respiratory syndrome virus, based on the two antigenic proteins GP4D and GP5D, that induced specific humoral and cellular immune responses in immune pigs (An et al. 2018).

Several other plants, mainly cereal and edible species, have been tested principally for a use as platforms for the production of vaccines intended for the direct administration by animal feeding. A veterinary vaccine was developed in rice against ETEC in pigs inducing passive and active immunity (Takeyama et al. 2015). Black-eyed beans were used to produce the capsid VP2 protein of mink enteritis virus (Dalsgaard et al. 1997), while potato tubers were used to produce the VP60 protein of the rabbit haemorrhagic disease virus (Martín-Alonso et al. 2003) and the S1 glycoprotein of infectious bronchitis virus (Zhou et al. 2003). By these approaches, the animals orally immunized with the vaccines resulted to be partially and, in some cases fully protected from infection.

The majority of veterinary vaccines manufactured in plants are based on stable transformation with the accumulation of antigen either in whole plants or in leaves or seeds. However, transient expression is also commonly used. Several viral antigens have been successfully expressed transiently by agroinfiltration in *N. benthamiana*, such as the outer-capsid protein VP2 of the bluetongue virus (Thuenemann et al. 2013), the haemagglutinin (HA) protein of H5N1 zoonotic influenza virus that formed VLPs purified and tested in mice (Landry et al. 2010) and 4 structural proteins (VP2, VP3, VP5 and VP7) of the African horse sickness virus that formed VLPs purified and injected in pigs or horses raising neutralizing antibodies (Dennis et al. 2018). Viral expression vectors have not been well studied yet for the production of veterinary vaccines but some example exists for human. For example, VLPs derived from the Norwalk virus were produced using: a TMV derived transient expression system in *N. benthamiana* leaves (Santi et al. 2008), or geminiviral replication vectors in lettuce, selected because of its rapid growth, low metabolic content and edibility (Lai et al. 2012a).

Beside their use as biofactories plants can also provide carriers endowed of immunopotentiating properties.

## **The fragment crystallizable of immunoglobulin Y as vaccine delivery moiety**

Immunoglobulins are complex glycoproteins of the vertebrates immune system endowed with the property of specifically detect and neutralize antigens and made of two heavy (about 55 kDa) and two light (about 24 kDa) chains. Through disulphide bonds the four chains are linked together and the molecule assume a Y-shape organised in three main regions: the fragment crystallisable (Fc) region, formed by the C-terminal constant domains of the heavy chains and involved in immunoglobulin effectors functions, and two fragment antigen-binding (Fab) domains each formed by the N-terminal variable domains of an heavy and a light chain and involved in the interaction with the antigen (Cohen 1975). The Fab “pocket” of an antibody can specifically recognize only one antigen and, after binding, the immune complex is usually eliminated through phagocytosis by cells such as macrophage, monocyte or granulocytes. In the mammalian immune system, five different immunoglobulin isotypes have been described, namely IgG, IgA, IgE, IgM and IgD and each isotype, defined by the constant domains of the heavy chains, is involved in specific immune processes. IgA and IgM are involved, during the initial phase of infection, in pathogens elimination, IgE in the allergic reactions, IgD in the B cell maturation and IgG in the antigen neutralization (Senger et al. 2015).

### **IgG receptors**

IgG are the most represented immunoglobulin in blood circulation and two main classes of receptors have been described able to bind their Fc: the Fc $\gamma$  receptor (Fc $\gamma$ R) and the neonatal Fc receptor (FcRn). Fc $\gamma$ Rs can be classified, based on their binding affinity, in low-affinity (Fc $\gamma$ RIIa, Fc $\gamma$ RIIc, Fc $\gamma$ RIIIa, Fc $\gamma$ RIIIb) and high-affinity (Fc $\gamma$ RI) receptors. They are expressed on the plasma membranes of almost all leukocytes (macrophages, neutrophils, B cells, dendritic cells, NK cells) (Sondermann and Szymkowski 2016). They are activated by the binding to IgG Fc and their effector functions are antibody-dependent cellular phagocytosis



(ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC) and inflammation, which induce phagocytosis, microbe killing, and cytokines release. Only one receptor is known, FC $\gamma$ RIIb, whose binding induce the stimulation of counter-balancing (inhibitory) signalling pathways (Hayes et al. 2014). FcRn is a heterodimeric glycoprotein with a structure similar to that of MHC class I. FcRn is widely distributed in the organism, highly expressed on endothelial cells, epithelial cells and placental syncytiotrophoblasts and involved in the regulation of IgG half-life and transport across mucosal cells or from mother to foetus (Kuo et al. 2010). FcRn can specifically mediate bidirectional transcytosis of IgG across the intestinal epithelial barrier and can recycle IgG-antigen complexes to dendritic cells for processing (Challa et al. 2014).

Considering the essential role of IgG Fc in regulating immune responses and IgG transport across epithelial cells, the fusion of antigens to this moiety has been investigated as a possible strategy to enhance antigen immunogenicity and to develop innovative mucosal vaccines. The extracellular domain of the M2 protein (M2e) of the influenza A (H1N1) virus produced in *Lactobacillus plantarum* as fusion to the Fc region of IgG was demonstrated to be efficiently up-taken by FcRn inducing protective mucosal and cellular immune responses when used to orally vaccinate mice (Yang et al. 2017). Similarly, Fc fused to the gp120 envelope protein (Env) of the Human Immunodeficiency virus 1 (HIV-1) was demonstrated able to induce enhanced and persistent immune responses in rhesus macaques, represented by higher IgG titers, neutralizing antibodies and ADCC response (Shubin et al. 2017). An IgG Fc-receptor targeted vaccine produced in plants and consisting in the fusion of the domain III of the envelope protein of Dengue virus (cEDIII) to a modified IgG Fc carrying at the C-terminus an IgM fragment to result in hexameric structures, the polymeric immunoglobulin G scaffold (PIGS), was demonstrated able to induce neutralizing antibody responses and activation of cellular immunity (Kim et al. 2017).

**IgY**

In birds, only three isotypes of immunoglobulin are present: IgA, IgM and IgY. IgY is mainly produced during secondary humoral immune responses to pathogens infection and found in the serum at concentrations ranging between 5 and 15 mg/l. Similar to mammalian IgG, IgY concentration in the blood quickly rises after the synthesis of IgM the main mediator of primary humoral immune responses. IgY is produced by B lymphocytes that in birds differentiate in the Bursa of Fabricius. The IgY-producing cells are not detected outside the Bursa before day 4 after hatching and before day 8 in the spleen (Erhard and Schade 2001). Circulating IgY are specifically and actively transported from the hen blood to the egg where they are stored in large quantities. During the development of the avian oocyte, the membrane receptors allow the accumulation of large amounts of IgY that are stored in the sterile yolk (up to 200 mg/egg at the moment of laying). The IgY concentration into the egg is proportional to the concentration found into the maternal serum. On the contrary, IgM and IgA are transferred to the egg together with other proteins and are stored specifically in the albumen at low concentrations (Behn et al. 2001).

IgY shares functional and structural homologies to both mammalian IgG and IgE (Table 2) of which they have been hypothesized to be the common ancestor. The IgY precursor, diversified during evolution in the two immunoglobulins, is structurally closer to IgE but functionally similar to IgG and this is the reason why it has been called chicken IgG for a long time. IgY can be found in birds, reptile and amphibians but their identification and detailed characterization have been carried out in chickens (Zhang et al. 2017).

Similarly to IgG, they are result by the assembly of four polypeptides, two heavy (approximately 67 kDa each) and two light (approximately 25 kDa each) chains, associated to form the typical Y-shaped structure (about 180 kDa) organised in the fragment crystallisable (FcY) and two Fab. Each heavy chain is folded in four domains named C<sub>v1-2-3-4</sub> similarly to IgE

and one more of IgG heavy chains. The sequences close to the boundaries between C $\nu_1$  and  $\nu_2$  and C $\nu_2$  and  $\nu_3$  contain proline and glycine residues that give some flexibility to the structure even in the absence of the hinge region present in mammalian IgG. The two heavy chains are linked together through three disulfide bonds (inter-chains linkages) that involve two cysteines located on the C $\nu_2$  (C252 and C340) and one on the C $\nu_3$  (C347). A model assumes the existence of two inter-chains linkages between two C $\nu_2$ -C $\nu_2$  (C252 with C340) determining an X shape, and an inter-chains linkage between two C $\nu_3$ -C $\nu_3$  (C347 with C347) (Figure 6) (Parvari et al. 1988; Suzuki and Lee 2004).

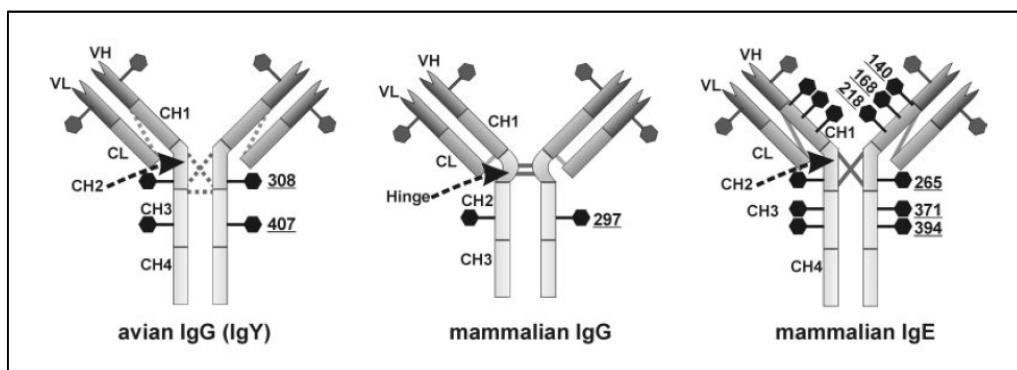
IgY, whose isoelectric point varies between 5.7 and 7.6, is resistant to pH values comprised between 4.0 and 11.0 and loses irreversibly its conformation below a pH value of 3.5. Also the temperature affects the molecule stability and functionality that are both lost if IgY are maintained above 60°C for more than 15 minutes (Shimizu et al. 1992; Gilgunn et al. 2016).

|                            | IgG             | IgE              | IgY                                      |
|----------------------------|-----------------|------------------|------------------------------------------|
| Species                    | Mammals         | Mammals          | Birds, reptiles, amphibians and lungfish |
| Molecular weight (kDa)     | 150             | 200              | 180                                      |
| Isoelectric point (pI)     | 6.4–9.0         | 5.2–5.8          | 5.7–7.6                                  |
| Concentration (mg/mL)      | 10–12 (serum)   | 10 <sup>-4</sup> | 8–10 (Serum) 15–25 (Egg Yolk)            |
| Number of constant domains | 4 (3 H and 1 L) | 5 (4 H and 1 L)  | 5 (4 H and 1 L)                          |
| Hinge                      | Yes             | No               | No                                       |
| Complement binding         | Yes             | No               | No                                       |
| Rheumatoid factor binding  | Yes             | No               | No                                       |
| Fc receptor binding        | Yes             | No               | No                                       |
| Mediates anaphylaxis       | No              | Yes              | Yes                                      |
| Binding to protein A/G     | Yes             | No               | No                                       |

**Table 2:** Comparison of the features of IgG, IgE and IgY (from Gilgunn et al. 2016)

IgY contains two N-glycosylation sites on the Fc region, one located on the C $\nu_2$  domain (Asn308) and the second one on the C $\nu_3$  domain (Asn407), and one on the Fab region (position not determined). IgY glycosylation pattern is more similar to that of IgE than to IgG (Figure 6). In IgY, both the C $\nu_2$  and the Fab are decorated by complex-type N-glycans. On the contrary, only high mannose N-glycans are present on the C $\nu_3$  site while on the homologous site of IgG

present on the CH<sub>2</sub> domain (Asn297) only complex-type N-glycans are observed. It has been hypothesized that the particular glycosylation pattern of IgY is a consequence of protein folding. Indeed, after the addition in the ER of high mannose-type sugars to the Asn407 site, the C<sub>v</sub><sub>3</sub> domain rapidly folds and the glycosylation site becomes inaccessible to glycan-processing enzymes in the Golgi apparatus. Conversely, the C<sub>v</sub><sub>2</sub> N-glycosylation site is not buried when the protein folds and undergoes correct Golgi-processing (Suzuki and Lee 2004). The IgY glycosylation pattern impacts thermal stability and physicochemical properties. The removal of the oligo-saccharide chains reduces the storage stability of IgY, decreases the resistance to enzymatic proteolysis and exposes the protein to a conformational change that alters the protein structure. Moreover, the glycosylation affects the circulating half-life, immunogenicity and binding to Fc-receptors (Sheng et al. 2017).



**Figure 6:** Structure of IgY, IgG and IgE highlighting N-glycosylation sites. CH<sub>1-2-3-4</sub>: heavy chain constant domains; VH: heavy chain variable domain; CL: light chain constant domain; VL: light chain variable domain (from Suzuki and Lee 2004).

### IgY receptors

Also the Fc region of IgY plays a major role in immunomodulatory functions through the binding to three different receptors discovered and currently characterized: the chicken immunoglobulin-like receptor AB1 (CHIR-AB1), the chicken yolk sac receptor (FcRY) and the

*Gallus gallus* Fc receptor (ggFcR). The binding to CHIR-AB1 and FcRY involves the C<sub>v3</sub>-C<sub>v4</sub> inter-domain whereas the binding to ggFcR involves the C<sub>v2</sub>-C<sub>v3</sub> inter-domain (Zhang et al. 2017).

CHIR-AB1 is expressed on monocytes, macrophages, B lymphocytes and NK cells and binds with high affinity to IgY through a site that is structurally homologous to the site that in mammalian Fc $\alpha$ RI is involved in binding to IgA. It is a bifunctional receptor with a well-characterized activatory activity and a potent but not yet elucidated inhibitory activity. The receptor/ligand ratio has a stoichiometry of 2:1. The receptor is involved in the uptake of IgY by chicken immune cells and may also be required for leukocytes activation and B-cell activation and maturation (Viertlboeck and Göbel 2011).

The ggFcR is structurally homologous to the mammalian counterparts Fc $\epsilon$ RI and Fc $\gamma$ R. The receptor is structurally organized in four Ig-like domains and a transmembrane region containing a charged residue important for the association of adapter molecules. It is expressed on blood cells including macrophages and thrombocytes but the effects of its stimulation have not been elucidated yet even if a current hypothesis correlates its function to leukocyte activation. IgY binding affinity to both CHIR-AB1 and ggFcR is modulated by the glycosylation at Asn407 on the C<sub>v3</sub> (Schreiner et al. 2012).

FcRY is the structural homologous of the phospholipase A2 receptor, member of the mannose receptor family, and is functionally similar to the FcRn. After IgY transport from the maternal blood to the egg yolk by oocyte membrane receptors that have not been isolated yet, it is involved in the transport of IgY from the egg yolk to the embryo. Like FcRn/IgG interaction, the binding is pH-dependent and allows the uptake of IgY from intracellular vesicles where the pH value is around 6.0 and their release in the blood where the pH value is around 8.0. FcRY structure is characterized by 10 extracellular domains (*i.e.* an N-terminal cysteine-rich domain, a fibronectin type II domain, and eight C-type lectin-like domains), a transmembrane region

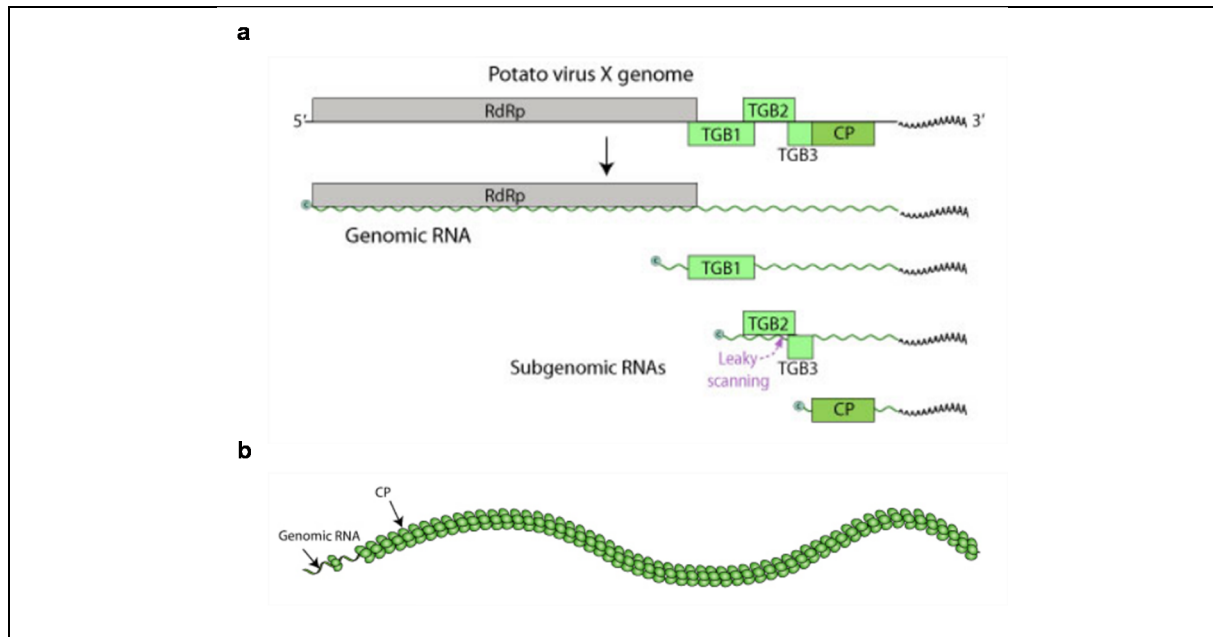
and a cytoplasmic tail. The expression of FcRY in polarized mammalian epithelial cells is functional in endocytosis, bidirectional transcytosis, and recycling of chicken IgY demonstrating that the receptor may be involved in the transfer of IgY across the mucosal epithelial cells similarly to mammalian FcRn (Tesar et al. 2008; Tian and Zhang 2012; Murai et al. 2013).

On the basis of this information, it is possible to hypothesize that, similarly to the IgG-Fc, FcY may be used as fusion moiety for antigens in innovative subunit vaccines constructions in order to activate APCs through targeting and uptake by receptors such as FcRY and CHIR-AB1. In a recent publication, antigens fused to the IgY constant region were demonstrated able to enhance specific immune responses in chickens, reducing morbidity and mortality (Wang et al. 2017). Moreover, the outer membrane protein A of *Bordetella avium* expressed in *Pichia pastoris* as fusion to FcY was able to activate avian macrophages enhancing immune responses in chickens (Dong et al. 2016). Transgenic *Eimeria mitis* chicken parasites, stably expressing FcY and used as vaccine in chicken, demonstrated to have stronger immunogenicity compared to unmodified *E. mitis* with a better protection provided in case of further *E.mitis* infection probably because the FcY fragment facilitates the parasite antigen uptake during vaccination (Qin et al. 2016).

## Potato virus X as vaccine delivery platform

Potato virus X (PVX) belongs to the family *Alphaflexiviridae* and is the type member of the genus *Potexvirus* (Adams et al. 2004). It infects herbaceous plants of the *Solanaceae* family such as potato, tomato and tobacco and is transmitted by mechanical contact. It is considered a pathogen with important economic impact (Baulcombe et al. 1994).

The virus has a 6.4 kb ss(+)RNA genome with a 5'-methylguanosine cap and a polyadenylated 3'-end and contains five ORFs (Figure 7a) (Dolja et al. 1987). The first ORF encodes the RdRp, a 166-kDa RNA-dependent RNA polymerase required for viral replication. Three overlapping ORFs, also known as the triple gene block (TGB), encode the viral multifunctional proteins TBGp1, TBGp2 and TBGp3. These proteins are translated from two subgenomic RNAs (sgRNA) with sgRNA1 encoding the TBGp1 (25 kDa) and sgRNA2 the transmembrane proteins TBGp2 (12 kDa) and TBGp3 (8 kDa) (Morozov et al. 1991). The last ORF, with a size of 0.9 kb, encodes the coat protein (CP) which is involved in the encapsidation of the viral RNA and also in viral movement (Chapman et al. 1992). The RdRp is responsible for viral RNA capping through a methyltransferase type 1 domain and in replication through an helicase domain and a replicase domain (Rozanov et al. 1992; Longstaff et al. 1993; Davenport and Baulcombe 1997). The TBGp1 act as a silencing inhibitor, contributes to the translational activation of the virions and increases the plasmodesmata size exclusion limit together with TBGp2 and TBGp3 that are also involved in the formation of viral replication vesicles, known as X-bodies, by the remodelling of the ER membranes (Verchot-Lubicz et al. 2007; Verchot-Lubicz et al. 2010).



**Figure 7:** Schematic representation of Potato virus X **(a)** genome and **(b)** virion (from <https://viralzone.expasy.org>)

The virion is composed of the genomic viral RNA wrapped in approximately 1270 units of the CP. It has helical symmetry and each helix is composed of 8.9 CP units (Kendall et al. 2008). The structure is flexible and filamentous, 500 nm in length and 15 nm in diameter, with a deeply grooved and highly hydrated surface (Figure 7b) (Nemykh et al. 2008).

### **The virus behaviour**

After penetration into plant cells, the serine and threonine residues at the exposed N-terminus of each CP subunit undergo phosphorylation mediated by cellular enzymes and this induces particles unpacking (Atabekov et al. 2001). Once released, the viral genome is translated to produce the RdRp that initiates the replication into “viral factories” called X-bodies, recruited close to the nucleus by the TBGp1 protein. The X-bodies are isolated environments, organised in compartments, formed by actin filaments and remodelled cellular membranes originating from the ER and the Golgi apparatus. The neo-synthesized genome as well as the CP and the movement proteins, are produced inside X-bodies while the particle assembly occurs at the



periphery of these structures (Tilsner et al. 2012). The virus moves from cell-to-cell through the plasmodesmata and spreads throughout the host through the phloem. The CP is essential for both cell-to-cell and systemic movement but the assembly of the virus particle is compulsory only for phloematic movement (Santa Cruz et al. 1998; Betti et al. 2012). The symptoms revealing the presence of the virus in the leaves are vein clearing, shrinking, chlorosis, ringspot and mosaic.

### **Use of PVX as a vaccine delivery platform**

Different strategies to use PVX as a scaffold for biomedical or nanomaterial applications exploiting the CP are available resulting in either permanent or reversible functionalization of the virus (Lico et al. 2015). The CP has a pivotal role in infection efficiency and spreading and defines the morphology and the stability of the virus particle. It has been determined that the C-terminus of the protein is located inside the virion whereas the N-terminus is exposed on the outer surface and for this reason represents an excellent site for the fusion of heterologous peptides/proteins or for the linking to chemicals.

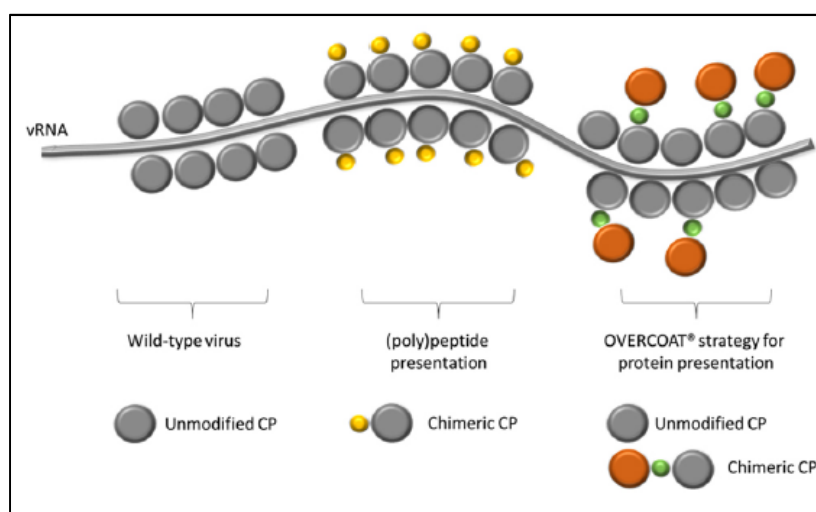
The first strategy exploits the surfaces grooves and the polar amino acids of the virus to spontaneously attach drug molecules by hydrophobic interactions and  $\pi$ - $\pi$  stacking. It has allowed to produce PVX particles useful for targeted drug delivery carrying the chemotherapeutic drug doxorubicin (Le et al. 2017) and taking advantage of the PVX enhanced tumour homing characteristic (Shukla et al. 2013). A second strategy consists in exploiting solvent-exposed cysteine or lysine residues of the CP to chemically conjugate through maleimide or N-hydroxysuccinimide (NHS) chemistry whole proteins, polymers or small molecules such as fluorescent dyes (Röder et al. 2019). The disadvantage of using these two chemical methods is that the reaction times are very long and poorly efficient.

To overcome these bottle necks, a third approach has been designed that consists in genetically modifying the CP encoding sequence in order to obtain chimeric PVX particles displaying the polypeptide of interest on the surface as fusion to the N-terminus of each CP subunit (Figure 8). To this purpose, a viral expression vector is needed carrying the cDNA corresponding to the complete viral genome and engineered to easily insert foreign sequences at the 5'-end of the *cp* gene. It has been defined that to increase the chance to obtain stable chimeric PVX particles, the first parameter to consider is the size of the heterologous polypeptide as long sequences risk to sterically interfere with CP folding and particle assembly. The probability to get the display of polypeptides longer than 60 amino acids is very low even if recently an exceptionally long sequence (*i.e.* the 113 amino acids long iLOV protein) has been directly fused to the CP without consequences on the virus behaviour, even if the systemic infection was delayed and the infection symptoms were mild (Röder et al. 2018). Also the isoelectric point and the amino acid composition of the heterologous polypeptide are important because these parameters may interfere with viral movement and replication sometimes resulting in the selection of compensatory deletion/substitution mutants (Lico et al. 2006).

A further approach consists in the insertion between the N-terminus of the CP and the foreign protein sequence of a 16 amino acids long peptide, known as 2A, derived from the Foot-and-mouth disease virus (FMDV) (OVERCOAT strategy) (Figure 8) (Lico et al. 2015). The presence of this sequence may induce a ribosomal skip during translation with a frequency that depends on the nature of the nucleotides immediately before the 2A cleavage site, and determines the production of chimeric and w.t. CP that will both participate to the assembly of the PVX particle (Minskaia and Ryan 2013). Several examples are present in the literature of chimeric PVX particles displaying proteins too large to be fused to all CP subunits and produced exploiting this strategy: the capsid protein of the rotavirus VP6 (O'Brien et al. 2000), a lipase

(Carette et al. 2007), a fluorescent protein (Shukla et al. 2014) or the ESAT-6 antigen of tuberculosis (Zelada et al. 2006).

Another approach that allows circumventing issues related to peptide size and composition consists in fusing in frame with the 5'-end of the *cp* gene the nucleotide sequence encoding the 13 amino acid long SpyTag peptide. The SpyTag and the SpyCatcher protein (12.3 kDa) come from the splitting in two of the CnaB2 domain of the fibronectin-binding protein of the *Streptococcus pyogenes* and bind spontaneously together through an isopeptide bond formation. Therefore, in parallel, a protein of interest is separately expressed fused to the SpyCatcher protein so that it will bind to the SpyTag when it will be mixed with chimeric PVX with a covalent attachment and a higher and faster coupling efficiency compared to chemical conjugation (around 70% of attachment). However, this system requires longer development and more expression steps compared to the OVERCOAT method (Röder et al. 2017; Röder et al. 2019).



**Figure 8:** Schematic representation of PVX particles with unmodified (grey) or genetically engineered CP. The chimeric virus can display the heterologous polypeptides (yellow) on all or only on some CP units (OVERCOAT strategy) (from Lico et al. 2015)

Chimeric PVX particles have been demonstrated to work excellently as carriers for the delivery of antigens of interest for vaccine formulations. In animal models, the modified PVX particles are able to induce the production of antibodies specific for the displayed polypeptide and also of immune responses mediated by MHC class I-restricted CD8<sup>+</sup> T cells (Lico et al. 2009). Interestingly, these properties are evident also when PVX is delivered without adjuvant probably due to the complex particulate structure of the virus and to the presence of the genomic RNA that may interact with TLRs stimulating innate immunity activation (Jobsri et al. 2015). In addition to their immunogenic properties, PVX particles are also efficiently, quickly and easily purified resulting in high purity and yields.

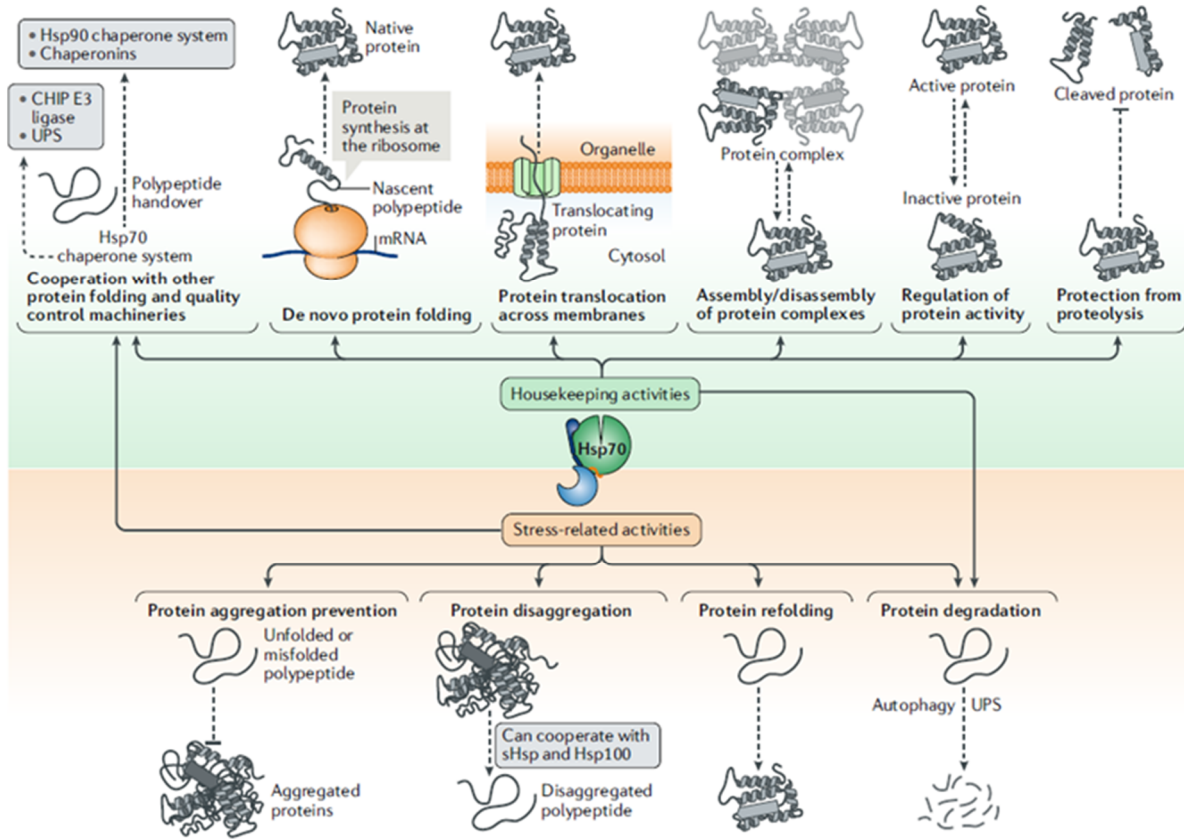
## **HSP70 as vaccine delivery platform**

Heat shock proteins (HSP) are a wide and heterogeneous group of ubiquitous proteins, abundant and constitutively expressed in all eukaryotic and prokaryotic cells and considered as the most phylogenetically conserved (Joly et al. 2010). Their expression was initially associated to cell responses to stressful conditions such as heat shock, harmful chemicals or pathogen infections but it is now clear that they also have a pivotal role in cell homeostasis by helping protein folding, preventing non-specific aggregation and activating the autophagy pathway to eliminate degradation products. Several different types of HSP have been identified and classified based on molecular size. The most studied groups are HSP100 (100kDa), HSP90 (90 kDa), HSP70 (70 kDa), HSP60 (60 kDa) and small HSP (sHSP, 12-40 kDa). HSP diversity varies among organisms and cell types as some HSP are associated with some tissues or cell compartments and expressed with or without stress exposure (Basu and Matsutake. 2019).

### **HSP70**

HSP70 family, the central player among HSP, is present in all living organisms and in all cellular locations and is the most structurally and functionally conserved. These molecular chaperones have a size comprised between 68 and 78 kDa and facilitate a multitude of cellular housekeeping activities helping to maintain the protein cell machinery under favourable conditions in the presence of extreme situations such as heat or oxidative stress (Figure 9). They act at every stage of the protein life from synthesis to degradation and therefore help to maintain protein homeostasis. HSP70 control the folding of newly synthesized polypeptides, but also protein translocation into organelles, disassembly of protein complexes, regulation of protein activity and protection from proteolysis. In order to protect the cell against stress conditions, they also prevent the aggregation of unfolded or misfolded proteins, promote protein disaggregation and help to refold or degrade misfolded protein. Thus, HSP70 re-equilibrate

protein homeostasis imbalance and reduce the related deleterious effects (Rosenzweig et al. 2019).

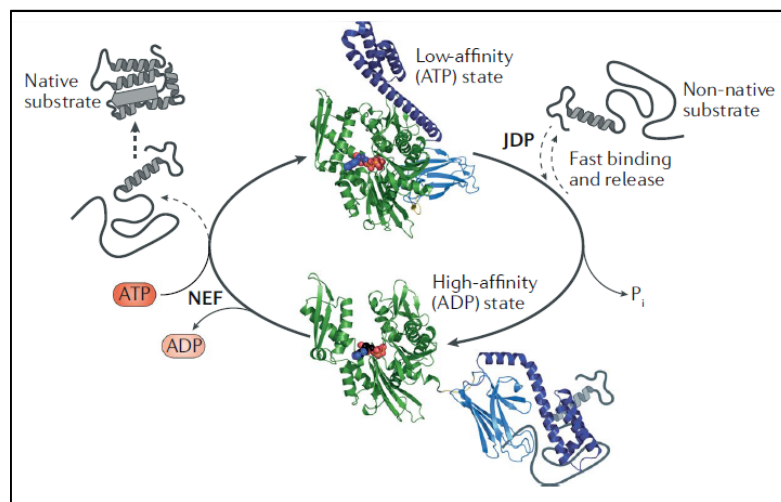


**Figure 9:** The diverse housekeeping and stress-related activities of HSP70 (from Rosenzweig et al. 2019)

### **HSP70 structure and mechanism of action**

HSP70 are folded into two domains, the 45-kDa N-terminal actin-like nucleotide-binding domain (NBD) and the 30-kDa C-terminal substrate-binding domain (SBD) that are connected by a conserved flexible and hydrophobic linker. The NBD consists of four subdomains forming two lobes separated by a cleft. The SBD consists of a 15 kDa  $\beta$ -sandwich subdomain (SBD $\beta$ ) containing the polypeptide binding cavity, a 10 kDa  $\alpha$ -helical lid subdomain (SBD $\alpha$ ) and a disordered C-terminal tail with variable length. In eukaryotic cytosolic and nuclear HSP70, the tail is generally composed of a conserved charged motif (Glu-Glu-Val-Asp) which can interact

with specific co-factors (Mayer and Gierasch 2019). All the HSP70 share the same common fundamental mechanism of action modulated by the binding and hydrolysis of adenosine triphosphate (ATP) that gives to the chaperone the ability to realize fast association and release of polypeptides (Figure 10). During the allosteric cycle, the HSP70 exist in two different and stable states. In the ATP-bound state, also considered as the open state, the NDB is bound by an ATP and the SBD is free of the substrate (polypeptide). When ATP is hydrolysed to adenosine diphosphate (ADP), HSP70 bind non-covalently to the polypeptide substrate and move to the “closed” state. In the ADP-bound state, the NBD and SBD are independent from each other and the substrate-binding affinity of the SBD is high with low-association and dissociation rates. Finally, the exchange of ADP with ATP by nucleotide exchange factors release the substrate from the chaperone that reaches again the open conformation with low substrate affinity (Clerico et al. 2019). The polypeptides that are bound to HSP70 are the fingerprinting of the proteins expressed by the cell.



**Figure 10:** Functional cycle of HSP70 (from Rosenzweig et al. 2019)

**Vaccine use**

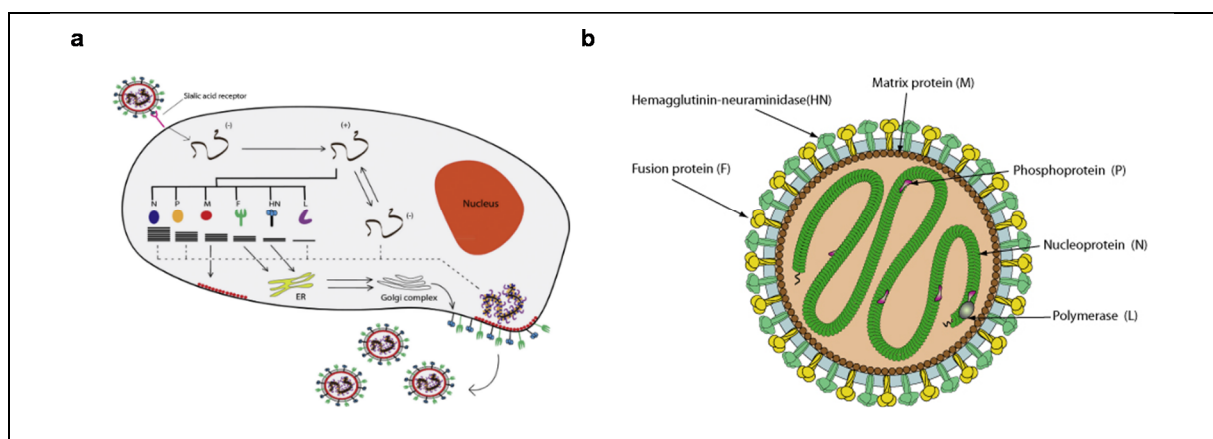
HSP70 demonstrated to have interesting adjuvant properties due to the ability to activate both innate and adaptive immune responses (Bolhassani and Rafati 2008). Released into the extracellular environment in case of cell stress, damage or death, they may act as an alarm messenger for the immune system (Calderwood et al. 2007). They are able to induce the release of cytokines and chemokines and to stimulate the maturation of dendritic cells activating the innate immune system. Extracellular HSP also demonstrated to have the inherent capacity, when associated with peptides, to interact with APCs and their uptake enhance in particular the cross-presentation of antigens through MHC class I molecules inducing the activation of cell-mediated adaptive immune responses (Taha et al. 2019). HSP-based vaccines are also regarded as an effective anti-tumour tool. HSP-peptides complexes derived from the tumour cells of a patient, when delivered, stimulate the immune system eliciting responses that are effective in specifically targeting the cancer cells (Parmiani et al. 2004). For these reasons, HSP70 are regarded as a promising tool for vaccination purpose both as antigen carrier in an HSP-peptide complex form or as adjuvant free of peptides (McNulty et al. 2013). In addition to their immunogenic properties, HSP70 are also efficiently, quickly and easily purified resulting in high purity and yields. The specific recovery of HSP70-peptides complexes is possible through ADP-based chromatography while ATP-based chromatography allows to recover HSP70 free of polypeptides (Ménoret 2004). Because of the very high conservation of HSP70 between different organisms, the possibility to use plant HSP70 (pHSP70) as vehicle of polypeptides derived from antigens transiently expressed in plants has been evaluated. A model of pHSP70 structure was designed by homology modelling and demonstrated to be superimposable to the crystal structure of the mammalian protein (Buriani et al. 2011). On the basis of the strong structural similarity, the nucleoprotein (NP) of the influenza A virus was transiently expressed in plants and leaves used to extract and purify pHSP70 by ADP-agarose chromatography with a



recovery of approximately 23 µg per gram of fresh tissue. When injected in mice without adjuvant the purified HSP70-polypeptide complexes were demonstrated able to activate both humoral and cell-mediated NP-specific immune responses encouraging their use for the development of innovative vaccination strategies (Buriani et al. 2012).

## Infectious Bursal Disease virus (IBDV)

IBDV belongs to the genus *Avibirnavirus*, family *Birnaviridae*. It is a non-enveloped virus characterized by an icosahedral capsid with size ranging from 60 to 70 nm and symmetry with a triangulation number (T) of 13 (Coulibaly et al. 2005). The viral genome consists of two linear ds-RNA segments, designated as A and B, with a total length of 6 kb. Segment A is about 3.2 kb long and contains two partly overlapping ORF (Figure 11a) (Müller et al. 1979a). The smaller ORF encodes a non-structural protein named VP5 (17 kDa) involved in virus release and dissemination but not essential in replication while the larger encodes a 110 kDa polyprotein auto-catalytically cleaved into three proteins: VP2 (42 kDa) and VP3 (32 kDa) that are structural, and VP4 (28 kDa) that is a serine protease (Mahgoub 2012). VP4 cleaves the polyprotein to its N and C termini and releases VP3 and the precursor of VP2 (pVP2) into the infected cell. The pVP2 precursor (also identified as VPX) is further truncated by VP4 four times at its C-terminus to obtain the mature VP2 protein. The viral capsid results from the assembly of the VP2 and VP3 proteins (Figure 11b) (Saugar et al. 2010). The segment B (2.8 kbp) of the viral genome contains an ORF that encodes the RdRp protein named VP1 (97 kDa) that associates to the RNA and circularizes segments A and B (Lombardo et al. 1999).

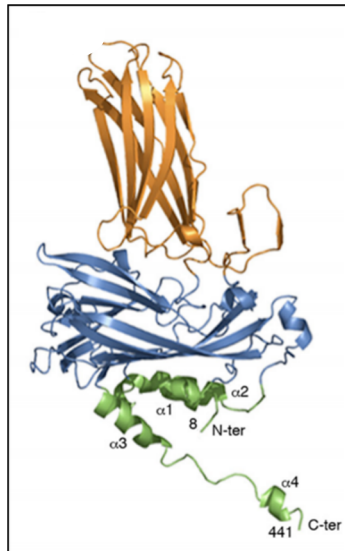


**Figure 11:** Schematic representation of the *Birnaviridae* (a) genome and (b) virion (from <https://viralzone.expasy.org>)

**VP2 protein**

The processing of the pVP2 is essential to obtain assembled virions. The VP2 proteins interact to form trimers and 260 trimers assemble to form the external surface of the viral shell for a total of 780 copies of the protein. The trimers are linked to the VP3 which forms the internal surface of the capsid and is involved in the formation of the ribonucleoprotein complex together with the VP1 protein and the RNA genome (Berg 2000). The T=13 icosahedral capsids are the most represented in infected cells but other minor and aberrant structures such as tubular and smaller T=1 capsids (23 nm) can also be found. It has been demonstrated that when expressed alone the VP2 spontaneously assembles in T=1 subviral particles formed by 20 VP2 trimers, whereas the expression of the pVP2 leads to poorly ordered tubular structures. It has been established that to get the formation of the T=13 capsids, the link between the VP2 C-terminal end and the VP3 plays an important role (Saugar et al. 2005).

When assembled into T=13 capsids, VP2 is folded into three domains disposed radially (from the outer capsid surface to the inner): the projection (P), the shell (S) and the basis (B) (Figure 12). The N- and C- terminal ends of the protein form the B domain and the P domain is formed by a loop between the S and the B domains. While the alignment of the VP2 sequences of different viral strains demonstrate that the S and the B domains are relatively well conserved, it appears evident that the sequence of the domain P is hypervariable (Letzel et al. 2007). Deletion mapping and selection with neutralizing monoclonal antibodies of IBVDV escape mutants indicate that the two outmost and hydrophilic regions of P, namely the P<sub>BC</sub> loop (amino acid positions 210 to 225) and the P<sub>HI</sub> loop (amino acid positions 312 to 325) harbour the main neutralizing conformational epitopes (Fahey et al. 1989).



**Figure 12:** X-ray model of the VP2 protein (from Saugar et al. 2010)

### **The disease**

Infectious Bursal disease, also known as Gumboro disease, is a worldwide distributed chicken infection which has been described for the first time in the '60s in the city of Gumboro, Delaware, USA (Sharma et al. 2000). There are two different viral serotypes: serotype 1, pathogenic in poultry, and serotype 2 isolated from turkey and non-pathogenic (McFerran et al. 1980). It is an immunosuppressive disease because the virus targets mainly the lymphoid organs causing, in particular, the inflammation and atrophy of the Bursa of Fabricius (BF) where the differentiation and maturation of B lymphocytes occurs in birds. The immunosuppression increases the sensitivity of the animals to opportunistic bacterial, protozoan and other viral diseases, especially in intensive production conditions (Ingrao et al. 2013). The virus causes also high morbidity and mortality producing important economic losses for the poultry industry. In the field, the major mode of transmission is oral. The virus then infects gut-associated macrophages and lymphoid cells, enters the portal circulation and reaches the BF where it actively replicates. Thereafter, the virus enters the bloodstream and spreads overall the body causing severe clinical signs and mortality (Etteradossi and Saif 2013). The BF, the principal

organ affected by the disease, initially increases in size doubling its weight at 3 days post infection (d.p.i.) to then turn haemorrhagic and becoming atrophic within 7-10 d.p.i. with a reduction in the immune response. Dehydration and discolouration of the kidneys, as well as muscles haemorrhages, can also be observed during *post mortem* examination. As a consequence of these severe organs alterations, the chickens display several visible clinical signs such as ruffled feathers, anorexia, diarrhoea and dehydration, depression, distress but also mortality. The intensity of the symptoms varies with the virulence of the infecting IBDV strain but the surviving chickens rapidly recover after the incubation period of about 2-4 days. 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 (Ingrao et al. 2013). There are different levels of virulence and replication efficiency. Different strains have been described and classified as classical (low virulence) or very virulent (vvIBDV). Up to 1987, only low virulent strains were observed inducing 1-2% of death into the field. The vvIBDV strains emerged in Europe and Japan after 1987. These strains display stronger symptoms severity and higher mortality rates (up to 100% in Specific Pathogen Free (SPF) chickens, 60% in laying hens and 35% in broilers). Nowadays, vvIBDV strains are diffused all over the world and represent a major threat in poultry farming, causing great losses for the chicken market (Mahgoub 2012).

### **Vaccines against IBDV**

There are no specific treatments for IBDV infection, and disease diffusion is mostly controlled with vaccination programs. Until 1987, the disease was controlled by hyper-immunisation of breeders before the point of lay using live/inactivated vaccines (Müller et al. 2012). The offspring was protected by Maternally Derived Antibodies (MDA) received via the yolk sac during the first weeks of life when birds are the most susceptible to virus infection before

decrease of this maternal immunity with time. The situation has changed since the emergence of IBDV variants in the USA and of vvIBDV. To control the diffusion of these viral strains and to limit the severity of the disease, chicks are now vaccinated with live-attenuated vaccines at an early stage of life when the MDA have declined (2-3 weeks of age). However, the MDA level not only protects against the field challenge but also neutralises live vaccines when given too early, and it is challenging to establish exactly at which time after birth vaccination produces immunity avoiding MDA interference (Al-Natour et al. 2004). Live attenuated vaccines used for young chick immunization are classified into three categories. The mild ones are highly attenuated and are no longer applicable in the commercial environment because of their inability to break through MDA levels to provide protection to the young chicks. Vaccination is therefore performed using live intermediate and/or “intermediate plus” vaccines because these vaccines have higher breakthrough titers and can break earlier through maternal immunity. Nonetheless, the safety and efficacy of this type of vaccines remain a major concern (He et al. 2009; Pikuła et al. 2018). To overcome these issues, new vaccines have also been developed during the last decades based on the use of antigen-antibody complexes or on recombinant viral vectors. The immune complex based vaccine (Icx) consists of a suspension of live-attenuated virus mixed in well-defined proportions, and according to well-defined procedures, with an antiserum of defined avidity prepared in hyper-immunised chickens (Whitfill et al. 1995). Antibodies in the mixture are not sufficient to neutralize the virus but are sufficient to delay its pathological effects. Although the mechanism of action of this vaccine is still poorly understood, IBDV complexed with specific antibodies causes a delay in virus detection of approximately 5 days (Ignjatovic et al. 2006). It decreases the viral load in the BF and consequently the bursa lesions with a remarkably low depletion level of B lymphocytes into the organ. The Icx vaccine allows young chicks to be vaccinated with strains that would be too virulent in normal conditions for the use *in ovo* or at hatching. It is also effective in the

presence of MDA and can be delivered by subcutaneous injection at 1-day old chickens in the hatchery. If an egg injection equipment is available, it can also be used for *in ovo* vaccination at day 18 of incubation (Iván et al. 2005). Genetically engineered live IBDV vaccines have also been investigated in order to obtain modified viruses providing a strong immune response without virulence. Some attenuated IBDV mutants were obtained by site-directed mutagenesis but, similarly to the viruses used in live attenuated vaccines, these mutants are not stable and sometimes revert to virulence. In order to get a safe engineered IBDV attenuated vaccine, several mutations are necessary to obtain a stable engineered virus but no product has yet reached the market (Müller et al. 2012). Inactivated vaccines, because of the higher production costs and the necessity to use adjuvants, are only used for the vaccination of high-value birds, such as future breeders, and have been proved to be more efficient in a prime-boost regimen after priming with a live attenuated vaccine. Subunit vaccines are also under development and mainly focused on the production of the recombinant version of VP2 protein, the main neutralizing antigen, using different expression systems, such as *E. coli* (Jiang et al. 2016), insect cells (Ge et al. 2015), yeasts (*Pichia pastoris*) (Taghavian et al. 2013) and plants (Lucero et al. 2019). The VLPs-based strategy is particularly interesting due to high immunogenicity of the assembled VP2 and may represent a valid alternative to vaccination with inactivated IBDV. Recently, the use of DNA or live vectored vaccines has also been considered. An HVT vectored vaccine carrying the VP2 gene demonstrated efficient in protecting animals from different IBDV strains, including the very virulent, also when used *in ovo* and has been recently commercialized (VAXXITECH HVT+IBD) (Darteil et al. 1995; Dobner et al. 2019). However, this vaccine requires storage in liquid nitrogen and this prevents its wide distribution. The development of novel, efficacious and low-cost vaccines against vvIBDV strains and combining straightforward administration with high efficacy and few side effects is therefore essential.

### **Plant-based vaccines against IBDV**

VP2 represents the most important antigenic protein for vaccine development. For this reason, in the last 15 years, there have been several examples of the production of VP2 in different plant species and different expression strategies were adopted ranging from the generation of transgenic plants to the use of novel transient expression systems. VP2 was expressed preferentially in its native form but there are two interesting examples in which antigenic portions of this protein were fused to protein carriers. In most cases, the plant-produced subunit vaccines were delivered systemically, but successful results were also obtained using the oral (mucosal) administration route. In this paragraph, are detailed the highly differentiated approaches that have been used for the production of IBDV subunit vaccines in plants highlighting the advantages and weaknesses of each methodology.

#### **VP2-based oral vaccines using transgenic plants**

The first attempt to produce an edible plant-derived subunit vaccine against IBDV was carried out in *A. thaliana* by investigating the expression of the antigenic capsid protein VP2 and its potential immunogenicity in chickens (Wu et al. 2004a). Transgenic plants were obtained expressing the recombinant VP2 in leaves with expression levels in the range of 0.5 - 4.8% of total soluble proteins (TSP). After oral immunization with the crude leaf extract, the sera of chickens were demonstrated to contain antibodies specifically recognizing the VP2 protein. Further studies were aimed to explore the potential protective properties of the plant-derived antigen used either as a main immunogen or to boost the immune response in animals primed with a commercial vaccine (Wu et al. 2004b). SPF chickens were treated with different immunization schedules and delivery routes (oral or subcutaneous). The efficacy of protection, measured evaluating the atrophy of the BF after challenge with a classical IBDV strain, was in line with the antibody titers results and indicated that the resistance to infection observed in the



animals primed with the commercial vaccine and orally boosted with the plant extract was comparable to that observed in birds receiving two doses of the vaccine.

Another interesting example of a potentially safe and inexpensive plant-based IBDV edible vaccine was obtained using rice plants (*Oryza sativa*) as biofactories (Wu et al. 2007). In this case, the stable expression of the VP2 was specifically directed in the seed endosperm using the strong Glutelin A promoter, obtaining expression levels that ranged from 0.7% to 4.5% of TSP corresponding in the best-expressing line to about 40 µg of VP2 per grain. All the chickens fed with the VP2-containing seeds produced IBDV-specific neutralizing antibodies with titers rising as a function of the received dose. Interestingly, the group fed with the highest quantity of seeds (5 g) responded similarly to the group injected with the commercial attenuated vaccine. Also, the protection from challenge with a classical viral strain was higher in the group of animals immunized with the highest antigen dose in terms of bursal lesions score. Overall, these results demonstrated that the plant-produced VP2 is able to elicit IBDV-specific immune responses and does not lose this ability even if subjected to the harsh environment of the avian digestive tract. Interestingly oral induced protection is acquired without adjuvant. Nevertheless, plant-production of vaccines using this approach still presents some, drawbacks such as the low expression levels that are generally obtained in transgenic plants/seeds and the necessity to develop a quantification and administration protocol of the antigen that allows standardizing the amount of vaccine to be delivered orally to each animal.

### **IBDV vaccines produced in plants using transient expression systems**

In the case of rapidly evolving pathogens such as IBDV, it is very important to reduce the manufacturing cycle time since there is the need to rapidly produce new vaccines to efficiently limit the spreading of newly emerging virulent strains.

In a recent work, the VP2 protein fused at its C-terminus to the His-tag was transiently produced in the leaves of *N. benthamiana* plants (Taghavian 2013) to obtain VLPs similarly to that previously obtained in other heterologous systems (Saugar et al. 2005). However, in spite of the high expression levels reached (260 µg/g of Fresh Weight, FW) only VP2 trimers could be detected into plant cells. The protein was thus purified from the leaf extracts by Immobilized Metal Ion Affinity Chromatography (IMAC) and its immunogenicity tested by intramuscular (IM) delivery to mice (seven injections 50 µg/dose) with an adjuvant. The results of these experiments evidenced that this immunization schedule was able to induce the production of anti-VP2 antibodies. In a different work, four doses of the crude extract of *N. benthamiana* leaves transiently expressing the mature VP2 (12 µg/dose) delivered IM in SPF chickens together with Freund's adjuvant were able to induce the production of anti-VP2 antibodies. These were endowed with neutralizing activity and were able to protect the birds from the challenge with a classic viral strain as shown by the reduction in bursal lesions and T-cell infiltration compared to the negative control group (Gómez et al. 2013). In an attempt to evaluate the efficacy of the plant-produced VP2 in mucosal vaccination, intranasal and oral administration were also investigated (Lucero 2016). However, two doses of an extract containing 7.5 µg of VP2 when administrated to SPF chickens were unable to induce antibody production and protection from challenge. Conversely, the same extract, when injected 2 times IM without adjuvant, was able to induce neutralizing antibodies and to protect chickens from IBDV infection.

The *N. benthamiana* leaf extracts expressing VP2 were also evaluated as a possible boost of pre-immunized breeder hens, in alternative to the commonly used inactivated vaccines (Lucero et al. 2019). Hyper-immunization of breeder hens is a commonly adopted procedure in the laying period in order to provide passive immunity to the progeny through MDA that ensure protection in the first weeks of life. To this aim, a plant crude extract containing 30 µg of VP2

was injected IM to hens pre-immunized with the live attenuated Bursine 2 commercial vaccine and humoral response investigated in the offspring demonstrated that the plant-VP2 boost was as efficient as the inactivated vaccine. Furthermore, anti-VP2 antibodies were detected in chicks up to 3 weeks after hatching similarly to the inactivated vaccine control group. The MDA were endowed of neutralizing activity and conferred protection against the challenge as indicated by the absence of BF damage and by the low viral titers observed inside this organ. The passive immunity obtained with the plant VP2, without the need of adjuvant, was as effective as that achieved with the conventional inactivated vaccine.

The same immunogen was also tested in chickens either in a homologous or in a heterologous prime-boost immunization scheme in combination with a recombinant modified vaccinia Ankara virus carrying the VP2 mature gene (rMVA-VP2) (Richetta et al. 2017). Plant extracts containing approximately 8 µg of the VP2 protein were IM injected once (as prime or boost in combination with the rMVA-VP2) or twice in SPF chickens. All immunization schemes were able to elicit the production of significant anti-VP2 antibody titers endowed with IBDV neutralizing activity and to protect animals to virus challenge. The heterologous prime-boost immunization scheme did not show clear evidence of superiority compared to the homologous prime-boost scheme. Overall, these results demonstrate that transient expression systems allow obtaining higher yields of VP2 compared to stable expression and that either the purified antigen or the crude plant extract elicits in chickens the production of antibodies able to neutralize IBDV. It is remarkable that also when administered IM the VP2-containing plant extracts are effective even if the adjuvant is not included in the formulation. Indeed, it has been already demonstrated that the plant extract contains some components such as polysaccharides that could act as inducers of dendritic cells maturation positively modulating the immune response (Di Bonito et al. 2009; Yang et al. 2015; Feng et al. 2017).

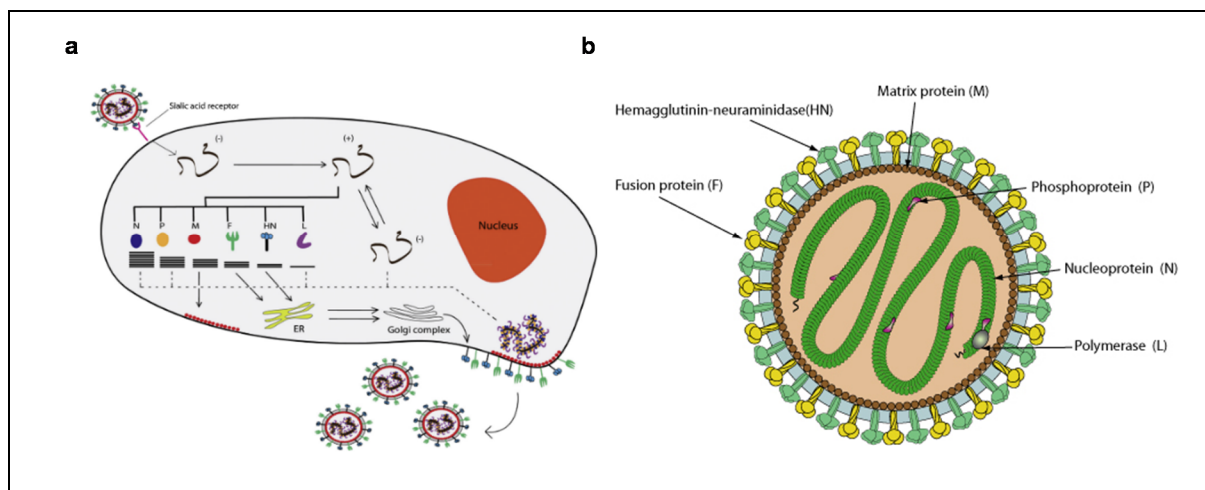
**VP2 domains fusions to immunopotentiated protein carriers**

With the aim of improving the intrinsic immunogenic properties of subunit vaccines, novel recombinant fusion molecules with enhanced immunogenic potential were obtained by conjugating VP2 antigenic portions to immunogenic carriers such as a plant virus coat protein (CP).

The first example was aimed to transiently produce the BC loop of the VP2 as a fusion with the CP of the Bamboo mosaic virus (BaMV) using a strategy known as epitope presentation (Chen et al. 2012). By using a recombinant BaMV-based viral vector, BaMV chimeric particles displaying the VP2-derived antigen on the surface were produced in plants (0.26-0.28% TSP of the chimeric CP in *Chenopodium quinoa*). SPF chickens IM injected with 600 µg of the purified chimeric virus particles together with Freund's incomplete adjuvant produced antibodies against IBDV and were protected from challenge with vvIBDV in terms of survival. This work demonstrated that chimeric plant virus particles displaying a neutralizing epitope of VP2 protect from IBDV infection and that high doses of these BaMV particles do not affect animal health.

**Newcastle Disease virus (NDV)**

NDV is a member of the genus *Avulavirus* in the family *Paramyxoviridae*. It belongs to the serotype 1 and is a pleomorphic enveloped virus with a diameter of about 200 nm. NDV genome is a negative sense (-), non-segmented, ss-RNA of 15.2 kilobases (kb). The genome is flanked at its 3'-end by a 55 nucleotides (nt) long leader sequence and at its 5'-end by a 114 nt long trailer sequence. These sequences accommodate the regulatory signals for transcription and replication (Dortmans et al. 2011). The genome is composed of six genes five of which are monocistronic and encode the nucleocapsid protein (N), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase protein (HN), and the large protein (L) that is the RdRp. The sixth gene is transcribed by RNA editing into three different mRNAs encoding the structural phosphoprotein (P) and the non-structural V and W proteins (Figure 13a) (Ganar et al. 2014). For protection against nucleases, the NDV genome is encapsidated by the N protein and forms a helical structure that is used as a template for a polymerase complex composed by the L and P proteins. The process results in the formation of a herringbone-like ribonucleoprotein complex (RNP) essential for mRNAs synthesis and for viral replication. The phosphorylated P protein helps to stabilize the polymerase L protein in the P-L complex that carries out genomic replication. The M protein is layered between the nucleocapsid and the viral membrane and interacts with both N, HN and F (Cox and Plemper 2017). The non-structural V protein modulates viral replication *via* interaction with the N protein. The non-structural W protein is produced similarly to the V protein via RNA editing but its exact role has not been established yet (Lamb and Parks 2007).



**Figure 13:** Schematic representation of (a) NDV replication (from Ganar et al. 2014) and (b) structure (image from <https://viralzone.expasy.org>)

### **Membrane glycoproteins HN and F**

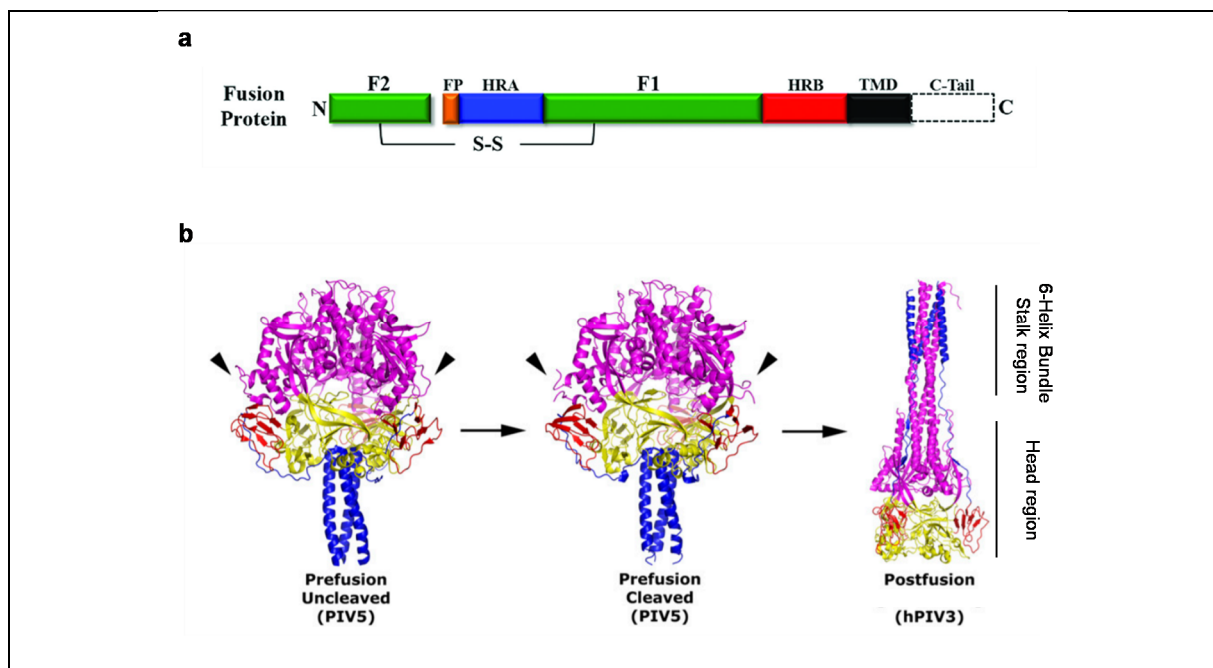
NDV is delimited by a lipid envelope derived from the host cell plasma membrane in which two glycoprotein-complexes, HN and F, are anchored and exposed on the outer surface (Figure 13b). The two complexes are required for infection, as they are responsible for the binding of the virus to the target cell and for the entry of the genomic material through the fusion of the viral envelope to the target cell membrane (Smith et al. 2009). Both are recognized by the immune system and induce the production of neutralizing antibodies (Kumar et al. 2011).

HN is assembled in a homotetramer and is endowed of two activities responsible for the attachment of the virion to the host cell (Aguilar et al. 2016). The first is the haemagglutinin activity and relates to the binding of the HN protein complex to sialic acid-containing receptors exposed on the surface of the target cell. HN binding is mediated by two sialic acid binding sites, one located on the globular head of each monomer and the second formed at the interface between two monomers and involving residues from both (Porotto et al. 2012). The neuraminidase activity relates to the removal by cleavage of sialic acid derivatives from glycoproteins on both the viral envelope and the target cell membrane. This activity prevents viral self-agglutination during budding and assists the detachment of the viral particles from the

infected cells reducing the virus re-entry (Bossart et al. 2013). The HN protein is also known to interact with the second glycoprotein inserted on the viral envelope, the F protein, and to assist its function (Jardetzky and Lamb 2014).

The F protein is involved in the fusion of the virus envelope with the plasma membrane of the host cell. It is a type I viral fusion protein and it assembles in homotrimers on the surface of the virion in a precursor form identified as F0 (Colman and Lawrence 2003). F0 is cleaved by host-derived proteases into two subunits, F1 and F2 that are linked together via a disulfide bond (Figure 14a) (Chen et al. 2001a). The F protein is generally considered as a molecular marker of NDV virulence that varies as a function of the amino acid composition of the F0 cleavage site. Virulent isolates (mesogenic and velogenic) are characterized by the presence of multiple basic amino acids (arginine and lysine) and by a phenylalanine in position 117. This feature allows the cleavage by furine-like proteases ubiquitously present in the chicken extracellular environment. On the other hand, low virulent and avirulent isolates (lentogenic) are characterized by the presence at the F cleavage site of monobasic or dibasic amino acids and by a leucine in position 117, and for this reason are preferential targets of extracellular trypsin-like proteases found mainly in the gastrointestinal and the respiratory systems. Acidic conditions are not required to proceed to the cleavage and the activation of the F protein depends only on the presence of the specific proteases (Panda et al. 2004). The viral fusion with the host cell membrane as well as the consequent tissue necrosis and virus spreading are therefore controlled only by the sensitivity of the strain to differently distributed proteases. The propagation of avirulent strains is thus limited to the respiratory and enteric tracts resulting in low viral activity whereas virulent strains are active in a wide range of environments and this results in systemic spread and acute infections. The cleavage step is a prerequisite to obtain a protein folded into a metastable pre-fusion state, biologically active and able to mediate fusion and infection (Figure 14b) (Connolly et al. 2006).

The HN protein, after binding to sialic-acid containing receptors, interacts with the F protein in its pre-fusion state and induces major irreversible conformational changes that activate the fusion. The mechanism of the interaction between the two glycoproteins is not totally understood but it is clear that it determines the refolding of the F protein and the formation of a fusion pore (Figure 14b) (Swanson et al. 2010).



**Figure 14:** Paramyxovirus F protein structure and presentation of its different conformational states

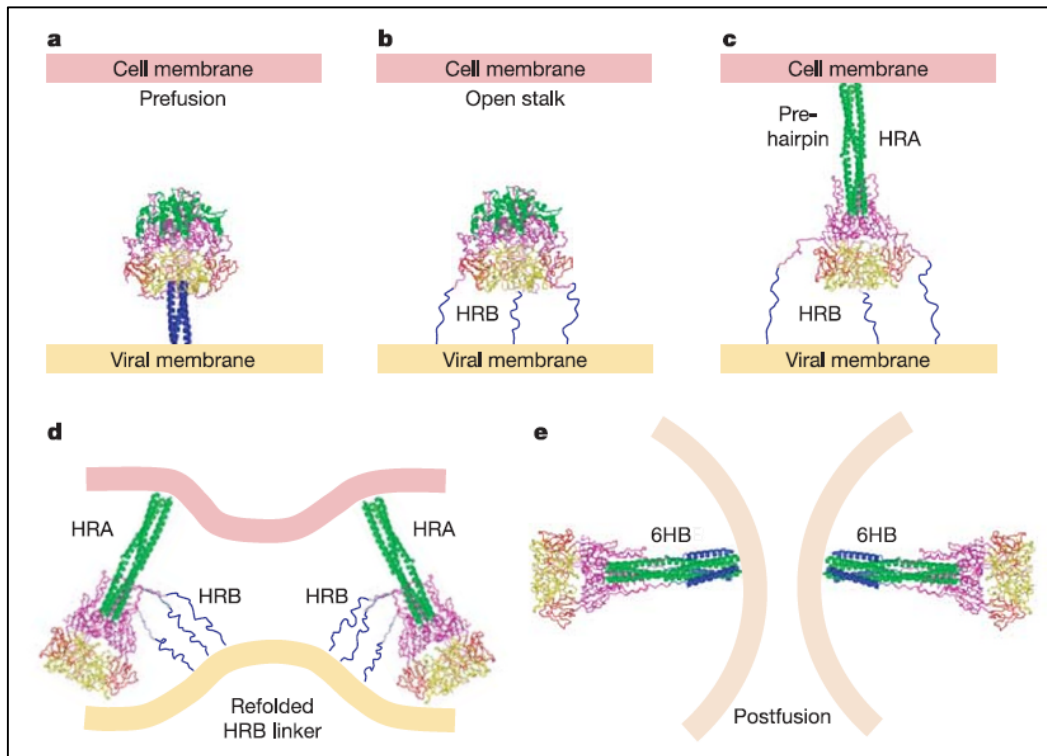
(a) Schematic representation of F protein domains (FP: fusion peptide; HRA and HRB: heptad repeat sequence A and B; TMD: transmembrane domain; C-Tail: cytoplasmic tail; S-S: disulfide bonds) (from Smith et al. 2009). (b) Structure of distinct conformational states of the F protein ectodomain (PIV5: paramyxovirus parainfluenza virus 5; hPIV3: human parainfluenza virus 3). Arrows indicate the progression of F through its various conformational states. Each structure is color-coded by domain (domains of the PIV5 protein: DI (yellow), DII (red); DIII (magenta); HRB: blue; HRA are comprised in DIII) (from Welch et al. 2012).

In details, the cleavage of F<sub>0</sub> into the F<sub>1</sub> and F<sub>2</sub> subunits generates a new N-terminus on F<sub>1</sub> characterized by a hydrophobic region known as the fusion peptide. After this structural change,



induced by the interaction with HN, the fusion peptide is repositioned, exposed and promotes protein insertion into the target membrane that leads to the fusion process (Welch et al. 2012). The fusion of the viral and cellular membranes is not a spontaneous process and the necessary energy is stored in the metastable pre-fusion F trimer and provided through the conformational changes of the protein among which binding of the internal heptad repeat sequences A (HRA) and B (HRB) (Figure 15). In the pre-fusion state, HRA are located proximal to the N-terminus of the F1 subunits and HRB are proximal to the C-terminus while during the passage to the post-fusion state they assemble and bind each other to form a 6-helix bundle. This structural modification triggers the formation of a pore through which the viral RNA genome is transferred into the host cell cytoplasm (virus entry) (Connolly et al. 2006). In the post-fusion state, the F protein homotrimer is characterized by a cytoplasmic tail, a transmembrane domain and a head region. The tail and the transmembrane regions are linked to the globular head through a helix stalk region that gives to the overall F structure a golf-tee like configuration (Chen et al. 2001b). The stalk region is formed by the 6-helix bundle composed of HRA (3 helices) and HRB (3 helices) (Swanson et al. 2010).

Logically, the pre-fusion configuration has been identified to raise the majority of the neutralizing antibodies relative to the F protein in Paramyxoviruses. However, it has been determined that the post-fusion folding conserves, even if in a lower proportion, some neutralizing epitopes (Swanson et al. 2011; McLellan et al. 2011).



**Figure 15:** Model of paramyxovirus F-mediated membrane fusion (image from Yin et al. 2006)

### The disease

Newcastle disease (ND) is one of the major viral-induced poultry diseases and has detrimental effects on avian breeding and production worldwide with tremendous economic losses (Mayers et al. 2017). The disease, that is a threat for several avian species such as chicken, turkey or pigeon, has been officially reported for the first time in 1926 in Java and in 1927 in England in Newcastle upon Tyne from where it takes its name. ND is listed by the World Organisation for Animal Health (OIE) as one of the most economically and clinically damaging poultry diseases and requires immediate notification upon recognition (OIE 2018).

The incubation period of NDV varies significantly depending on the affected species, health status of the host, environmental conditions, virus strain pathogenicity and quantity of inoculate. Under field conditions, the incubation period is 2 at 15 days long with an average of 5-6 days but it extends for some species to more than 20 days with symptoms usually appearing around 21-28 days of age. Also the symptoms depend on different factors such as pathogenicity

of the strain, type and age of the host (Lamb and Parks 2007). Thus, the clinical signs alone are not sufficient to determine the ND diagnosis during an outbreak and five different categories have been identified based on the pathogenic and clinical signs in chickens. The least severe are the asymptomatic and lentogenic forms that are associated to no or mild disease signs and respiratory symptoms in young chickens and do not induce mortality. Acute signs of diseases are only present in case of co-infection with other agents. The mesogenic form induces very low mortality rate with neurological and respiratory symptoms and sometimes a drop in egg production in laying hens. The velogenic neurotropic form exhibits respiratory and neurological clinical signs. Contaminated chickens present head twisting, tremors, torticollis, spasms and paralysis with a very high mortality rate. The velogenic viscerotropic form is characterized by a mortality rate close to 100 % associated with various symptoms such as conjunctivitis, tremors, nasal discharges, paralysis, prostration, ruffled feathers, weakness, diarrhoea. A dramatic drop in egg production occurs in case of contamination of layers and breeders. The incubation period for velogenic forms can be short with sudden death without previous observation of clinical signs (Bello et al. 2018).

NDV classification is based on *in vivo* pathogenicity tests. These tests are useful in ND diagnosis in order to rapidly and efficiently identify the NDV strains contributing to contain the infection. The most used parameters are the mean death time (MDT) and the IntraVenous Pathogenicity Index (IVPI). However, the accuracy and the reliability of these two data are not sufficient to reliably characterize NDV isolates in case of outbreaks. Therefore, the IntraCerebral Pathogenicity Index (ICPI) which is more sensitive and accurate is generally preferred to determine the virulence of an NDV strain (Dortmans et al. 2011).

A major problem relative to NDV source and transmission is the wide diversity of avian species that are often asymptotically contaminated by lentogenic isolates. This makes wild birds a reservoir of NDV as they can excrete and transmit the virus to breeding animals by shedding,

exhaled air or faeces contributing to its persistence in the farm environment (Brown and Bevins 2017). Furthermore, the persistence of NDV lentogenic isolates in poultry without clinical signs exposes to a risk of emergence of new mutated pathogenic and virulent strains. The maintenance and the continuous circulation of NDV constitute a huge threat associated with important economic losses (Gould et al. 2001).

The invisible transmission of the NDV lentogenic strains combined with the massive increase of international trade resulted in their worldwide distribution. On the contrary, velogenic isolates that appeared more recently, are still poorly distributed. They are endemic in Central and South America and for the moment only widely spread in Asia, Middle East and Africa (Ganar et al. 2014).

### **Vaccines against NDV**

Vaccination is the main strategy used in the poultry industry for the prevention and control of ND. Even if NDV is constantly evolving all the isolates are grouped into a single serotype identified as the avian paramyxoviruses type 1. This characteristic of NDV implies that, theoretically, immunity induced by a strain should be able to cross-protect poultry from challenge with any other. Since 70 years, immunity has been induced by vaccines formulated with inactivated or live-attenuated viruses (Allan et al. 1978). These types of vaccines are effective in preventing the appearance in the birds of the clinical symptoms of the disease thus reducing its economic impact. By the way, inactivated vaccines because of the low efficacy have been quickly overcome by live-attenuated vaccines. The first viral strains used for this kind of formulations were of the mesogenic type but, even if good protection was obtained, they conserved the ability to initiate the disease and cause mortality in vulnerable poultry. Nowadays, they are used only in countries where the infection is endemic and where the control of the spread of the disease is weak. Live attenuated vaccines currently available on the market

are based on lentogenic strains such as LaSota, F or Hitchner B1 (Winterfield et al. 1957). These vaccines are able to stimulate mucosal and systemic immune responses and to induce very strong protection against field challenge. However, live attenuated vaccines are mainly formulated with viral strains phylogenetically divergent from the current prevalent genotypes and, even if all the NDV isolates are grouped into the same serotype, the antigenic difference between the strains present in the vaccine and those circulating on the field results in insufficient immunity and do not prevent viral shedding (Miller et al. 2007).

The development of new vaccination technologies in the last years may result in the overcome of the drawbacks of classic vaccination methods. Last generation vaccines based on DNA (Firouzmandi et al. 2016), VLPs (Pantua et al. 2006) or viral vectors (Kumar et al. 2011) have been developed and are giving promising results. A viral-vectored vaccine against NDV is already available on the market. It is formulated with the Herpesvirus of Turkey (HVT), which is related to the Marek disease virus (MDV), the causative agent of the Marek's disease, and is genetically engineered to carry the gene encoding the whole F protein. This vaccine that is able to simultaneously confer protection against NDV and MDV is one of the most promising and effective and can be delivered *in ovo* inducing long term immunity. However, similarly to the HVT vectored vaccine against IBDV, its efficacy strictly depends on storage in liquid nitrogen and this severely prevents its wide use especially in poor countries (Palya et al. 2012).

### **Plant-based vaccines against NDV**

Several examples of production of HN and F, the most important NDV antigens, in plants are present in the literature (Thomas and Walmsley 2018). The two proteins were expressed either alone or together preferentially in their native form (*e.g.* F rather than F1 or F2) but interesting examples are also present in which smaller sequences were produced alone or fused to viral carriers. The plant-produced antigens were delivered either systemically or orally (mucosal) for

example by feeding. In the following paragraphs, an overview of the different approaches adopted is provided.

### **Expression of the HN protein**

#### *Transient expression of the whole HN*

The transient expression of HN fused to the c-Myc tag and to two different signal peptides separately, one from the HN gene and the other one from the sea anemone equistatin, was induced by agroinfiltrating *N. benthamiana* leaves and the expression levels and subcellular localisation were defined (Gómez et al. 2009). The effect of the fusion of the two different signal peptides were compared and similar levels of HN accumulation were observed in WB analysis using an anti-c-Myc monoclonal antibody. However, the addition of the KDEL peptide at the C-terminus of the HN protein targeted and retained the recombinant antigen in the ER enhancing its accumulation level.

#### *Stable expression of the whole HN*

HN has been stably expressed in potato leaves to develop an edible vaccine (Berinstein et al. 2005). The transgenic plant lines were demonstrated to correctly express the recombinant protein, at levels ranging from 0.3 to 0.6 µg/mg of TSP, by enzyme-linked immunosorbent assay (ELISA) using anti-NDV specific antibodies. Mice intraperitoneally injected four times with the TSP extracted from 20 mg of leaves and emulsified in Incomplete Freund Adjuvant (IFA) or orally immunized by feeding five times per week for approximately one month with 0.5 g of leaves were demonstrated positive for the presence in the serum of anti-NDV antibodies. In addition, in the orally immunized animals, the presence of anti-NDV IgA in the gut was also evidenced. A more accurate analysis of the mucosal immune response confirmed the presence in the intestinal tissue of plasma cells secreting the anti-NDV IgA, with an increase in the numbers of IgA+ B cells and of T cells subpopulations (CD4+, CD8+, TCR γδ+, TCRαβ+)

(Gómez et al. 2008). Potato plants were also transformed with the construct containing the KDEL peptide used to transiently express the whole HN in *N. benthamiana* (see paragraph: Transient expression of the whole HN) and that was previously determined to enhance the transient expression of the antigen. The aim of the experiment was to observe a similar improvement in case of stable expression. The results revealed that by this approach the expression was increased to 3 µg per mg of TSP (Gómez et al. 2009).

The stable expression of the HN antigen was also investigated in tobacco plants (Hahn et al. 2007). The insertion of the transgene into the plant genome was verified by PCR and Southern Blot analyses and the transcription in mRNA was validated by Northern blot. The presence of the protein was validated by WB and ELISA using anti-HN antibodies and the highest expression levels obtained were of about 0.069% TSP. Chickens were fed with 2 g of lyophilised transgenic leaves (containing approximatively 20 µg of HN) and the presence in collected sera of specific anti-NDV antibodies verified. Conversely, the nasal delivery of the protein extracts failed to induce the production of antibodies.

The HN was also expressed in *N. tabacum* cell lines (NT-I cells) generated by *A. tumefaciens* stable transformation (Mihaliak et al. 2004). The goal of this study was to determine for the first time if a vaccine antigen produced in plant cell lines may be able to provide protection from NDV infection in animals. HN expression was verified by ELISA while its functionality was tested by haemagglutination inhibition assay. Large scale antigen preparations obtained by culturing the transgenic plant cell line suspension in a bioreactor demonstrated, when injected subcutaneously two times in chickens with doses ranging between 3 and 33 mg, able to induce the production of HN specific antibodies protective against NDV challenge. This production strategy was adopted by Dow AgroSciences to develop the first plant-made vaccine approved for commercialization by the US Department of Agriculture (USDA) (Vermij and Waltz 2006).

The full length-HN protein was also expressed in the medicinal plant *Centella asiatica* transformed by particle bombardment (Lai et al. 2012b). The plants were screened by RT-PCR for the presence of HN mRNA transcripts and by Dot blot with an anti-NDV chicken serum for protein expression but the immunogenic properties of the produced protein were not tested *in vivo*.

#### *Stable expression of HN ectodomain*

The stable expression in *N. benthamiana* BY-2 cells of the ectodomain of the HN antigen (eHN) fused to the His tag was also investigated (Lai et al. 2013). The transgenic cell lines were verified to express the eHN mRNA transcripts by RT-PCR. The presence of the eHN protein was also tested by WB analysis using an anti-His-Tag antibody. The antigen was mainly accumulated in the cytosol with yields of about 0.2-0.4% TSP. Mice injected four times with 5 µg of the protein purified through the His-Tag and supplemented with IFA produced anti-NDV antibodies in the serum.

#### **Expression of the whole F protein**

In the same publication discussing HN expression (see paragraph: Stable expression of the whole HN, Berinstein et al. 2005), F has been stably expressed in potato leaves to develop an edible vaccine. The transgenic plant lines were demonstrated to express the protein at levels ranging from 0.3 to 0.6 µg/mg of TSP. Mice intraperitoneally injected four times with the TSP extracted from 20 mg of leaves and emulsified in IFA or receiving orally five doses per week, for approximately one month with, of 0.5 g leaves were demonstrated positive for the presence in the serum of anti-NDV antibodies. In addition, in the orally immunized animals, the presence of anti-NDV IgA in the gut was also demonstrated. A more accurate analysis of the mucosal immune response confirmed the presence in the intestinal tissue of plasma cells



secreting the anti-NDV IgA, with an increase in the numbers of IgA+ B cells and of T cells subpopulations (CD4+, CD8+, TCR  $\gamma\delta$ +, TCR $\alpha\beta$ +) (Gómez et al. 2008).

The F glycoprotein was also stably expressed in transgenic maize plants transformed by particle bombardment, and accumulated in maize kernels (Guerrero-Andrade et al. 2006). To this aim, the expression was driven by the maize ubiquitin promoter and the presence of the transgene was verified by Southern Blot. The F content ranged between 0.9 and 3% TSP. Chickens orally immunized once or twice with 3 g (containing 100  $\mu$ g of the F protein) or 15 g of the transgenic kernels or allowed to feed ad libitum, were positive for the presence of anti-NDV antibodies in the serum and protected from challenge with the viscerotropic NDV.

The stable expression of F was also investigated under the control of the maize ubiquitin or the rice glutelin promoters in transgenic rice (Yang et al. 2007). Twelve lines were obtained with the insertion of the transgene verified by PCR. Expression and accumulation of the protein were evidenced for both in leaves and seeds at levels ranging from 0.25 to 0.55% TSP. Mice immunized two times by intraperitoneal injections with 400  $\mu$ g of TSP from leaves or seeds supplemented with complete Freund's adjuvant (CFA) in the first dose and with IFA in the second, were demonstrated to have anti-NDV antibodies in the serum.

### **Expression of F and HN polypeptides**

In order to develop a cost-effective anti-NDV subunit vaccine in plant, a neutralizing epitope derived from F (amino acid (aa) 65-81: LLPNMPKDKEACAKAPL) and one derived from HN (aa 346-353: DEQDYQIR) were fused to the capsid protein of Cucumber Mosaic virus (CMV) (Zhao and Hammond 2005). The sequences encoding the epitopes were separately inserted as fusion with the sequence encoding the viral capsid protein (CaP) into a plasmid (pUC3M) containing a cDNA copy of the RNA 3 of CMV. A third plasmid was also prepared by inserting two times the encoding sequence of the HN epitope to obtain a duplicated version (HN2) of the

epitope as fusion to the viral CaP gene. Infectious transcripts were prepared from the three plasmids and used to inoculate *N. benthamiana* plants in order to obtain modified CMV displaying either the F epitope, the HN epitope or a polypeptide corresponding to the two HN epitopes in sequence (HN2). The transcript encoding the chimeric CMV bearing either the F or the HN epitopes induced the formation of infectious particles, even if the symptoms appeared slower than expected, differently from the transcript encoding the duplicated HN epitope (HN2) that was not infectious. However, it was observed that deletions of one to several aa of the epitopes could occur and the F epitope was not stable in successive infections. Interestingly, the HN-bearing CMV particles were recognized by an anti-NDV chicken serum and about 1.2 -1.5 mg could be purified from 3.5 or 7 g of *N. benthamiana* and *N. tabacum* leaves after a second inoculation. In a second time, a system based on a PVX recombinant vector carrying the modified CaP-encoding sequence was developed and investigated to produce CMV chimeric particles. The previous NDV epitopes were fused again to the capsid protein separately or together in a tandem HN-F (Natilla et al. 2006; Natilla and Nemchinov 2008). The viral constructs remained stable over time (up to 2 to 4 weeks) and after successive infections in *N. benthamiana* leaves. Symptoms of the PVX infection were also observed, undistinguishable from w.t. PVX 5-7 days post-inoculation. The expression of the epitopes onto the CaP was validated by WB specific anti-F or -HN epitope antibodies. Chimeric CMV particles were successfully purified to a yield ranging from 0.3 to 1.8 mg/ml by sucrose-gradient fractionation and concentration. Transmission electron microscopy revealed the correct formation of CMV VLPs indistinguishable from w.t. CMV virions. Chickens were immunized only with fractions containing the purified CMV-HN particles because they visually contained the higher quantity of VLPs compared to the other constructs and antibodies against CMV and HN were detected in sera. F and HN peptides were also investigated in a display strategy with Maize rayado fino virus (MRFV) VLPs (Natilla and Hammond 2011). The peptides were cross-linked by NHS-

PEG4-Maleimide (see paragraph: Use of PVX as a vaccine delivery platform) to purified VLPs modified to provide an anchor for chemical modifications. The resulting particles were recognized by WB analysis with anti-F and anti-HN antibodies, respectively, demonstrating the use of the platform for bioconjugation to peptides of interest and potential interest for vaccination.

Four tandem repeats of HN epitope followed by 3 tandem repeats of F epitope fused N-terminally to the His-Tag and C-terminally to the KDEL peptide were also produced in tobacco by stable transformation (Shahriari et al. 2016). The best expression levels of the fusion protein in the transgenic plants reached 0.44% of TSP. When four doses of crude leaf protein extracts prepared from the transgenic plants and containing 500 µg of TSP each, were injected in rabbits emulsified in IFA the production of NDV-specific antibodies was induced (Shahriari et al. 2019).

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## Chapter 2:

### **Functional characterization of a plant-produced Infectious Bursal Disease Virus antigen fused to the constant region of avian IgY immunoglobulins**

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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.



## 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 occurs 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 classified as classical or very virulent (vvIBDV). The vvIBDV strains, which show stronger severity of symptoms and higher mortality rates compared to classical strains, emerged in Europe and Japan at the end of the 80's. 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 auto-catalytically 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 labour-intensity and costs, while attenuated vaccines may induce bursa lesions, 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 vaccines 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. 2004; Wu et al. 2007), or transiently transformed *Nicotiana benthamiana* (Gómez et al. 2013; Richetta et al. 2017). In all 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, 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) (Yoshida et al. 2004; Tesar et al. 2008). 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% weight /volume (w/v) ratio, and centrifuged at 4,500 x 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 sera against vvIBDV. For the production of a chicken polyclonal serum against vvIBDV, formalin-inactivated bursal homogenates were inoculated in 6 weeks 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 (Figure 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- $\Omega$  (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  $\Omega$  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) harbouring 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 4,000 x g for 10 min and resuspended in leaf infiltration buffer [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM MgCl<sub>2</sub>, pH 5.8]. The *A. tumefaciens* suspensions were mixed to a final optical density at 600 nm (OD<sub>600</sub>) of 0.4 each, before being used for either pressure infiltration with a needle-less syringe (small-scale production) or vacuum infiltration (large-scale production) of *N. benthamiana* leaves. The large-scale production was made submerging 6 weeks 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 (w/v) ratio. The extracts were centrifuged at 20,000 x g for 20 min, the supernatants 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 x g for 10 min to obtain clear fluids corresponding to IF that were directly analysed.

For PD-FcY purification, leaves were ground in liquid nitrogen in the presence of 4% weight/weight (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 x 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 x g for 10 min. The supernatant was then adjusted to pH 7.5 and centrifuged again at 20,000 x 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 x g for 20 min, and the supernatant 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 x 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 extraction buffer supplemented with potassium sulfate in the case of HiTrap IgY (20 mM sodium phosphate, 0.5M potassium sulfate, pH 7.5) or in PBS in the case of the GenScript IgY resin. Extracts were then filtered through a 0.45  $\mu$ M PES membrane before purification.

In the case of affinity chromatography based on thiophilic adsorption, the clarified extract passed through the HiTrap IgY HP column (GE Healthcare, Uppsala, Sweden) following manufacturer's instructions. Proteins were eluted from the column using 20 mM sodium phosphate buffer pH 7.5, collecting 1.5 ml fractions.

In the case of affinity chromatography based on peptoid ligands that specifically bind IgY (Ligatrap<sup>TM</sup> Technologies, Raleigh, NC, USA), the clarified extract was passed through the Ligatrap IgY column 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 manufacturer's instructions. TSP content in the extracts or column eluted samples was determined by Bradford colorimetric assay (Biorad, Hercules, CA, USA).

In the case of the GenScript IgY resin protocol clarified extract was incubated with the resin (GenScript, Piscataway, NJ, USA) at r.t., for 30 min. The resin was recovered by centrifugation at 5,000 x 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 5,000 x g for 30 s.

### **SDS-PAGE and Western Blot (WB) analysis**

Proteins were separated by 10-12% SDS-PAGE under non-reducing and reducing conditions, the latter obtained by adding 3%  $\beta$ -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 Polyvinylidene Difluoride (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<sup>2</sup> 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 a 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% volume/volume (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 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%), 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  $\mu$ ZipTipC18 pipette tips (Millipore). Protein digests were analysed 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  $\times$  75  $\mu$ m internal diameter, 2- $\mu$ m particles, 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, 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,  $3 \times 10^5$  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  $\mu$ g/ml), a chicken IgY (10  $\mu$ g/ml), the partially purified PD-FcY protein (50  $\mu$ g/ml of total purified proteins containing 10  $\mu$ g/ml of PD-FcY), or a similarly processed p19 plant extract (50  $\mu$ g/ml of purified plant proteins). The  $\beta$ -galactosidase activity was measured using 130  $\mu$ l/well chlorophenolrot- $\beta$ -D-galactopyranoside (CPRG, Roche, Mannheim, Germany) as substrate, and quantified by optical density reading at 575 nm 4 h after incubation. Values of three independent assays were combined to calculate the mean  $\pm$  SD.

### **Animal studies**

Fifteen 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  $\mu$ l of partially purified PD-FcY (40  $\mu$ g of total purified proteins containing 8  $\mu$ g of PD-FcY) emulsified in 80  $\mu$ l of Montanide ISA 71 VG (Seppic, France) kindly provided by Biopharma Laboratories (Rabat, Morocco). Similarly, the second group was injected intramuscularly with 120  $\mu$ l of p19 Negative Control (40  $\mu$ g of total purified plant proteins) emulsified in 80  $\mu$ l 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 oculo-nasal 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<sub>50</sub> (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.

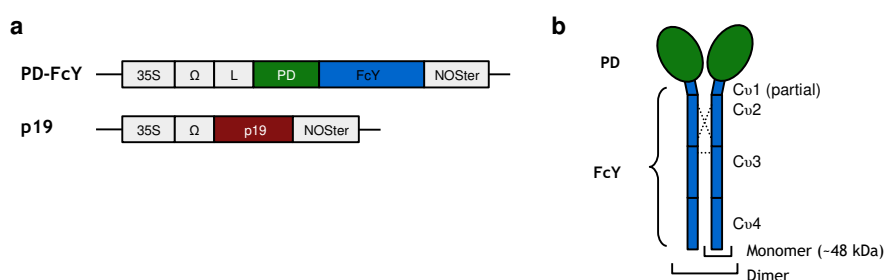
### **Statistical analysis**

Statistical analysis was performed using Student's t-test on R Software (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](http://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 (Figure 1a and b). The p19 silencing suppressor protein was used to enhance protein accumulation.

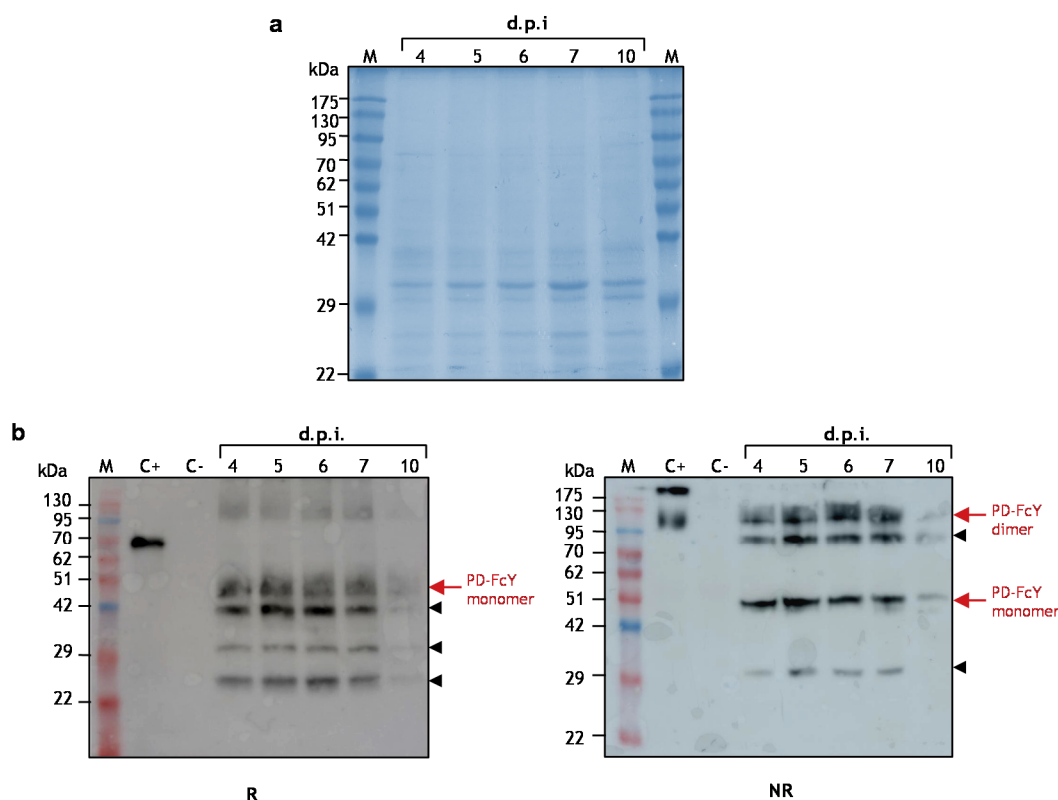


**Figure 1:** Schematic representation of plant expression constructs

(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. Cu 1→4: constant region domains of avian IgY. Dotted lines: disulphide bonds.

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 2a) was assessed by SDS-PAGE followed by WB analysis using an anti-IgY antibody (Figure 2b). 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 6 d.p.i.. Under reducing conditions (Figure 2b, 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 (Figure 2b, 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. Bands of higher molecular mass values (>100 kDa) were also present, possibly indicating the formation of multimers.

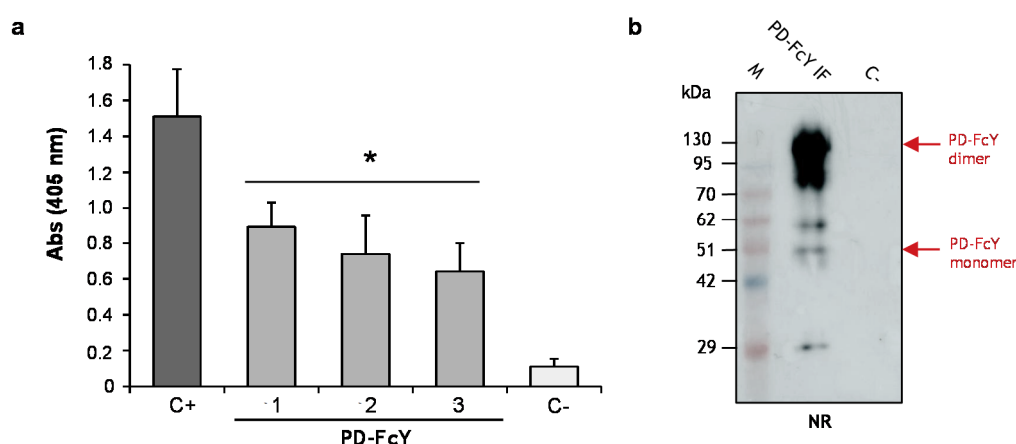


**Figure 2:** Analysis of PD-FcY expression in *N. benthamiana* leaves

(a) Coomassie staining of protein extracts prepared from PD-FcY agroinfiltrated leaves at different days post infiltration (d.p.i.) and separated by 12% SDS-PAGE under reducing conditions. Total soluble protein (TSP) content in the extracts was determined by Bradford colorimetric assay (Biorad, Hercules, CA, USA). Five  $\mu$ g of TSP were loaded in each well. M: molecular weight marker. (b) WB 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  $\mu$ g of TSP were loaded in

each well. M: molecular weight marker; C-: leaves infiltrated only with *A. tumefaciens* harbouring the p19 construct; C+: purified chicken IgY (100 ng). Black arrow heads: putative PD-FcY degradation products.

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 (Figure 3a). 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 (Figure 3b). 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).

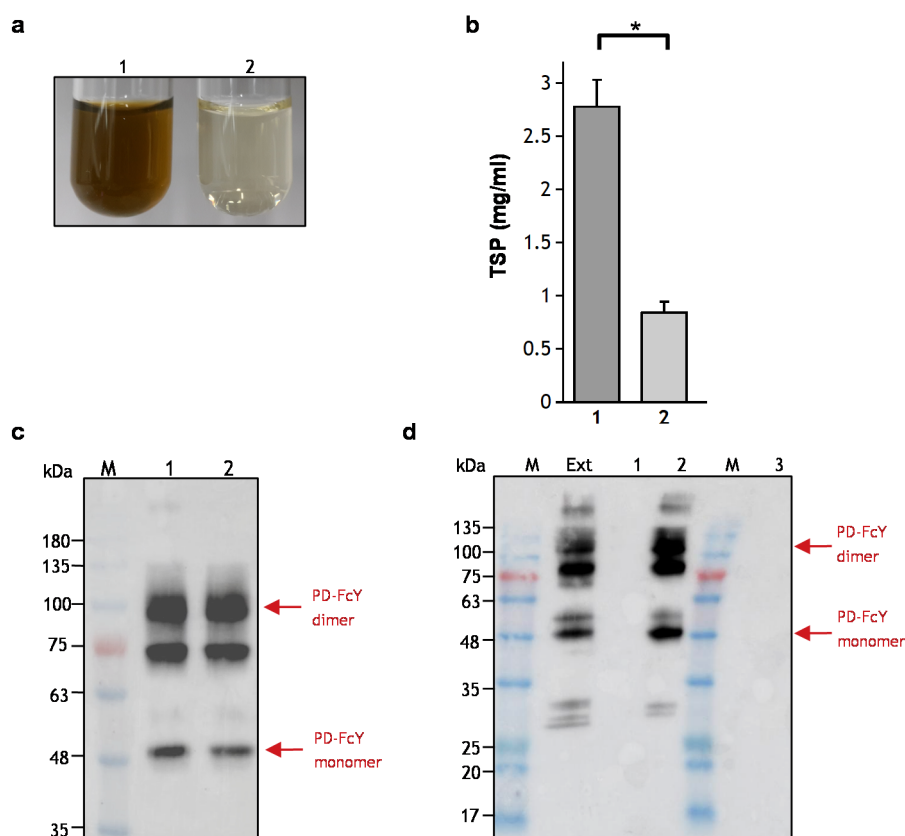


**Figure 3:** Analysis of PD-FcY expression in *N. benthamiana* leaves

**(a)** DAS ELISA of PD-FcY plant extracts. Capture antibody: chicken anti-IBDV serum. C+: inactivated IBDV virus (0.5  $\mu$ l); 1, 2, 3: PD-FcY plant extracts (100  $\mu$ l, 50  $\mu$ l, 25 $\mu$ l, respectively); C-: p19 plant extract (100  $\mu$ l). Detection antibody: rabbit anti-VP2 polyclonal antibody. Values are the mean  $\pm$  standard deviation (SD) of triplicate wells. \*: Student's t-test,  $p < 0.05$  for PD-FcY samples versus C-. **(b)** WB analysis of proteins present in the intercellular fluids (IF) of agroinfiltrated leaves at 6 d.p.i.. Twenty  $\mu$ l were separated by 10% SDS-PAGE under non-reducing conditions. C-: IF from leaves infiltrated only with *A. tumefaciens* harbouring p19 construct; PD-FcY-IF: IF from leaves infiltrated with *A. tumefaciens* strains harbouring PD-FcY and p19; M: molecular weight marker.

### **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 homogenised 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 colourless aspect (Figure 4a), the TSP content was significantly reduced (Figure 4b), while only a minor loss of PD-FcY was observed (Figure 4c). 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 4d).



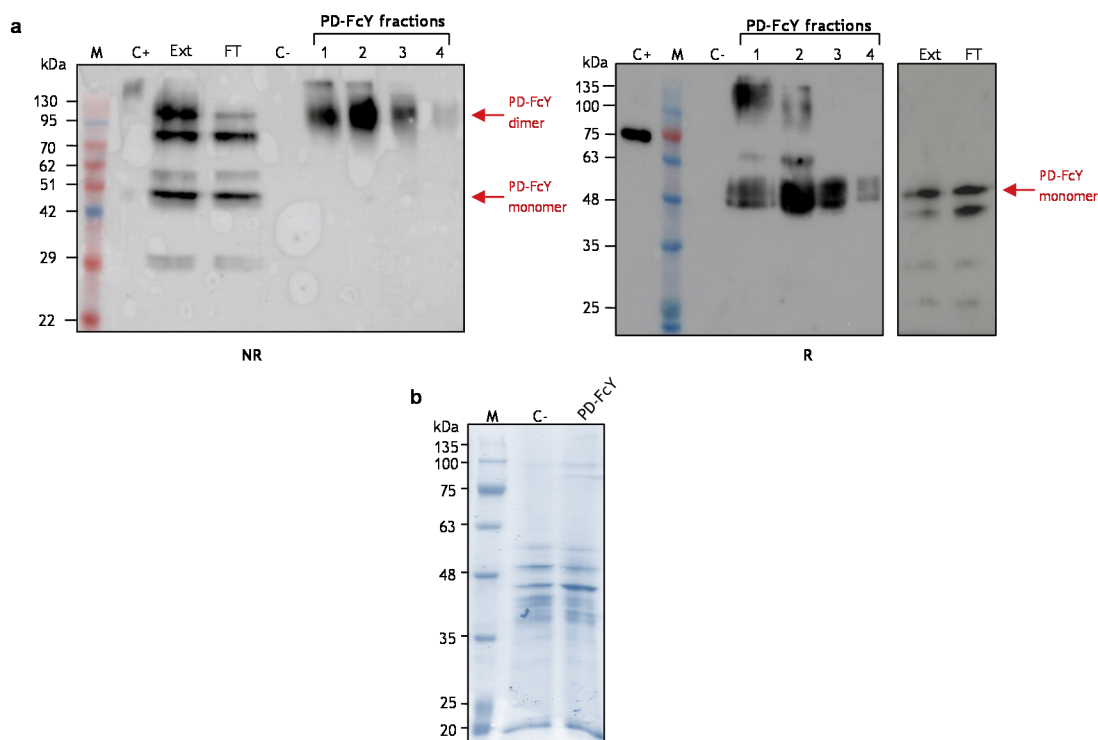
**Figure 4:** Analysis of the clarification of PD-FcY crude plant extract



Two different extraction protocols were compared in which leaves were: i) ground in liquid nitrogen and homogenized in the presence of extraction buffer (20 mM sodium phosphate) (protocol 1); ii) ground in liquid nitrogen in the presence of 4% (w/w) polyvinylpolypyrrolidone (PVPP), and further processed by adding extraction buffer supplemented with 1% (w/v) ascorbic acid and 4% (w/v) PVPP and then adjusted to pH 7.5 (protocol 2). All extracts were then clarified by centrifugation before further analysis. **(a)** Visual aspect of the plant extracts obtained with the two different protocols (1, 2). **(b)** Total soluble protein (TSP) content in the two different extracts (1, 2) as determined by Bradford colorimetric assay (Biorad, Hercules, CA, USA). Values are the mean  $\pm$  SD of triplicate wells. \*: Student's t-test,  $p < 0.05$  for sample 1 versus sample 2. **(c)** WB analysis with an anti-IgY antibody of protein extracts obtained with the two different procedures and separated by 10% SDS-PAGE under non-reducing conditions. Twenty  $\mu$ L of extract was loaded in each well. M: molecular mass marker; 1: protocol 1; 2: protocol 2. **(d)** WB analysis with an anti-IgY antibody of protein extracts obtained using protocol 2 and precipitated with ammonium sulfate. PD-FcY clarified extracts (20  $\mu$ L per well) were separated by 10% SDS-PAGE under non-reducing conditions. M: molecular mass marker; Ext: extract before ammonium sulfate precipitation; 1: Protein pellet obtained by precipitation at 0-15% saturation; 2: Protein pellet obtained by precipitation at 15-50% saturation; 3: Supernatant of the protein pellet precipitated at 50% saturation.

To purify PD-FcY from the clarified extract, three affinity-purification methods (HiTrap IgY, Ligatrap IgY and GenScript IgY) were then tested. WB analysis of the fractions eluted from the HiTrap IgY column separated under non-reducing conditions revealed the presence of two bands migrating at about 100 kDa and above (Figure 5a, left panel). Identical experiments performed under reducing conditions showed only two major bands migrating at about 100 kDa and 48 kDa, corresponding to intact forms of PD-FcY in multimeric and monomeric configurations, respectively (Figure 5a, right panel). The absence of difference in the intensity of the signal observed in the extract (Ext) and in the flow through (FT) demonstrated the very low binding efficiency of the resin. The best HiTrap IgY fraction was further characterized by SDS-PAGE performed under reducing conditions followed by Coomassie staining (Figure 5b). 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. Moreover, no bands migrating at about 48 kDa (PD-FcY monomer) were evident only in the PD-FcY sample (Figure 5b).

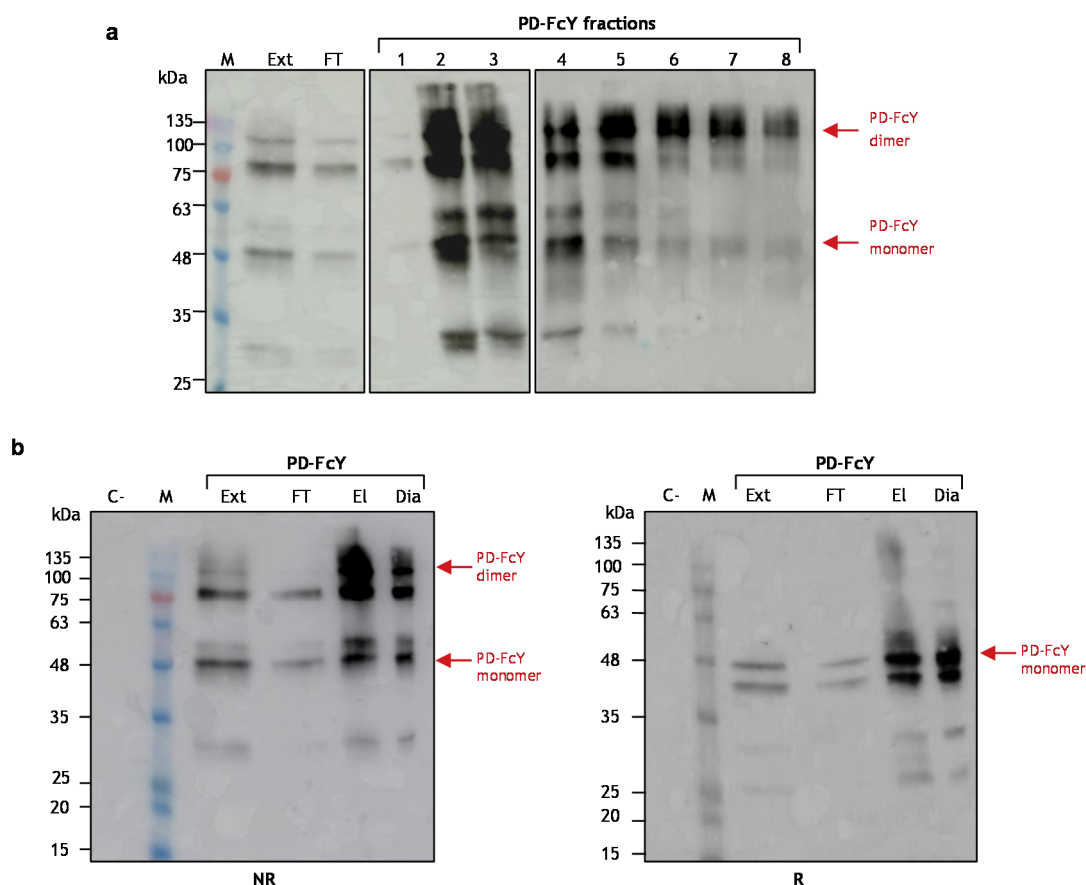


**Figure 5:** Purification of PD-FcY using HiTrap IgY affinity chromatography method

**(a)** WB analysis with an anti-IgY antibody of the fractions (1 to 4) eluted from the HiTrap IgY column. Proteins were separated by 10% SDS-PAGE under non-reducing (NR, left panel) and reducing (R, right panel) conditions. Five  $\mu$ l of each fraction were loaded per well. C+: 100 ng purified chicken IgY; M: molecular weight marker; C-: pool of the fractions (1 to 4) eluted from the HiTrap column loaded with p19 plant extracts; Ext: clarified extract (20  $\mu$ l); FT: column flow through (20  $\mu$ l). **(b)** Coomassie staining of proteins eluted from HiTrap IgY column and separated by 12% SDS-PAGE under reducing conditions. Twenty  $\mu$ l were loaded in each well. M: molecular weight marker; C-: proteins eluted from HiTrap column loaded with p19 plant extracts.

Fractions eluted from the Ligatrap IgY column containing the highest concentration of PD-FcY (fractions 2 to 7; Figure 6a) were pooled together. WB 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 (Figure 6b, left panel). Identical experiments performed

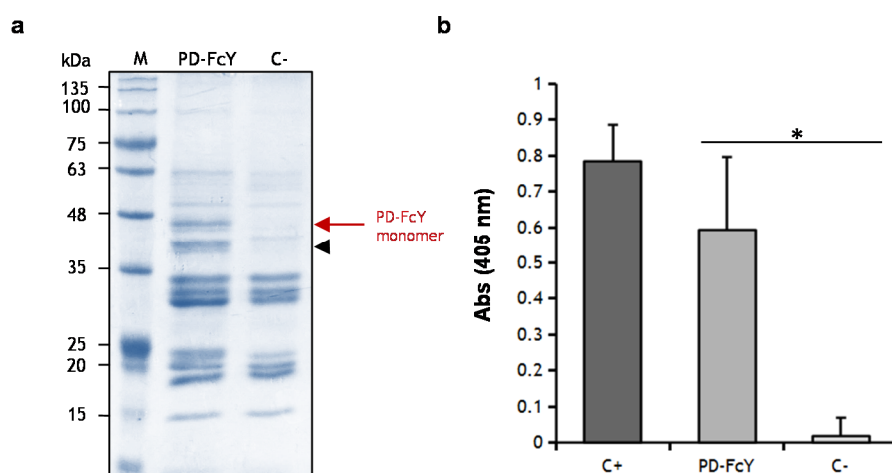
under reducing conditions showed only two major bands migrating at about 48 and 45 kDa, corresponding to intact and degraded forms of PD-FcY, respectively (Figure 6b, right panel). The difference in the intensity of the signal observed in the extract (Ext) and in the flow through (FT) demonstrated the binding efficiency of the resin. The Ligatrap IgY purified sample was then dialysed, concentrated (without significant recombinant protein loss) (Dia; Figure 6b).



**Figure 6:** Purification of PD-FcY using Ligatrap IgY affinity chromatography method

**(a)** WB analysis, with an anti-IgY antibody, of fractions eluted from Ligatrap IgY column separated by 10% SDS-PAGE under non-reducing conditions. M: molecular mass marker; Ext: plant extract (20  $\mu$ L); FT: column flow through (20  $\mu$ L); 1 to 8: eluted fractions (10  $\mu$ L). **(b)** WB analysis with an anti-IgY antibody of a pool of the fractions (2 to 7) eluted from the Ligatrap IgY column (El). Ten  $\mu$ l were 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  $\mu$ l); M: molecular mass marker; Ext: clarified extract (20  $\mu$ l); FT: column flow through (20  $\mu$ l); Dia: PBS dialysed PD-FcY eluted fractions (concentrated two times) (5  $\mu$ l).

The Ligatrap IgY dialysed sample was further characterized by SDS-PAGE performed under reducing conditions followed by Coomassie staining (Figure 7a). 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 (Figure 7a). 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. 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 and dialysed sample (Figure 7b).

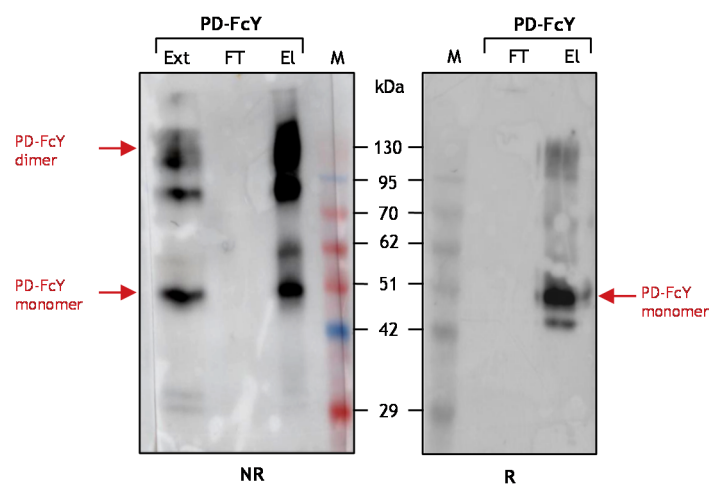


**Figure 7:** Analysis of the PD-FcY purified and dialysed sample

**(a)** Coomassie staining of proteins eluted from the Ligatrap IgY column and separated by 12% SDS-PAGE under reducing conditions. Twenty  $\mu$ l (7  $\mu$ g of total purified proteins) were 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. **(b)** Double antibody sandwich ELISA (DAS) of the Ligatrap purified PD-FcY. Capture antibody: chicken serum anti-IBDV. PD-FcY: dialysed fractions eluted from the Ligatrap IgY column (40  $\mu$ l); C-: dialysed fractions eluted from the Ligatrap IgY column loaded with the p19 plant extracts (40  $\mu$ l); C+: inactivated IBDV (5  $\mu$ l). Detection antibody: rabbit anti-VP2 polyclonal

antibody. Values are the mean  $\pm$  standard deviation (SD) of triplicate wells. \*: Student's t-test,  $p < 0.05$  for PD-FcY sample versus C-.

WB analysis of the sample eluted from the GenScript IgY resin (El; Figure 8) 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 (Figure 8). 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.

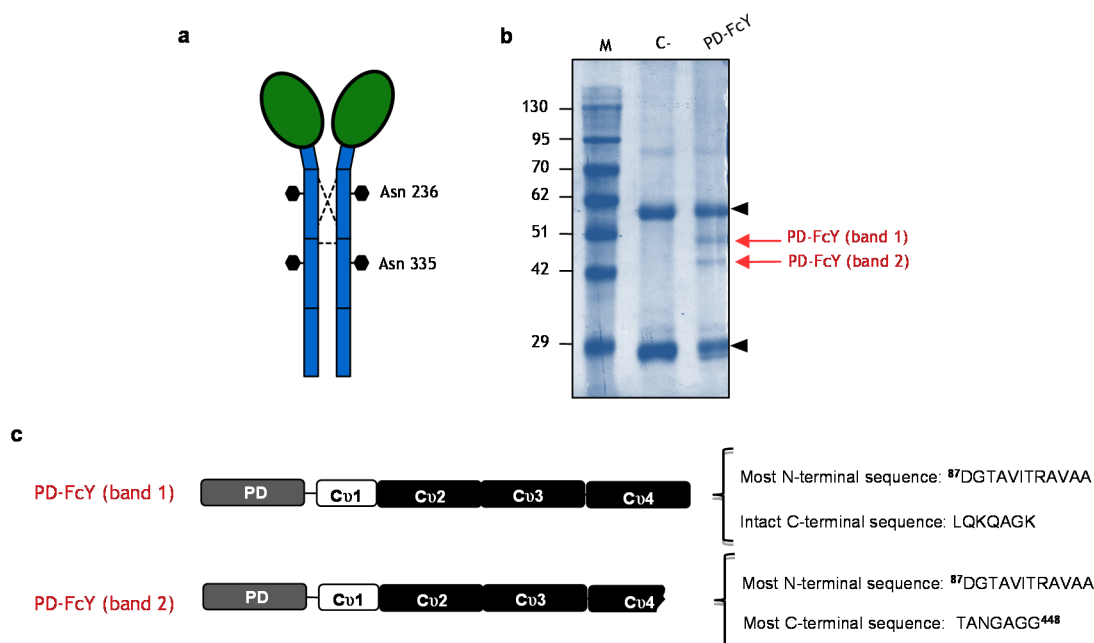


**Figure 8:** Purification of PD-FcY using GenScript IgY affinity chromatography method

WB 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  $\mu$ l); FT: resin flow through (20  $\mu$ l); El: resin elution (5  $\mu$ l).

### Proteomic and glycosylation analysis

In order to characterize the modification status of the two putative *N*-glycosylation sites occurring in PD-FcY (Figure 9a), 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 (Figure 9b), 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 9c, Annex 1), based on peptide mapping data showing the absence of specific peptides at protein C-terminus in the latter species.



**Figure 9:** 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  $\mu$ l). 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) Sequence coverage of the two PD-FcY forms (PD-FcY band 1 and band 2) from 10% SDS-PAGE as obtained by nLC-ESI-Q-Orbitrap MS/MS analysis of the endoprotease Asp-N proteolytic mixtures. Cleavage specificity at N-terminal of aspartic and glutamic residues was set as searching parameter. A schematic representation of the structural characterization results by mass spectrometry is reported. In the case of PD-FcY band 1, MS experiments identified the intact C-terminus while in PD-FcY band 2 a truncated C-terminus was found. The indicated amino acids numbering comprises the secretory signal peptide sequence.

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<sup>TM</sup> 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 C<sub>v2</sub> and C<sub>v3</sub> of the Fc part of PD-FcY, respectively (Figure 9a). Specifically, glycosylation analysis revealed the presence of the peptide SLSSRVNVSGT (Asn236) bearing plant-type *N*-linked glycostructures including  $\alpha$ -1,3-fucose (F) and  $\beta$ -1,2-xylose (X) residues, as well as of the peptide EHFNGTYSASSAVPVSTQ (Asn335), showing an uncommon plant-type glycoform with high-mannose moieties (Annex 2). A low percentage of non-glycosylated forms was 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). 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 in Table 1. 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).

| Peptide (230-240) SLSSRVWVSGT |                      |                               |                                       |                        |
|-------------------------------|----------------------|-------------------------------|---------------------------------------|------------------------|
| Glycan composition            | Glycoform            | Glycan modification mass (Da) | Observed mass (MH <sup>+</sup> ) (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 <del>M</del> GTYSASSAVPVSTQ |                                |                               |                                       |                        |
|---------------------------------------------------|--------------------------------|-------------------------------|---------------------------------------|------------------------|
| Glycan composition                                | Glycoform                      | Glycan modification mass (Da) | Observed mass (MH <sup>+</sup> ) (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                    |

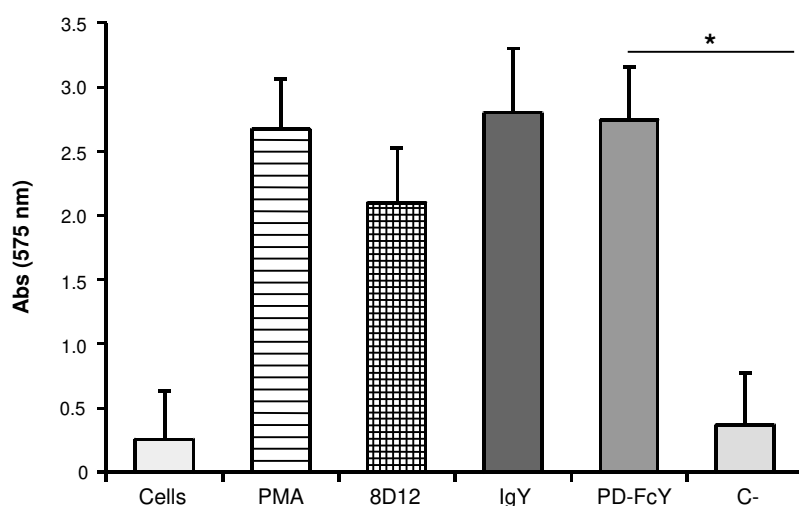
**Table 1:** Glycosylation analysis of the PD-FcY fusion protein

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].



### **PD-FcY binding to the CHIR-AB1 avian receptor**

To evaluate the binding of PD-FcY to the Fc receptor, an assay based on  $\beta$ -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  $\beta$ -galactosidase activity were observed (Figure 10). 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.



**Figure 10:** Binding of PD-FcY to the CHIR-AB1 avian receptor

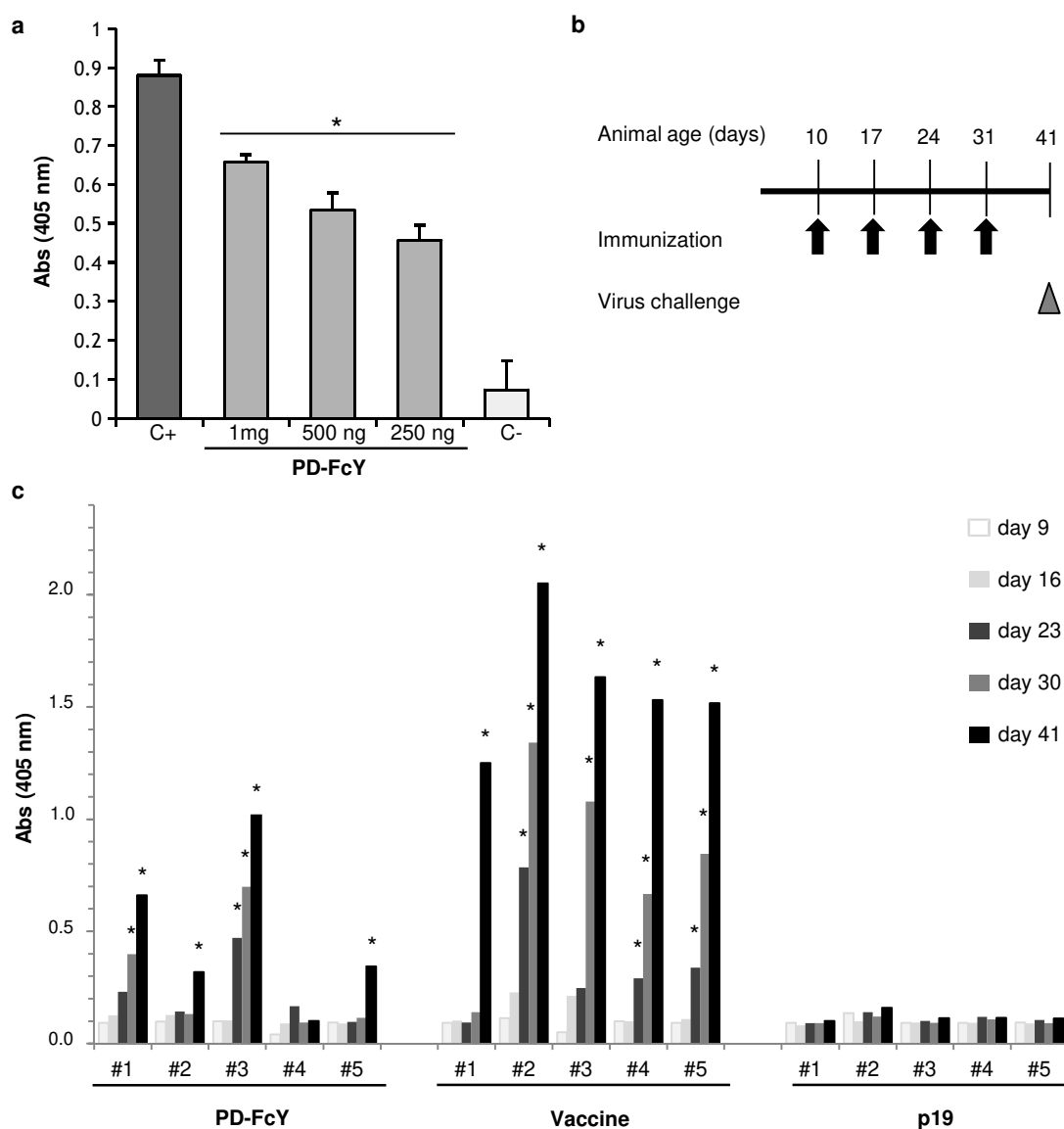
Measurement of  $\beta$ -galactosidase activity in BWZ.36-reporter cells expressing on their surface the chicken Fc receptor CHIR-AB1 stimulated with PD-FcY (PD-FcY) or the purified p19 extract (C-) used as a negative control. As positive controls, cells were stimulated with PMA, CHIR-AB1 specific mAb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

### **Immunogenicity and viral challenge studies**

To verify the antigenic potential of the plant-produced chimeric protein, an ELISA 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 (Figure 11a).

Antigenicity of partially purified plant-produced PD-FcY was further tested by intra-muscular administration to 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) (Figure 11b). Two groups of chickens were injected following the same immunization schedule with the commercial inactivated IBDV 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 one 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 to 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 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 (Figure 11c). 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 #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.



**Figure 11:** 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  $\mu$ g, 500ng and 250 ng); C-: fractions eluted from the Ligatrap IgY column loaded with the p19 plant extracts (12.5  $\mu$ l); IBDV: inactivated IBDV (1  $\mu$ l). Values are the mean  $\pm$  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 one 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 ( $OD_{405} \pm SD$  of triplicate wells for each animal. \*:  $p < 0.05$  compared to the p19 control samples (two tailed, unpaired Student's t-test and one-way ANOVA).

## 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 to traditional ones, mainly represented by their partial immunogenic and protective efficacy, and by the labour 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<sub>BC</sub> loop (portion 210-225) and the P<sub>HI</sub> 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 homologue 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 “stabilise” 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 the plant expressed PD-FcY. 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 (GenScript 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 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 characterisation 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 C $\nu$ <sub>4</sub> 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 characterised 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 C $\nu$ <sub>2</sub>) showing typical plant complex sugars, while the second (in C $\nu$ <sub>3</sub>) 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 (Suzuki and Lee 2004; Sheng et al. 2017). 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 C $\nu$ <sub>3</sub> 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 C $\epsilon$ <sub>3</sub> 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 (Purzel et al. 2009; He and Bjorkman 2011). 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 to IgY from chicken serum or to 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 to 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 (Pitcovski et al. 1999; Letzel et al. 2007), 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 to 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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**Annex 1: Identification details from Proteome Discoverer analysis of nLC-ESI-Q-Orbitrap MS/MS data from endoprotease Asp-N hydrolysis of PD-FcY products from bands 1 and 2 in 10% SDS-PAGE (Figure 9b).**

|        | Accession  | Description                    | Coverage [%]                       | # Peptides | # PSMs              | # AAs    | MW [kDa]  | calc. pI  | Score  | Mascot     | Found in Sample: [S1] F1: Sample | Found in Sample: [S2] F2: Sample |      |       |
|--------|------------|--------------------------------|------------------------------------|------------|---------------------|----------|-----------|-----------|--------|------------|----------------------------------|----------------------------------|------|-------|
|        | PD-FCY     | 496aa                          |                                    | 48         | 21                  | 90       | 496       | 53,1      | 5,68   | 2676       | High                             | High                             |      |       |
|        | Confidence | Sequence                       | Modifications                      | # PSMs     | # Missed (Theo. MH) | Found in | Found in  | Found in  | Charge | m/z [Da]   | DeltaM [ppm]                     | DeltaM/z                         | Ions | Score |
|        |            |                                |                                    |            |                     |          |           |           |        |            |                                  |                                  |      |       |
| BAND 1 | High       | DAKLRLVVNLPS                   | 1xCarbamidomethyl [C6]             | 6          | 0                   | 1484,825 | High      | High      | 2      | 742,9151   | -1,64                            | -0,00122                         | 40   |       |
|        | High       | DAKLRLVVNLPS                   | 1xCarbamidomethyl [C6]; 1xDeamidat | 3          | 0                   | 1485,809 | High      | High      | 3      | 495,94171  | 0,81                             | 0,0004                           | 38   |       |
|        | High       | DAKLRLVVNLPSDSSLSVTWTREKSGNLRP | 1xCarbamidomethyl [C6]             | 2          | 2                   | 3498,849 | High      | High      | 5      | 700,57629  | 1,09                             | 0,00076                          | 77   |       |
|        | High       | DAKLRLVVNLPSDSSLSVTWTREKSGNLRP | 1xCarbamidomethyl [C6]; 1xDeamidat | 1          | 2                   | 3499,833 | High      | Not Found | 4      | 875,71503  | 1,63                             | 0,00143                          | 30   |       |
|        | High       | DGAQSCSPIQLYAIPPSPG            | 1xCarbamidomethyl [C6]             | 1          | 0                   | 1957,932 | Not Found | High      | 2      | 979,46826  | -1,61                            | -0,00158                         | 32   |       |
|        | High       | DGTAVITRAVAA                   |                                    | 1          | 0                   | 1144,632 | High      | Not Found | 2      | 572,81879  | -1,54                            | -0,00088                         | 31   |       |
|        | High       | DGVGGLLVASQSPAVRSGYSTLSRVNVSGT |                                    | 2          | 0                   | 3108,592 | High      | High      | 3      | 1036,86926 | 0,4                              | 0,00041                          | 44   |       |
|        | High       | DHRAVPATEFVTAVLP               |                                    | 4          | 1                   | 1823,965 | High      | High      | 2      | 912,48706  | 1                                | 0,00091                          | 61   |       |
|        | High       | DHRAVPATEFVTAVLPE              |                                    | 3          | 2                   | 1953,008 | High      | High      | 2      | 977,00519  | -2,31                            | -0,00226                         | 37   |       |
|        | High       | DHRAVPATEFVTAVLPEERTANGAGG     |                                    | 7          | 3                   | 2766,381 | High      | High      | 4      | 692,349    | -2,31                            | -0,00159                         | 65   |       |
|        | High       | DHRAVPATEFVTAVLPEERTANGAGG     | 1xDeamidated [N23]                 | 6          | 3                   | 2767,365 | High      | High      | 3      | 923,12671  | 0,37                             | 0,00034                          | 69   |       |
|        | High       | DSSLSVTWTR                     |                                    | 8          | 0                   | 1151,569 | High      | High      | 2      | 576,28796  | -0,41                            | -0,00024                         | 46   |       |
|        | High       | DSSLSVTWTREKSGNLRP             |                                    | 23         | 1                   | 2033,041 | High      | High      | 3      | 678,34857  | -4,86                            | -0,00329                         | 63   |       |
|        | High       | DSSLSVTWTREKSGNLRP             | 1xDeamidated [N15]                 | 1          | 1                   | 2034,025 | High      | Not Found | 4      | 509,26334  | 3,17                             | 0,00161                          | 47   |       |
|        | High       | DSSLSVTWTREKSGNLRPDPMLVQ       | 1xOxidation [M21]                  | 1          | 2                   | 2732,367 | High      | Not Found | 2      | 1366,69165 | 3,23                             | 0,00441                          | 32   |       |
|        | High       | DWLSGERFTCTVQH                 | 1xCarbamidomethyl [C10]            | 2          | 1                   | 1735,786 | High      | High      | 2      | 868,39655  | 0,08                             | 0,00007                          | 42   |       |
|        | High       | DWLSGERFTCTVQHE                | 1xCarbamidomethyl [C10]            | 1          | 2                   | 1864,828 | High      | Not Found | 2      | 932,91748  | -0,32                            | -0,00029                         | 36   |       |
|        | High       | EALPMRFSQRTLQKQAGK             |                                    | 2          | 0                   | 2089,134 | High      | High      | 2      | 1045,07141 | 0,98                             | 0,00102                          | 36   |       |
|        | High       | EALPMRFSQRTLQKQAGK             | 1xDeamidated [Q13]; 1xOxidation [M | 1          | 0                   | 2106,112 | High      | Not Found | 3      | 702,70667  | -3,32                            | -0,00233                         | 34   |       |
|        | High       | EHFNGTYSASSAVPVSTQ             | 1xDeamidated [N4]                  | 2          | 0                   | 1882,845 | High      | High      | 2      | 941,92657  | 0,27                             | 0,00026                          | 68   |       |
|        | High       | EKSGNLRP                       |                                    | 2          | 0                   | 900,4898 | High      | High      | 2      | 450,74716  | -3,01                            | -0,00135                         | 37   |       |
|        | High       | EKSGNLRPDPMLVQ                 |                                    | 3          | 1                   | 1583,821 | High      | High      | 2      | 792,4162   | 2,6                              | 0,00206                          | 45   |       |
|        | High       | EKSGNLRPDPMLVQ                 | 1xOxidation [M11]                  | 1          | 1                   | 1599,816 | Not Found | High      | 2      | 800,41144  | -0,2                             | -0,00016                         | 35   |       |
|        | High       | EITQPITSIKLEIVTSKSGGQAG        |                                    | 1          | 2                   | 2357,292 | High      | High      | 3      | 786,43445  | -1,46                            | -0,00115                         | 52   |       |
|        | High       | ETAKWNGGTVFACMAVH              | 1xCarbamidomethyl [C13]            | 2          | 0                   | 1878,863 | High      | High      | 3      | 626,95691  | -3,39                            | -0,00213                         | 32   |       |
|        | High       | EVQVLHASSCTPSQS                | 1xCarbamidomethyl [C10]            | 1          | 0                   | 1629,754 | High      | Not Found | 2      | 815,37872  | -2,17                            | -0,00177                         | 38   |       |
|        | High       | EVQVLHASSCTPSQSESV             | 1xCarbamidomethyl [C10]            | 2          | 1                   | 1944,897 | High      | High      | 2      | 972,95239  | 0,39                             | 0,00038                          | 35   |       |
| BAND 2 | Accession  | Description                    | Coverage [%]                       | # Peptides | # PSMs              | # AAs    | MW [kDa]  | calc. pI  | Score  | Mascot     | Found in Sample: [S3] F3: Sample | Found in Sample: [S4] F4: Sample |      |       |
|        | PD-FCY     | 496aa                          |                                    | 48         | 21                  | 107      | 496       | 53,1      | 5,68   | 3280       | High                             | High                             |      |       |
|        | Confidence | Sequence                       | Modifications                      | # PSMs     | # Missed (Theo. MH) | Found in | Found in  | Found in  | Charge | m/z [Da]   | DeltaM [ppm]                     | DeltaM/z                         | Ions | Score |
|        | High       | DAKLRLVVNLPS                   | 1xCarbamidomethyl [C6]             | 8          | 0                   | 1484,825 | High      | High      | 2      | 742,91571  | -0,82                            | -0,00061                         | 47   |       |
|        | High       | DAKLRLVVNLPS                   | 1xCarbamidomethyl [C6]; 1xDeamidat | 4          | 0                   | 1485,809 | High      | High      | 3      | 495,94226  | 1,92                             | 0,00095                          | 43   |       |
|        | High       | DAKLRLVVNLPSDSSLSVTWTR         | 1xCarbamidomethyl [C6]             | 2          | 1                   | 2617,377 | High      | High      | 4      | 655,10083  | 1,85                             | 0,00121                          | 37   |       |
|        | High       | DAKLRLVVNLPSDSSLSVTWTREKSGNLRP | 1xCarbamidomethyl [C6]             | 5          | 2                   | 3498,849 | High      | High      | 5      | 700,57581  | 0,39                             | 0,00027                          | 78   |       |
|        | High       | DGAQSCSPIQLYAIPPSPG            | 1xCarbamidomethyl [C6]             | 1          | 0                   | 1957,932 | High      | Not Found | 2      | 979,46991  | 0,07                             | 0,00007                          | 39   |       |
|        | High       | DGTAVITRAVAA                   |                                    | 1          | 0                   | 1144,632 | Not Found | High      | 2      | 572,82086  | 2,08                             | 0,00119                          | 32   |       |
|        | High       | DGVGGLLVASQSPAVRSGYSTLSRVNVSGT |                                    | 2          | 0                   | 3108,592 | High      | High      | 3      | 1036,87036 | 1,46                             | 0,00151                          | 65   |       |
|        | High       | DHRAVPATEFVTAVLP               |                                    | 4          | 1                   | 1823,965 | High      | High      | 2      | 912,4881   | 2,14                             | 0,00195                          | 56   |       |
|        | High       | DHRAVPATEFVTAVLPE              |                                    | 6          | 2                   | 1953,008 | High      | High      | 2      | 977,00385  | -3,69                            | -0,0036                          | 54   |       |
|        | High       | DHRAVPATEFVTAVLPEERTANGAGG     |                                    | 4          | 3                   | 2766,381 | High      | High      | 3      | 922,79297  | -5,85                            | -0,0054                          | 61   |       |
|        | High       | DHRAVPATEFVTAVLPEERTANGAGG     | 1xDeamidated [N23]                 | 4          | 3                   | 2767,365 | High      | High      | 3      | 923,12604  | -0,36                            | -0,00033                         | 69   |       |
|        | High       | DHVGKCPDGAQSCSPIQLYAIPPSPG     | 2xCarbamidomethyl [C6; C13]        | 1          | 1                   | 2751,287 | High      | High      | 3      | 917,76978  | 3                                | 0,00275                          | 35   |       |
|        | High       | DSSLSVTWTR                     |                                    | 8          | 0                   | 1151,569 | High      | High      | 2      | 576,28894  | 1,28                             | 0,00074                          | 44   |       |
|        | High       | DSSLSVTWTREKSGNLRP             |                                    | 32         | 1                   | 2033,041 | High      | High      | 3      | 678,35229  | 0,63                             | 0,00043                          | 58   |       |
|        | High       | DSSLSVTWTREKSGNLRP             | 1xDeamidated [N15]                 | 4          | 1                   | 2034,025 | High      | High      | 3      | 678,68195  | 3,06                             | 0,00208                          | 54   |       |
|        | High       | DWLSGERFTCTVQH                 | 1xCarbamidomethyl [C10]            | 2          | 1                   | 1735,786 | High      | High      | 2      | 868,39545  | -1,19                            | -0,00103                         | 54   |       |
|        | High       | DWLSGERFTCTVQHE                | 1xCarbamidomethyl [C10]            | 2          | 2                   | 1864,828 | High      | High      | 2      | 932,9176   | -0,18                            | -0,00017                         | 39   |       |
|        | High       | EELPLPLSKSVYRNTGPTTPLIYFAPHPE  |                                    | 2          | 2                   | 3460,815 | High      | High      | 4      | 865,95709  | -2,48                            | -0,00214                         | 48   |       |
|        | High       | EELPLPLSKSVYRNTGPTTPLIYFAPHPE  | 1xDeamidated [N14]                 | 1          | 2                   | 3461,799 | Not Found | High      | 4      | 866,20532  | 0,09                             | 0,00008                          | 47   |       |
|        | High       | EHFNGTYSASSAVPVSTQ             | 1xDeamidated [N4]                  | 2          | 0                   | 1882,845 | High      | High      | 2      | 941,927    | 0,72                             | 0,00068                          | 63   |       |
|        | High       | EITQPITSIKLEIVTSKSGGQAG        |                                    | 2          | 1                   | 2357,292 | High      | High      | 3      | 786,43445  | -1,46                            | -0,00115                         | 48   |       |
|        | High       | EKSGNLRP                       |                                    | 2          | 0                   | 900,4898 | High      | High      | 2      | 450,74646  | -4,57                            | -0,00206                         | 33   |       |
|        | High       | EKSGNLRPDPMLVQ                 |                                    | 3          | 1                   | 1583,821 | High      | High      | 2      | 792,41498  | 1,06                             | 0,00084                          | 40   |       |
|        | High       | EVQVLHASSCTPSQS                | 1xCarbamidomethyl [C10]            | 2          | 0                   | 1629,754 | High      | High      | 2      | 815,38055  | 0,07                             | 0,00006                          | 64   |       |
|        | High       | EVQVLHASSCTPSQSESV             | 1xCarbamidomethyl [C10]            | 2          | 1                   | 1944,897 | High      | High      | 2      | 972,95099  | -1,05                            | -0,00102                         | 68   |       |

| Query #z | Protein Rank | Peptide                          | Glycans                       | Modification Type(s) | Observed m/z | z | Observed (M+H) | Calc. mass (M+H) | Mass error (ppm) | Starting position | Score | Protein Name  | Protein DB number | Comment                                        | Scan #                           | Scan Time |
|----------|--------------|----------------------------------|-------------------------------|----------------------|--------------|---|----------------|------------------|------------------|-------------------|-------|---------------|-------------------|------------------------------------------------|----------------------------------|-----------|
| 02789:2  | 1            | Y.SLSSRVNVSGT.D                  |                               |                      | 553,794      | 2 | 1106,580       | 1106,580         | 0,3              | 230               | 316,8 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6871.6871.2       | controllerType=0 controllerNumbr | 32,7      |
| 02787:2  | 1            | Y.SLSSRVNVSGT.D                  |                               |                      | 553,794      | 2 | 1106,580       | 1106,580         | -0,1             | 230               | 337,2 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.6888 | controllerType=0 controllerNumbr | 32,7      |
| 02672:2  | 1            | Y.SLSSRVN[+203.079]VSGT.D        | HexNAc(1)                     | N[+203]              | 655,332      | 2 | 1309,656       | 1309,659         | -2,5             | 230               | 398,5 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6787.6787.2       | controllerType=0 controllerNumbr | 32,5      |
| 02684:2  | 1            | Y.SLSSRVN[+203.079]VSGT.D        | HexNAc(1)                     | N[+203]              | 655,332      | 2 | 1309,657       | 1309,659         | -1,8             | 230               | 340,6 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.681  | controllerType=0 controllerNumbr | 32,5      |
| 02566:2  | 1            | Y.SLSSRVN[+1038.375]VSGT.D       | HexNAc(2)Hex(3)Fuc(1)         | N[+1038]             | 1072,979     | 2 | 2144,951       | 2144,955         | -2,0             | 230               | 172,6 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6696.6696.2       | controllerType=0 controllerNumbr | 32,3      |
| 02589:2  | 1            | Y.SLSSRVN[+1038.375]VSGT.D       | HexNAc(2)Hex(3)Fuc(1)         | N[+1038]             | 1072,979     | 2 | 2144,951       | 2144,955         | -1,9             | 230               | 168,9 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.672  | controllerType=0 controllerNumbr | 32,3      |
| 02517:2  | 1            | Y.SLSSRVN[+1170.417]VSGT.D       | HexNAc(2)Hex(3)Fuc(1)Pent(1)  | N[+1170]             | 1139,003     | 2 | 2276,999       | 2276,997         | 0,9              | 230               | 382,8 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6653.6653.2       | controllerType=0 controllerNumbr | 32,2      |
| 02525:2  | 1            | Y.SLSSRVN[+1170.417]VSGT.D       | HexNAc(2)Hex(3)Fuc(1)Pent(1)  | N[+1170]             | 1139,001     | 2 | 2276,994       | 2276,997         | -1,4             | 230               | 423,9 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.667  | controllerType=0 controllerNumbr | 32,2      |
| 02576:2  | 1            | Y.SLSSRVN[+1227.439]VSGT.D       | HexNAc(3)Hex(3)Pent(1)        | N[+1227]             | 1167,514     | 2 | 2334,020       | 2334,019         | 0,6              | 230               | 158,2 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6703.6703.2       | controllerType=0 controllerNumbr | 32,3      |
| 02550:2  | 1            | Y.SLSSRVN[+1241.454]VSGT.D       | HexNAc(3)Hex(3)Fuc(1)         | N[+1241]             | 1174,520     | 2 | 2348,033       | 2348,035         | -0,8             | 230               | 160,8 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6682.6682.2       | controllerType=0 controllerNumbr | 32,2      |
| 02563:2  | 1            | Y.SLSSRVN[+1241.454]VSGT.D       | HexNAc(3)Hex(3)Fuc(1)         | N[+1241]             | 1174,519     | 2 | 2348,030       | 2348,035         | -1,8             | 230               | 195,3 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.670  | controllerType=0 controllerNumbr | 32,3      |
| 02489:3  | 1            | Y.SLSSRVN[+1373.497]VSGT.D       | HexNAc(3)Hex(3)Fuc(1)Pent(1)  | N[+1373]             | 827,364      | 3 | 2480,077       | 2480,077         | -0,1             | 230               | 261,5 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6626.6626.3       | controllerType=0 controllerNumbr | 32,1      |
| 02488:3  | 1            | Y.SLSSRVN[+1373.497]VSGT.D       | HexNAc(3)Hex(3)Fuc(1)Pent(1)  | N[+1373]             | 827,363      | 3 | 2480,073       | 2480,077         | -1,4             | 230               | 339,2 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.665  | controllerType=0 controllerNumbr | 32,1      |
| 02481:2  | 1            | Y.SLSSRVN[+1576.576]VSGT.D       | HexNAc(4)Hex(3)Fuc(1)Pent(1)  | N[+1577]             | 1342,080     | 2 | 2683,153       | 2683,156         | -1,2             | 230               | 225,4 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.6620.6620.2       | controllerType=0 controllerNumbr | 32,1      |
| 02471:3  | 1            | Y.SLSSRVN[+1576.576]VSGT.D       | HexNAc(4)Hex(3)Fuc(1)Pent(1)  | N[+1577]             | 895,054      | 3 | 2683,148       | 2683,156         | -2,9             | 230               | 154,8 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.663  | controllerType=0 controllerNumbr | 32,1      |
| 06524:2  | 1            | Q.EHFN[+0.984]GTYSASSAVPVSTQ.    |                               | N[+1]                | 941,928      | 2 | 1882,849       | 1882,845         | 1,9              | 332               | 471,7 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.9842.9842.2       | controllerType=0 controllerNumbr | 39,7      |
| 06509:2  | 1            | Q.EHFN[+0.984]GTYSASSAVPVSTQ.    |                               | N[+1]                | 941,927      | 2 | 1882,846       | 1882,845         | 0,4              | 332               | 560,2 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.985  | controllerType=0 controllerNumbr | 39,8      |
| 05634:3  | 1            | Q.EHFN[+203.079]GTYSASSAVPVSTQ.  | HexNAc(1)                     | N[+203]              | 695,651      | 3 | 2084,939       | 2084,941         | -1,0             | 332               | 433,6 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.9127.9127.3       | controllerType=0 controllerNumbr | 38,0      |
| 05545:3  | 1            | Q.EHFN[+203.079]GTYSASSAVPVSTQ.  | HexNAc(1)                     | N[+203]              | 695,652      | 3 | 2084,940       | 2084,941         | -0,3             | 332               | 391,6 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.909  | controllerType=0 controllerNumbr | 38,0      |
| 04987:2  | 1            | Q.EHFN[+203.079]GTYSASSAVPVSTQ.  | HexNAc(1)                     | N[+203]              | 1042,976     | 2 | 2084,944       | 2084,941         | 1,5              | 332               | 459,0 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.865  | controllerType=0 controllerNumbr | 36,9      |
| 05527:2  | 1            | Q.EHFN[+203.079]GTYSASSAVPVSTQ.  | HexNAc(1)                     | N[+203]              | 1042,976     | 2 | 2084,945       | 2084,941         | 1,9              | 332               | 449,1 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.908  | controllerType=0 controllerNumbr | 37,9      |
| 05038:3  | 1            | Q.EHFN[+1540.529]GTYSASSAVPVSTQ. | HexNAc(2)Hex(7)               | N[+1541]             | 1141,464     | 3 | 3422,377       | 3422,390         | -3,8             | 332               | 246,0 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.869  | controllerType=0 controllerNumbr | 37,0      |
| 05070:3  | 1            | Q.EHFN[+1702.581]GTYSASSAVPVSTQ. | HexNAc(2)Hex(8)               | N[+1703]             | 1195,485     | 3 | 3584,440       | 3584,443         | -0,9             | 332               | 343,4 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8680.8680.3       | controllerType=0 controllerNumbr | 37,0      |
| 05011:3  | 1            | Q.EHFN[+1702.581]GTYSASSAVPVSTQ. | HexNAc(2)Hex(8)               | N[+1703]             | 1195,487     | 3 | 3584,448       | 3584,443         | 1,4              | 332               | 350,6 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.867  | controllerType=0 controllerNumbr | 37,0      |
| 05186:3  | 1            | Q.EHFN[+1864.634]GTYSASSAVPVSTQ. | HexNAc(2)Hex(9)               | N[+1865]             | 1249,506     | 3 | 3746,503       | 3746,496         | 2,0              | 332               | 263,0 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8782.8782.3       | controllerType=0 controllerNumbr | 37,2      |
| 04892:3  | 1            | Q.EHFN[+2026.687]GTYSASSAVPVSTQ. | HexNAc(2)Hex(10)              | N[+2027]             | 1303,523     | 3 | 3908,556       | 3908,548         | 1,9              | 332               | 220,9 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8545.8545.3       | controllerType=0 controllerNumbr | 36,7      |
| 05073:4  | 1            | Q.EHFN[+2026.687]GTYSASSAVPVSTQ. | HexNAc(2)Hex(10)              | N[+2027]             | 977,892      | 4 | 3908,544       | 3908,548         | -1,1             | 332               | 298,1 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8684.8684.4       | controllerType=0 controllerNumbr | 37,0      |
| 05161:3  | 1            | Q.EHFN[+2026.687]GTYSASSAVPVSTQ. | HexNAc(2)Hex(10)              | N[+2027]             | 1303,519     | 3 | 3908,542       | 3908,548         | -1,5             | 332               | 300,7 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8761.8761.3       | controllerType=0 controllerNumbr | 37,2      |
| 05075:3  | 1            | Q.EHFN[+2026.687]GTYSASSAVPVSTQ. | HexNAc(2)Hex(10)              | N[+2027]             | 1303,520     | 3 | 3908,547       | 3908,548         | -0,4             | 332               | 334,0 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.873  | controllerType=0 controllerNumbr | 37,1      |
| 05000:4  | 1            | Q.EHFN[+2026.687]GTYSASSAVPVSTQ. | HexNAc(2)Hex(10)              | N[+2027]             | 977,893      | 4 | 3908,550       | 3908,548         | 0,4              | 332               | 214,3 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2_180420061549.866  | controllerType=0 controllerNumbr | 36,9      |
| 05018:3  | 1            | Q.EHFN[+2188.740]GTYSASSAVPVSTQ. | HexNAc(2)Hex(11)              | N[+2189]             | 1357,539     | 3 | 4070,602       | 4070,601         | 0,3              | 332               | 213,4 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8640.8640.3       | controllerType=0 controllerNumbr | 36,9      |
| 05017:3  | 1            | Q.EHFN[+2204.772]GTYSASSAVPVSTQ. | HexNAc(4)Hex(5)Fuc(0)NeuAc(2) | N[+2205]             | 1363,217     | 3 | 4087,635       | 4086,634         | -0,5             | 332               | 292,9 | >PD-FCY 496aa | 1762              | PDFCY-extrZT-Aprile18_Banda2.8639.8639.3       | controllerType=0 controllerNumbr | 36,9      |

**Annex 2:** Details of mass spectrometry identification of glycopeptide (230-240) SLSSRVNVSGT (Asn236) and glycopeptide (332-349) EHFNGTYSASSAVPVSTQ (Asn335) from intact PD-FcY, as obtained through Bionic search of nLC-ESI-Q-Orbitrap MS/MS data of the corresponding endoprotease Asp-N digest.



## Chapter 3:

### **Production in hairy root cultures of a chimeric antigen for veterinary applications**

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## Abstract

Hairy roots (HR) are a promising plant tissue-based platform for the production of biopharmaceuticals based on the possibility to induce the secretion of recombinant proteins in the culture medium, thus simplifying the purification and recovery steps. In this study, *Nicotiana benthamiana* and *Brassica rapa* subsp. *rapa* (turnip) HR cultures were generated to produce the secretory version of an infectious bursal disease virus chimeric antigen (PD-FcY) of interest for the formulation of poultry vaccines, with the aim of optimizing protein production and purification. Recombinant protein accumulation kinetics in the medium after induction with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), whose effects were still poorly characterized, was firstly investigated using a HR clone expressing the red fluorescent protein. The results demonstrated that HR cultures induced once with 2,4-D and grown 21 days efficiently secrete the protein and, even after replacement of the growth medium with a hormone-free counterpart, maintain the same secretion rate for several days. This protocol applied to HR cultures expressing PD-FcY resulted, for *N. benthamiana*, in similar accumulation kinetics and allowed to obtain protein recovery concentrations of ~4 mg/l. Conversely, no accumulation was observed for *B. rapa* HR cultures that were discontinued. A downstream processing procedure consisting in recombinant product concentration through ultrafiltration followed by two sequential chromatographic steps allowed to obtain final yields of 0.8 mg/l of purified PD-FcY. The HR-derived product showed a similar glycosylation pattern compared to the counterpart obtained from agroinfiltrated *N. benthamiana*, but a higher quality in terms of purity and protein degradation. In conclusion, the proposed expression and purification strategy holds promises for the development of an innovative platform to produce low-cost subunit vaccines.

## Introduction

Conventional veterinary vaccines are commonly based on inactivated and live-attenuated viruses. Problems with these vaccines may derive from adverse events in which their use can be associated with a reversion to virulence or can induce the emergence of new pathogen strains (He et al. 2009). Recombinant subunit vaccines may represent a promising alternative to above-mentioned preparations, but in many cases the high costs hinder their diffusion. The use of plants as biofactories of recombinant proteins, including antigens for vaccine formulations, may offer important advantages in terms of safety and costs, as compared to more conventional systems (Liew and Hair-Bejo 2015). Currently available plant-based production platforms of veterinary vaccines are mainly represented by stable and transient expression strategies (Sheshukova et al. 2016). In stable transformation, the gene is inserted in the nuclear or in the plastid genome, and then transmitted to the progeny; for this reason, it is necessary to consider the safety issues associated with the growth of plants producing a pharmaceutical protein in open field. On the other hand, this approach allows targeting the expression of the recombinant product to specific organs, such as seeds, opening the possibility to orally deliver the antigen; this opportunity may be of particular interest in the case of veterinary vaccination. Transient expression systems require the antigen production in contained greenhouses but allow to produce high yields of the protein of interest in a short period of time (weeks) and the exogenous sequence is also not inherited by the offspring. In this context, the choice of the production platform is determined by different factors, such as the intended antigen delivery route (*e.g.* oral) or the need to quickly produce the vaccine (*e.g.* during pandemic events) (D'Aoust et al. 2010; Kolotilin et al. 2014). Also economic aspects are crucial, and the choice of the platform may determine the cost and competitiveness of the product on the market (Schillberg et al. 2019).

Along with the production of vaccines in whole plants, *in vitro* cultures of plant cells and tissues

may also be attractive as they ensure homogeneity of the final product and easier downstream purification processes (Schillberg et al. 2013). Since many years, plant cell suspensions as well as plant tissue cultures are exploited for the production of valuable secondary metabolites; recently, they have also been used for the production of heterologous proteins (Xu and Zhang 2014). For this type of application, the most used systems are cell suspensions derived from different plant species, such as *Nicotiana tabacum* (tobacco) or *Daucus carota* (carrot), and tobacco hairy root (HR) cultures, in which the heterologous gene is inserted into the nuclear genome exploiting *Agrobacterium tumefaciens* or *Rhizobium rhizogenes*, respectively (Donini and Marusic 2018). These systems offer several advantages such as the ability to propagate indefinitely without the need for sexual reproduction and the possibility to accumulate the protein of interest in the culture medium, facilitating the corresponding purification process.

HR cultures appear to be particularly well-suited for their genetic stability, the possibility to be grown under contained conditions, and the rapidity of proliferation in simple and easily-scalable experimental conditions (Hu and Du 2006). Probably the most interesting feature of HR cultures is represented by the possibility to induce the secretion of the heterologous functional protein in the culture medium by using hormones, which is a major advantage in the downstream processes facilitating recombinant product recovery while decreasing costs (Doran 2013). However, there are still some aspects of this platform to be improved, such as the secretion efficiency and the yield of the heterologous protein production as well as the definition of experimental conditions for large-scale production in bioreactors. In this context, different heterologous proteins have been successfully produced in HR cultures, among which antibodies (Lonoce et al. 2016), tissue plasminogen activator (t-PA) (Kim et al. 2012), murine cytokine interleukin-12 (Liu et al. 2009) and antigens for the formulation of human vaccines (Skarjinskaia et al. 2013). Nonetheless, only two examples of veterinary vaccines produced with HR cultures are present in the literature; in both cases, only the accumulation of the

recombinant antigen inside the roots was demonstrated (Gao et al. 2015; Singh et al. 2015).

The aim of the present work was to develop *N. benthamiana* and *B. rapa* subsp. *rapa* HR cultures stably expressing and secreting in the culture medium the chimeric antigen PD-FcY. This fusion construct contains the immunodominant projection domain (PD) of the VP2 envelope protein of infectious bursal disease virus (IBDV) and the crystallisable fragment of a chicken immunoglobulin Y (FcY), which is used to potentiate the immune response against the antigen and as a stabilizing partner to be exploited also as tag for the corresponding purification process. PD-FcY was previously expressed transiently in *N. benthamiana*, purified and tested in chicken, obtaining promising results concerning its immunogenicity (Rage et al. 2019). However, the final product was in part degraded and contained several unwanted plant protein contaminants. The HR culture production platform was here used, and a dedicated protocol was developed in order to optimize the accumulation of the intact chimeric antigen into the culture medium. Moreover, a downstream process was defined allowing to obtain a final product with improved quality.

## Materials and methods

### Plant-expression constructs

The pBI- $\Omega$ -PD-FcY plasmid (Rage et al. 2019) was designed to carry the *pd-fcy* encoding sequence (GenBank Accession Number MK908400) fused to 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 pBI- $\Omega$ -RFP plasmid (Lonoce et al. 2019) was designed to carry the *rfp* encoding sequence fused to the sequence encoding the apoplast secretory signal peptide from phaseolin (SP) (Hunter et al. 2007). In both plasmids, gene expression was under the control of the constitutive cauliflower mosaic virus 35S promoter (35S), the  $\Omega$  translational enhancer sequence from tobacco mosaic virus and the *A. tumefaciens* terminator sequence of the *nopaline synthase* gene (NOSter) (Figure 1).

### Generation of HR clones

*R. rhizogenes* (A4) (ATCC<sup>®</sup> 43057<sup>™</sup>, Manassas, VA, USA) electroporated with the pBI- $\Omega$ -PD-FcY was grown in 50 ml of Yeast Extract Beef medium (YEB) supplemented with 50 mg/l kanamycin. The bacterial culture was centrifuged at 3,000 x *g* for 10 min and the pellet resuspended to a final optical density at 600 nm (OD<sub>600</sub>) of 1 in Murashige and Skoog medium (MS) supplemented with 3% weight/volume (w/v) sucrose (MS/sucr) and 100  $\mu$ M 4'-hydroxy-3',5'-dimethoxyacetophenone, pH 5.8.

Wild type (w.t.) *N. benthamiana* leaves were sterilized by incubation in 0.5% sodium hypochlorite, for 10 min, followed by 4 washes in sterile water. The leaves were then cut into 1x1 cm squares, wounded on the abaxial side with a needle tip, and immersed into the *R. rhizogenes* suspension for 30 min, under swirling, in the dark. The fragments were then dried and positioned with the abaxial side upwards on Petri dishes containing MS agar medium



supplemented with 2 mM 2-(N-morpholino) ethanesulfonic acid (MES), 3% (w/v) sucrose, 300 mg/l each of kanamycin and cefotaxime (MS/agar/Kan/Cef), and transferred on to fresh substrate every two weeks.

*B. rapa subsp. rapa* (turnip) seeds were sterilized by 10 min incubation in 70% volume/volume (v/v) ethanol followed by 2 min incubation in 5% (w/v) sodium hypochlorite before 2 washes of 10 min in sterile water. Seeds were grown on solid medium ( $\frac{1}{2}$ MS, 1% (w/v) sucrose and 2 mM MES, pH 5.7) for 10 days at 25 °C with 16 h light and 8 h dark. Plant stems were then wounded with a sterile needle previously submerged in the *R. rhizogenes* suspension and incubated in the same conditions for 1 week.

HR clones eventually emerging from the leaves (*N. benthamiana*) or from hypocotyls (*B. rapa*) were excised and transferred singularly on fresh MS/agar/Kan/Cef (*N. benthamiana*) or  $\frac{1}{2}$ MS/agar/Kan/Cef (*B. rapa*) plates. In this case, the transfer was made every two weeks on fresh medium in which the concentration of cefotaxime was gradually reduced (250, 125 and 50 mg/l) until no antibiotic was added.

### **Growth of HR clones in liquid cultures and induction of protein secretion**

Selected HR clones were first transferred into a 50 ml-flask (Ø 51 mm, Duran® Erlenmeyer) containing 20 ml of MS/sucr (*N.benthamiana*) or Gamborg B5 medium supplemented with 3% (w/v) sucrose (B5/sucr, *B.rapa*). After 2 weeks of growth at 25 °C, in the dark, at 80 rpm on an orbital shaker, the roots positioned at the margin of the biomass were sterilely excised and transferred into a 250 ml-flask (Ø 85 mm) containing 100 ml of fresh medium. The cultures were then kept in the dark for 2 weeks, at 25 °C, on an orbital shaker at 100 rpm. To induce the secretion of the protein of interest, the culture medium was substituted with 100 ml of fresh MS/sucr (*N.benthamiana*) or B5/sucr (*B. rapa*) supplemented with 0.1 mg/l of the auxin hormone 2,4-dichlorophenoxyacetic acid (2,4-D) (D7299, Sigma). Medium samples (100 µl) were collected at 0, 2, 8, and 10 days after induction. In the non-induced control, the spent

medium was replaced with fresh medium without hormone, and the culture maintained for the same duration as the induced flasks.

In the case of *N. benthamiana* HR, to scale-up the recombinant protein production, roots (~15 cm<sup>3</sup>) from the margin of the root mass grown in the 250 ml-flask were excised and transferred in a 2 l-flask (Ø 166 mm) containing 800 ml of fresh MS/sucr. After 15 days of growth at 25 °C, in the dark, in an orbital shaker at 100 rpm, the medium was replaced with fresh MS/sucr supplemented with 0.1 mg/l of 2,4-D (T0). Aliquots of the culture medium (100 µl) were collected at T0, T7, T10, T12, T14, and T17 after induction. At T21, all the medium was collected and replaced with fresh MS/sucr without hormones. The HR culture was maintained for 21 more days collecting samples at T28, T31, T33, T35 and T38; at T42, the culture was finally sacrificed by collecting the medium.

### **Microscopy analysis**

Roots fragments were excised from the liquid culture, mounted on microscopy slides in water, and analysed with a photomicroscope (1.25X M420, WILD) capturing images with a Nikon Coolpix camera 995.

### **Recombinant protein concentration and purification from HR culture medium**

HR culture medium recovered at T21 and T42 after induction was pooled together and filtered through Miracloth (475855, Sigma). Protein precipitation was performed by adding ammonium sulfate up to 50% saturation, with gentle agitation at 4 °C, overnight. The sample was then centrifuged at 10,000 x g for 20 min, and the pellet resuspended in a volume of phosphate-buffered saline (PBS) (pH 7.5) 5-10-fold lower with respect to the initial one to obtain protein concentration. As an alternative, the culture medium was ultrafiltrated using PES (polyethersulfone) ultrafiltration discs (Biomax<sup>®</sup>, 10 kDa, Millipore, Temecula, CA, USA), in a stirred ultrafiltration apparatus (50 ml, model 8050, Millipore, Temecula, CA, USA)

containing N<sub>2</sub> at 2.0 bar, thus obtaining also in this case a 5-10-fold concentrated protein solution.

The concentrated medium containing PD-FcY was then filtered through a 0.45 µm PES Millex syringe filter (SLHP033N, Millipore, Temecula, CA, USA) before protein purification using the Ligatrap IgY column (Ligatrap™ Technologies, Raleigh, NC, USA). Elution from the column (2 x 1.5 ml fractions) was performed using 0.1 M sodium acetate, pH 4.0, and the pooled fractions were neutralized to pH 7.0 using 3 M Trizma base, pH 11.0. Finally, the fractions were concentrated and dialysed in PBS, pH 7.5, using Vivaspin 20 columns (PES membrane, 10,000 molecular weight cut-off) (Sartorius, Stonehouse, UK) following manufacturer's instructions.

### **Size-exclusion chromatography**

Size-exclusion chromatography (SEC) was performed on a calibrated Superdex™ 75 10/300 GL column (GE Healthcare, Sweden) thermostated at 20 °C, using PBS (pH 7.2) as eluent and an ÄKTA FPLC P920 instrument (GE Healthcare), at a 0.3 ml/min flow rate (Lombardi et al. 2010). Column calibration was performed using a gel filtration calibration kit (Low and High Molecular Weight, GE Healthcare) following manufacturer's instructions. Protein absorbance was measured at 280 nm and expressed as Absorption units (mAU). Peaks representing different proteins fractions were recovered using a fraction collector FRAC-920 (GE Healthcare).

### **HR extracts preparation, SDS-PAGE and Western Blot (WB) analysis**

To obtain protein extracts, 100 mg of HR tissue frozen in liquid nitrogen were grinded in an Eppendorf tube and homogenized in a 1:2 (w/v) ratio in PBS (pH 7.5) supplemented with a protease inhibitor cocktail (cOmplete™; Roche, Mannheim, Germany). The extracts were centrifuged at 20,000 x g for 20 min, at 4 °C, and the supernatants recovered for the analysis.

Proteins in HR extracts, culture medium and purified samples were separated by 10-12% SDS-PAGE (Mini-PROTEAN® TGX™ Precast gels, Biorad, Hercules, CA, USA) under non-

reducing and reducing conditions; the latter were obtained by adding 3%  $\beta$ -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). PiNK plus or BLUelf (GeneDireX, Las Vegas City, NV, USA) and a purified commercial RFP (Thermo Fisher Scientific, UK) were used as molecular mass (M) markers and as positive control, respectively. The gels were stained by incubation in Coomassie R250 (Sigma-Aldrich) solution at room temperature, overnight, before destaining them with 25% methanol and 10% acetic acid, or were stained by silver nitrate. In the case of purified PD-FcY protein quantification, densitometric analysis of the bands in the gel (the value was calculated as mean from three independent experiments  $\pm$  standard deviation, SD) was performed with the ImageQuant<sup>TM</sup> TL 7.0 Image analysis software (GE Healthcare), using bovine serum albumin (BSA) as a protein concentration standard.

Alternatively, proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Amersham<sup>TM</sup> Hybond® P 0.45  $\mu$ M, GE Healthcare, Freiburg, Germany) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare) for 50 min, at 1.4 mA/cm<sup>2</sup> and 30 V. Membranes were incubated in PBS containing 5% (w/v) skimmed milk (5% milk-PBS, blocking solution), at 4 °C, overnight. Purified chicken IgY (AC146, Millipore, Temecula, CA, USA) was used as positive control. Detection of PD-FcY 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<sup>TM</sup> Prime Western Blotting Detection (Amersham<sup>TM</sup>, GE Healthcare, Freiburg, Germany) and ImageQuant<sup>TM</sup> LAS 500 system (GE Healthcare) were used for chemiluminescence signal detection. Quantification was performed on three independent experiments (calculated as mean  $\pm$  SD) with the ImageQuant<sup>TM</sup> TL 7.0 Image analysis software (GE Healthcare) using as standard purified PD-FcY (obtained from HR medium) at different concentrations.

### **Fluorimetric and Bradford analysis of the RFP HR culture medium**

Samples of HR medium containing RFP were distributed in triplicate in CELLSTAR® 96-well plates (Greiner Bio-One GmbH) and then analysed using a fluorimeter GloMax® Discover (Promega, WI, USA) set-up with an excitation wavelength of 520 nm and an emission wavelength of 580-640 nm. Serial dilutions (500, 250, 125, 62.5, 31.25, 15.62, 7.8, and 3.9 ng/well) of commercial RFP (Thermo Fisher Scientific, UK) were used to construct a standard curve for quantification, while MS culture medium alone was used as negative control (calculated value represents the mean from three independent experiments  $\pm$  SD). Total soluble protein (TSP) content in the medium was determined in parallel by Bradford colorimetric assay (Biorad, Hercules, CA, USA) in triplicate on CELLSTAR® 96-well plates (Greiner Bio-One GmbH), and then measured at 600 nm using a GloMax® Discover. Serial dilutions of BSA were used to construct a standard curve for quantification.

### **Proteomic and glycosylation analysis**

Proteins were separated by reducing 10% SDS-PAGE, stained with a Coomassie G250 solution and finally destained; corresponding gel bands were excised, triturated, *in-gel* reduced and S-alkylated and finally digested with trypsin for identification of plant contaminants (Desiderio et al., 2019) or endoprotease Asp-N (Roche, Mannheim, Germany) (Salzano et al. 2013) for glycosylation analysis. 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  $\mu$ ZipTipC18 pipette tips (Millipore). Protein digests were analysed 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 chromatographer (Thermo Fisher Scientific). Peptides were separated on an Acclaim PepMap RSLC C18 column, 150 mm  $\times$  75  $\mu$ m internal diameter, 2- $\mu$ m particles, 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, at a nominal resolution of 70,000. Fragmentation of parent ions was controlled by a data-dependent scanning procedure over the 8 most abundant ions using 20 s dynamic exclusion. Mass isolation window and collision energy were set to  $m/z$  1.2 and 28%, respectively. NanoLC-ESI-Q-Orbitrap MS/MS data were searched using MASCOT algorithm (v.2.6.1) (Matrix Science, London, UK) against a database of *Viridiplantae* protein sequences from NCBI (6216320 sequences 12/2018) for protein identification experiments. For glycosylation analysis, raw data were searched using 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 C-terminal Lys/Arg or N-terminal Asp/Glu as cleavage specificity (depending on the protease used), allowing 2 or 3 missed cleavages as maximum value for trypsin or endoprotease Asp-N, and semi-specific cuts in the latter case. Cys carbamidomethylation was selected 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 20 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 the mean values of two independent measurements normalized to the total area of the ion chromatogram.

**Statistical analysis**

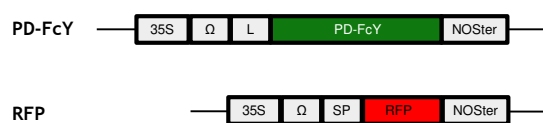
Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA, [www.graphpad.com](http://www.graphpad.com).

The  $p$ -values  $< 0.05$  were considered statistically significant.

## Results

### Generation of *N. benthamiana* HR expressing PD-FcY

*N. benthamiana* leaf fragments were inoculated with *R. rhizogenes* A4 strain carrying the PD-FcY-encoding construct to generate HR stably expressing the secretory version of PD-FcY recombinant protein (Figure 1).

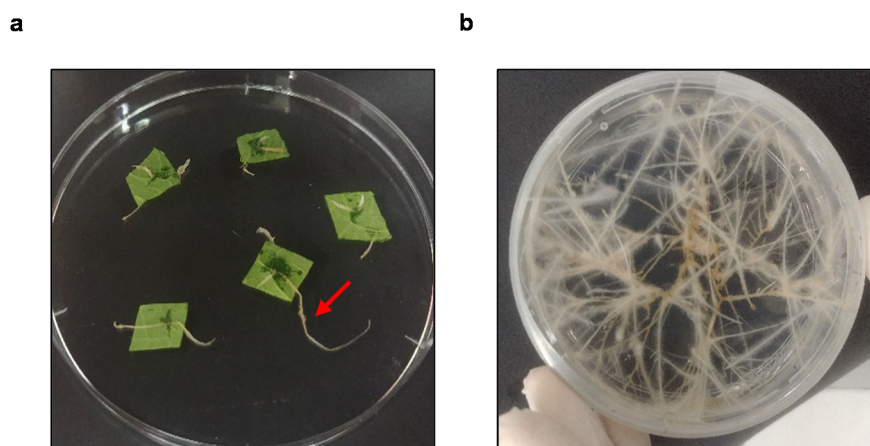


**Figure 1:** Schematic representation of plant expression constructs.

Schematic representation of the PD-FcY- and the RFP-encoding constructs used to generate HR cultures. Gene expression was under the control of the cauliflower mosaic virus 35S promoter (35S), the translation enhancer sequence of tobacco mosaic virus ( $\Omega$ ) and the *nopaline synthase* terminator sequence (NOSter). L: sequence encoding the secretion signal peptide from the heavy chain of an embryonic mouse immunoglobulin. SP: apoplast secretory signal peptide from phaseolin.

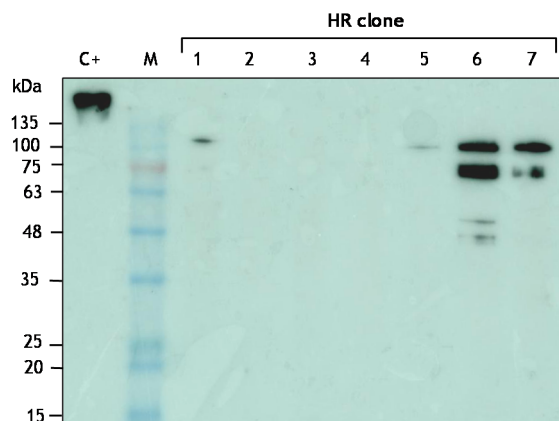
About 3 weeks after the infection, HR started to emerge from the wounding sites (Figure 2a) and a total of 75 HR clones were excised and cultured separately on solid medium (Figure 2b).





**Figure 2:** Example of a *N. benthamiana* HR clone **(a)** emerging from a wounding site (red arrow) and **(b)** growing on solid medium.

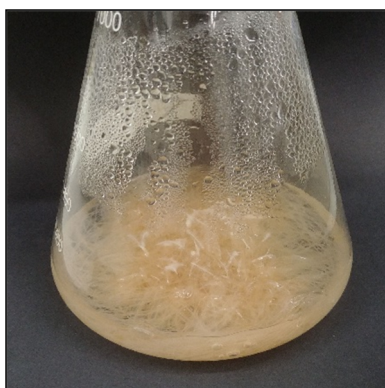
The accumulation of PD-FcY in the HR clones was assessed by WB analysis of HR protein extracts separated by SDS-PAGE under non-reducing conditions, using an anti-IgY antibody (Figure 3). The results demonstrated that 18 clones out of 60 (30%) expressed PD-FcY (hrPD-FcY) although with a different efficiency. All positive samples showed two major bands migrating at about 100 and 90 kDa, probably corresponding to dimers of intact and degraded PD-FcY, respectively. Lower bands were also present migrating at about 50 and 48 kDa, which probably corresponded to the non-assembled monomeric form of PD-FcY and to a degradation product, respectively.



**Figure 3:** Analysis of PD-FcY expression in *N. benthamiana* HR.

WB analysis with an anti-IgY antibody of the extracts (20  $\mu$ l) of seven representative HR clones grown on solid medium after separation by SDS-PAGE performed under non-reducing conditions. M: molecular mass marker; C+: purified chicken IgY (100 ng).

The four HR clones exhibiting the highest accumulation of PD-FcY were transferred in liquid medium (Figure 4).

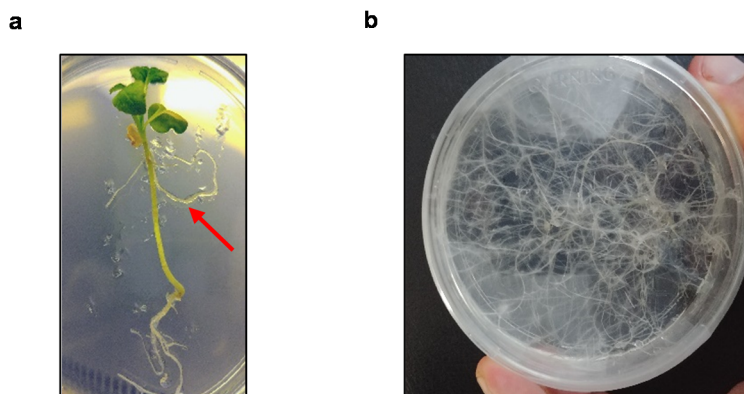


**Figure 4:** Liquid culture of a representative *N. benthamiana* hrPD-FcY clone.

### **Generation of *B. rapa* HR expressing PD-FcY**

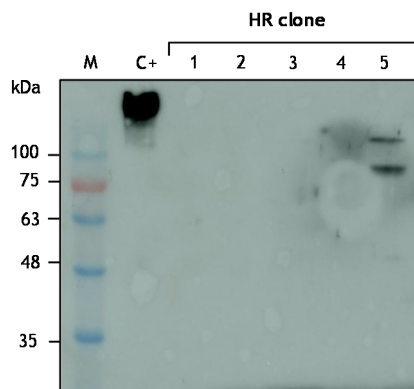
*B. rapa* plant stems were inoculated with *R. rhizogenes* A4 strain carrying the PD-FcY-encoding construct to generate HR stably expressing the secretory version of PD-FcY recombinant protein (Figure 1).

About one week after the infection, HR started to emerge from the wounding sites (Figure 5a) and a total of 150 HR clones were excised and cultured separately on solid medium (Figure 5b).



**Figure 5:** Example of a *B. rapa* HR clone (a) emerging from a wounding site (red arrow) and (b) growing on solid medium.

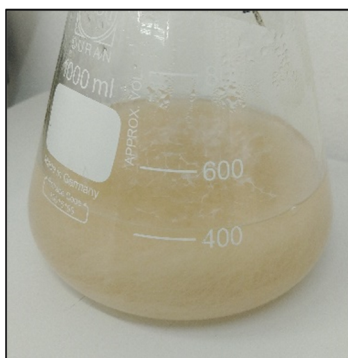
The accumulation of PD-FcY in the HR clones was assessed by WB analysis of HR protein extracts separated by SDS-PAGE under non-reducing conditions, using an anti-IgY antibody (Figure 6). The results demonstrated that 2 clones out of 20 (10%) expressed PD-FcY (hrPD-FcY) although with a low efficiency. The two positive samples showed two major bands migrating at about 100 and 90 kDa, probably corresponding to dimers of intact and degraded PD-FcY, respectively. Lower bands were not observed.



**Figure 6:** Analysis of PD-FcY expression in *B. rapa* HR.

WB analysis with an anti-IgY antibody of the extracts (20  $\mu$ l) of five representative HR clones grown on solid medium after separation by SDS-PAGE performed under non-reducing conditions. M: molecular mass marker; C+: purified chicken IgY (100 ng).

The two HR clones exhibiting accumulation of PD-FcY were transferred in liquid medium (Figure 7).

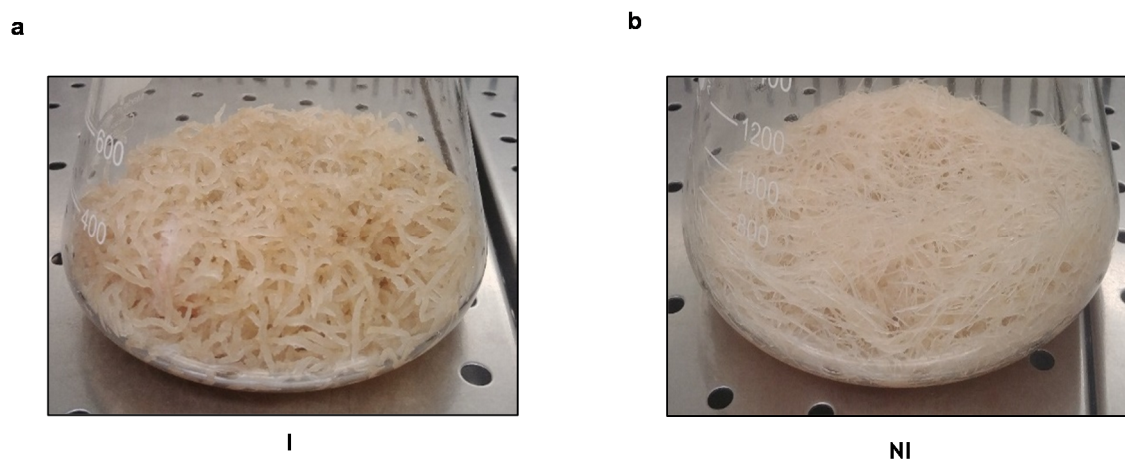


**Figure 7:** Liquid culture of a representative *B. rapa* hrPD-FcY clone.

### **PD-FcY secretion in HR culture medium**

The hrPD-FcY liquid cultures were scaled-up to 250 ml-flasks and recombinant protein secretion in the medium was induced by adding the synthetic auxin 2,4-D. Major macroscopic differences were observed in the induced HR cultures shortly (2-3 days) after the hormonal treatment, compared to the non-induced control. Roots at the margin of the mass lost adherence to the flask wall, and root elongation stopped. About 7 days after induction, the root mass started to become more compact, assuming a sponge-like aspect (Figure 8a) that persisted until the end

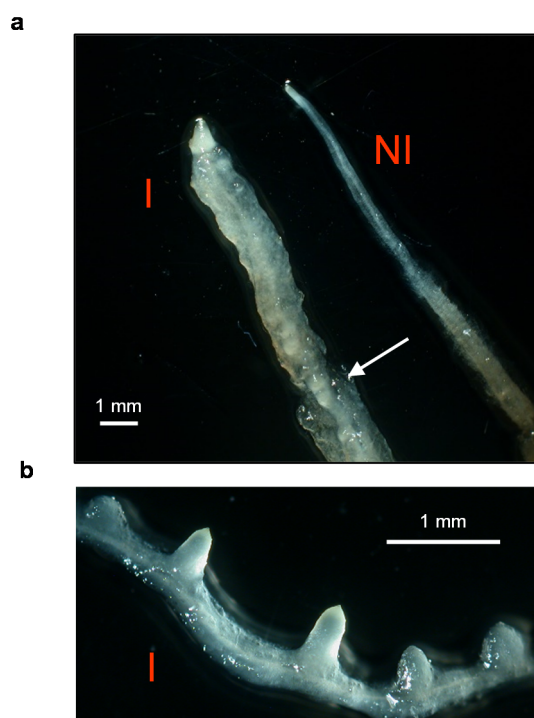
of the culture. In contrast, non-induced HR proliferated and intertwined together to form a compact mass actively growing mainly at its margins, where the younger roots adhered to the flask surface (Figure 8b).



**Figure 8:** Hormonal induction of PD-FcY secretion in liquid culture medium, macroscopic analysis.

Aspect of a representative *N. benthamiana* hrPD-FcY culture grown for two weeks in **(a)** MS/sucr supplemented with 0.1 mg/l of 2,4-D or **(b)** basic MS/sucr medium, respectively.

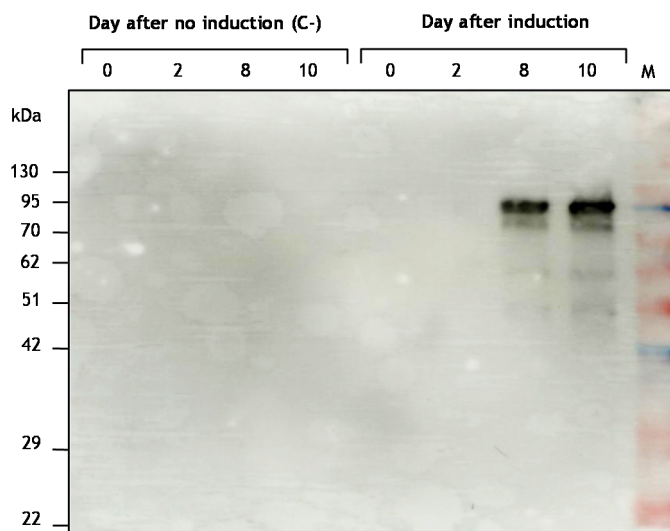
Samples of both induced and non-induced cultures were recovered 10 days after the inoculum; optical microscopy observations revealed major differences in the root tips that appeared to have a swollen aspect in induced cultures samples. Moreover, after hormonal treatment, the expanded root cortex appeared extremely fragile (Figure 9a), revealing the presence of peculiar emerging structures resembling lateral root primordia (Figure 9b).



**Figure 9:** Hormonal induction of PD-FcY secretion in liquid culture medium, microscopic analysis.

**(a)** Microscopic analysis of induced (I) and non-induced (NI) *N. benthamiana* hrPD-FcY. The arrow evidences the alteration of the cortex typically observed on the swollen induced HR. **(b)** Detail showing the peculiar structures (lateral root primordia) emerging from induced roots.

Medium samples were collected from hrPD-FcY cultures at different time points after induction (0, 2, 8, 12 and 10 days), and the presence of secreted PD-FcY was assessed by SDS-PAGE followed by WB analysis with the anti-IgY antibody. The results demonstrated that the recombinant protein was successfully secreted in the medium with a different efficiency by all induced clones of *N. benthamiana*, except one, and PD-FcY accumulation increased over-time reaching its maximum at day 10 (Figure 10). In all samples, the protein pattern was similar to the one observed in the root extracts, but with a more intense signal of the component migrating with an apparent mass of 100 kDa, which probably corresponded to the dimeric form of the protein. Clone 6 showing the best secretion levels was selected for HR culture scale-up experiments and recombinant protein purification.



**Figure 10:** Hormonal induction of PD-FcY secretion in liquid culture medium, protein analysis. WB analysis with the anti-IgY antibody of culture medium aliquots collected at different time points (0, 2, 8 and 10 days) in the induced or non-induced hrPD-FcY Clone 6 culture. Twenty  $\mu$ l were separated by 12% SDS-PAGE under non-reducing conditions; M: molecular mass marker.

On the contrary, no secretion of PD-FcY in the medium was observed for all induced clones of *B. rapa* but all the macroscopic and microscopic differences presented for *N. benthamiana* hrPD-FcY were also observed (data not shown). Therefore, *B. rapa* HR cultures were discontinued and focus was made on *N. benthamiana*.

### **Optimisation of PD-FcY secretion**

A *N. benthamiana* HR clone previously established in the laboratory (Lonoce et al. 2019) and expressing the red fluorescent protein (hrRFP), was used in this study as a model system to easily follow and define the conditions that maximize the secretion and accumulation of the recombinant protein in the culture medium. Liquid cultures of hrPD-FcY and hrRFP were established in 2 l-flasks with 800 ml of MS medium and induced two weeks later (T0) with 0.1 mg/l of 2,4-D. Before induction, the hrRFP showed the presence of high concentrations of the



recombinant protein in the root tips (Figure 11, upper panel), while only a few days after induction the RFP started to be released in the medium that gradually assumed a red colour (Figure 11, lower panel).

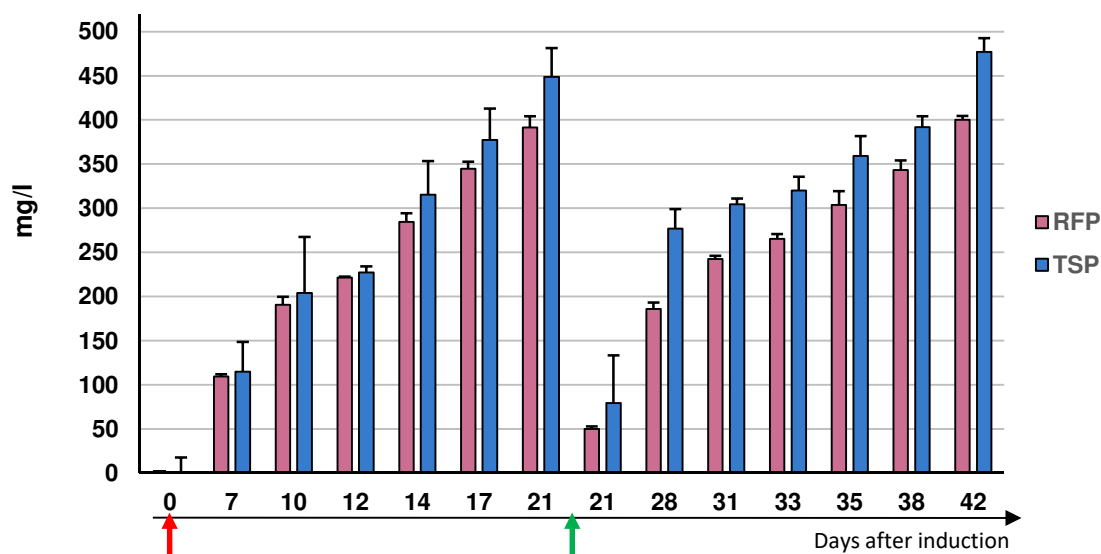


**Figure 11:** Macroscopic analysis of the RFP secretion in medium of induced HR culture.

hrRFP cultures in liquid medium before the induction (upper panel; accumulation of RFP in the root tips before induction was visible) and 21 days after induction (lower panel).

At day 21 after the induction (T21), the medium was replaced with fresh MS without hormone and the culture was grown up to T42. Interestingly, replacement of the medium did not alter the ability of the roots to secrete the RFP. All the medium samples collected at different time-points (from T0 to T42) were analysed for both RFP and TSP content (Figure 12). The results demonstrated that both concentrations increased constantly over time reaching maximum levels at T21 and T42, with a RFP accumulation in the medium of about 18 mg/l/day. The RFP/TSP ratio after induction was similar at all the time points, with RFP representing about 85% of the TSP. At both T21 and T42, RFP concentration was about  $400 \pm 20$  mg/l, corresponding to a total yield of about 640 mg/flask.

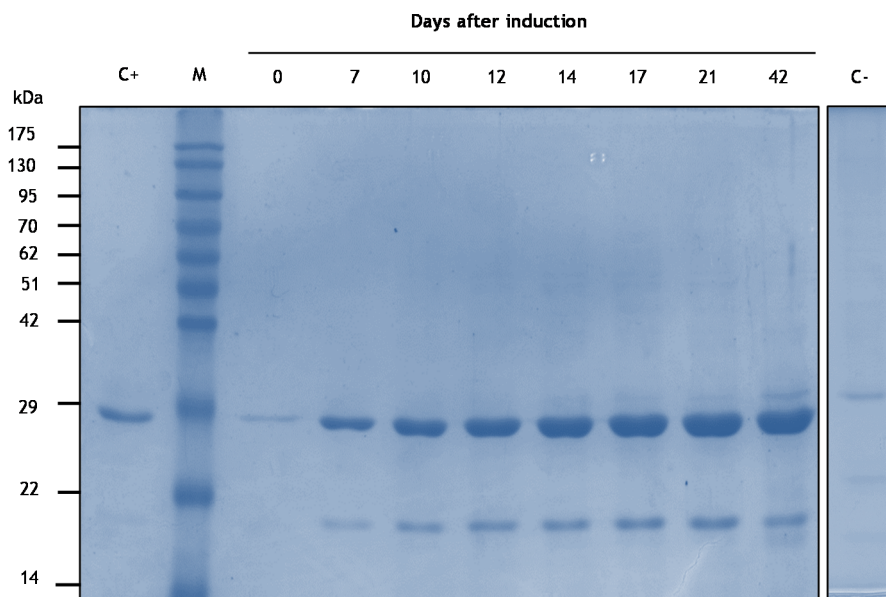




**Figure 12:** RFP and TSP secretion in medium of induced HR culture over-time.

Quantitative analysis of RFP and TSP content in the culture medium of induced hrRFP cultures as measured by fluorimetric analysis and Bradford assay, respectively. At day 0 (red arrow), the medium of the cultures was replaced with fresh one containing 2,4-D, while at day 21 after induction (green arrow) the medium was replaced with fresh one without the hormone. Data analysis was performed on results from three independent experiments and values represent the mean  $\pm$  SD.

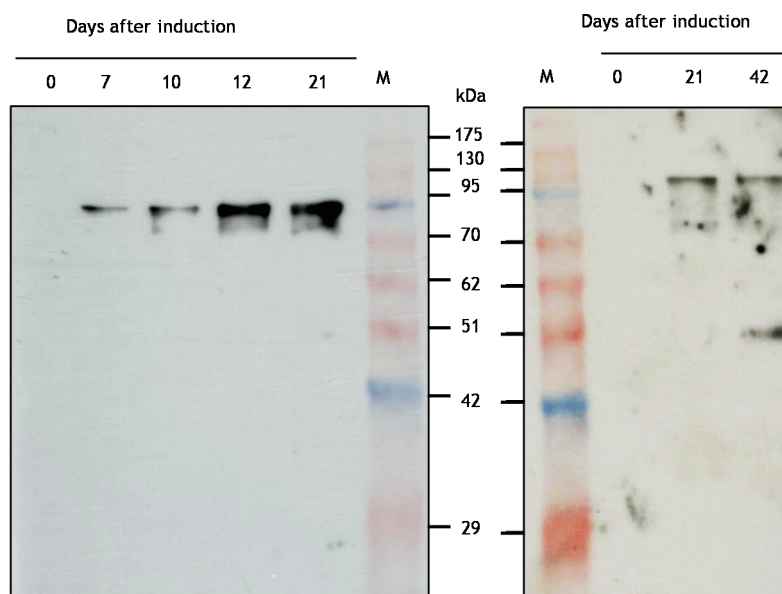
Medium samples were analysed for their protein content by SDS-PAGE performed under reducing conditions, which was followed by Coomassie staining (Figure 13). In line with previously published results (Lonoce et al. 2019), this analysis revealed the presence of two bands: a major band at 30 kDa corresponding to the intact RFP, and a minor band at 20 kDa corresponding to a RFP degradation product. A third faint band of about 32 kDa was visible mainly at T42; this was also present in the negative control, thus probably corresponding to a secreted plant protein.



**Figure 13:** Gel analysis of RFP secreted in medium of induced HR culture over-time.

SDS-PAGE under reducing conditions followed by Coomassie staining of the medium samples collected at different time points after hrRFP culture induction. Ten  $\mu$ l of medium were loaded in each well. M: molecular mass marker; C+: 50 ng purified commercial RFP; C-: medium (10  $\mu$ l) collected at T42 from a non-induced hrRFP culture.

Based on these results, the same scale-up and induction protocol was used to maximize the production of PD-FcY. The accumulation of the recombinant protein in the medium was analysed by WB analysis under non-reducing conditions at different time points after induction (T0, T7, T10, T12, T21 and T42). Results demonstrated that, similarly to RFP, PD-FcY accumulated over-time reaching at T21 (Figure 14) and, after medium change, at T42, similar yields of about  $4 \pm 0.6$  mg/l. Interestingly, the analysis showed that in T21 samples only two bands at about 100 and 90 kDa were detected, which corresponded to the PD-FcY dimer and its possible degraded form, respectively. At T42, an additional band around 50 kDa was also present, which corresponded to the PD-FcY monomer.

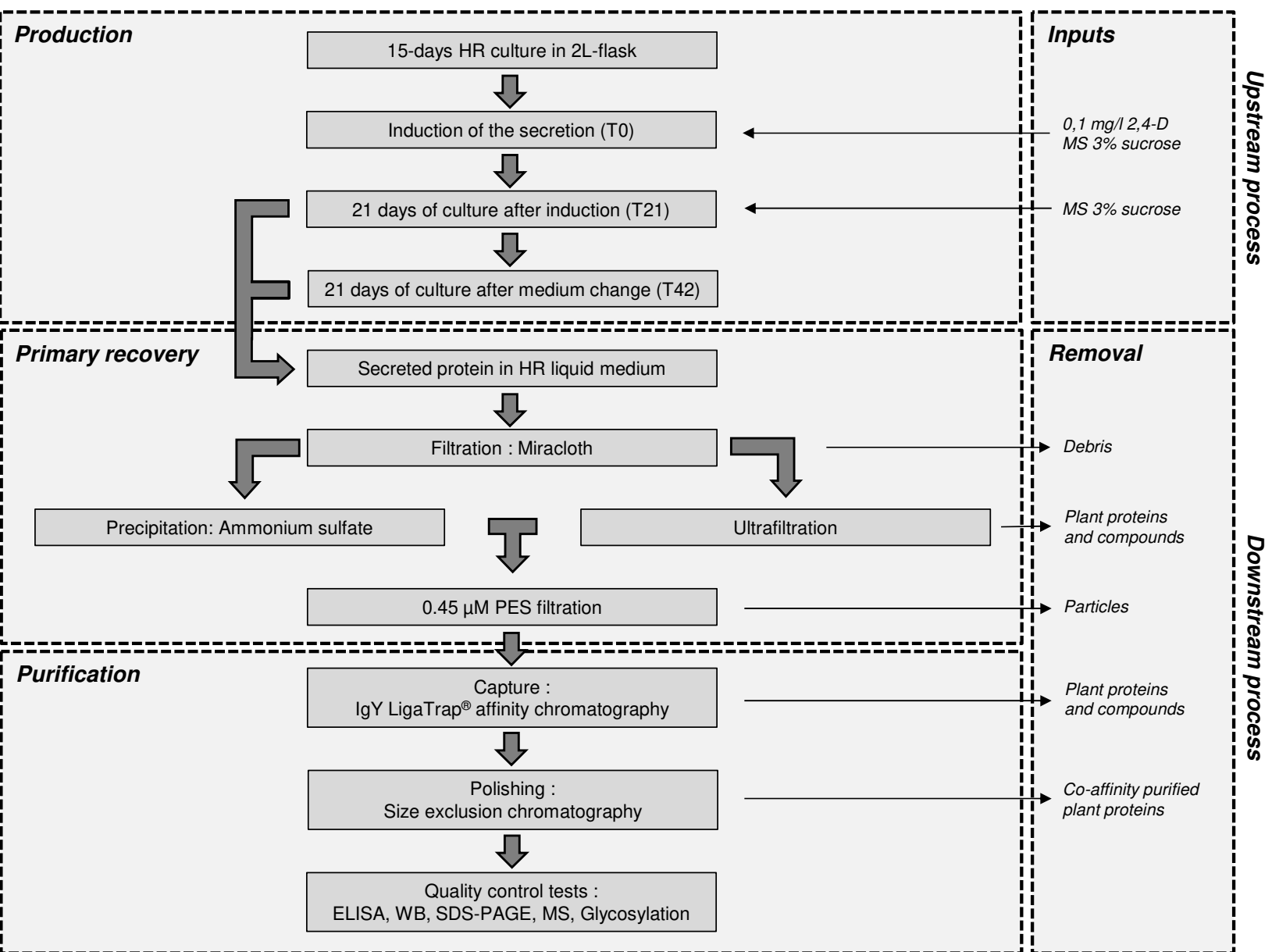


**Figure 14:** PD-FcY secretion in medium of induced hrPD-FcY culture over-time.

Non-reducing SDS-PAGE and WB analysis with an anti-IgY antibody of hrPD-FcY culture medium. Samples (20  $\mu$ l) collected at T0, T7, T10, T21 and T42 after induction. M: molecular mass marker.

### **Optimisation of the downstream process for the purification of PD-FcY from the HR culture medium**

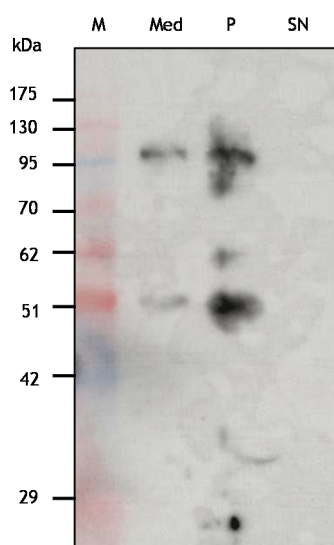
A pilot-scale downstream processing of PD-FcY was established using a pool of the culture medium collected at T21 and T42. A schematic representation of the workflow is illustrated in Figure 15.



**Figure 15:** Schematic representation of the upstream and downstream processes used to purify PD-FcY from HR culture medium.

As previously described, initial clarification and concentration steps are essential for the purification process to reduce the volume and the contaminants content (Rage et al. 2019). After a simple filtration through Miracloth, the medium exhibited a very clear colourless aspect and two different protein concentration methods were tested.

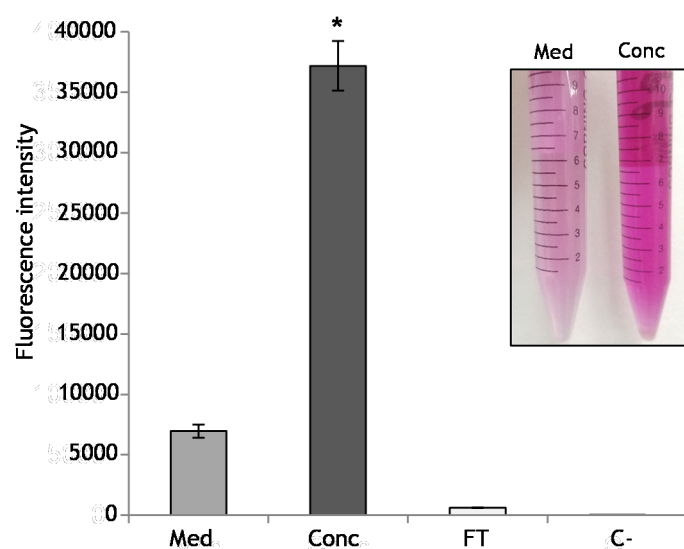
The first method consisted in inducing protein precipitation by 50% ammonium sulfate saturation and in resuspending the pellet in 1/10 of the starting volume of buffer. The analysis of an aliquot of this sample by SDS-PAGE performed under non-reducing conditions, followed by WB with the anti-IgY antibody, confirmed the presence of two major bands at about 100 kDa and 50 kDa. The intensity of the bands was very strong in the concentrated sample, while no signal was observed in the supernatant (Figure 16).



**Figure 16:** Concentration by ammonium sulfate precipitation of PD-FcY from hrPD-FcY culture medium

Non-reducing SDS-PAGE followed by WB analysis with the anti-IgY antibody of the sample obtained after ammonium sulfate precipitation of the hrPD-FcY culture medium (T21 and T42 pool). M: molecular mass marker; Med: induced HR culture medium (20  $\mu$ l); P: resuspended protein pellet (1/10 starting volume) obtained after precipitation of hrPD-FcY culture medium at 50% ammonium sulfate saturation (10  $\mu$ l); SN: supernatant of the hrPD-FcY culture medium after 50% ammonium sulfate precipitation (20  $\mu$ l).

The second approach consisted in the ultrafiltration of the culture medium using the hrRFP as a reference to set-up the protocol. An aliquot of the pool of the medium collected at T21 and T42, was clarified by filtration through Miracloth, and concentrated in a stirred ultrafiltration apparatus using a PES filter with a 10-kDa cut-off. The concentration of the RFP in the extract was easily monitored through the intensification of the red colour and fluorescence analysis (Figure 17). By using this approach, the culture medium was rapidly (about 30 min) concentrated up to ~5 times, without the formation of precipitates, and only a minor loss of RFP was observed in the flow-through.

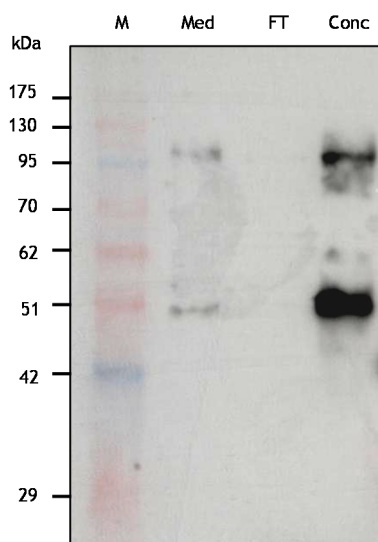


**Figure 17:** Concentration by ultrafiltration of RFP from HR culture medium

Fluorimetric analysis of the hrRFP medium collected at T21 and T42 days after induction, pooled together (Med) and concentrated about 5-fold by ultrafiltration (Conc). FT: ultrafiltration flow-through. C-: MS medium used as negative control. Data analysis was performed on results from three independent experiments and values represent the mean  $\pm$ SD. \*  $p < 0.05$  for Conc versus all other samples.

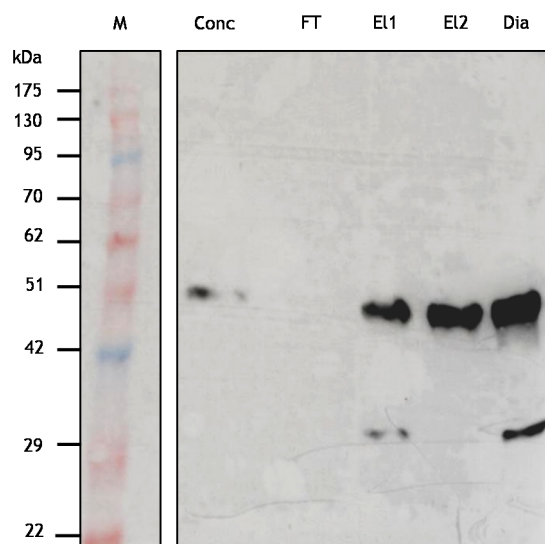
The same protocol was used to concentrate the culture medium containing PD-FcY, reducing 10-fold the initial volume. An aliquot of the obtained sample was separated by SDS-PAGE under non-reducing conditions, and analysed by WB using the anti-IgY antibody; this analysis

showed in this sample the presence of the same two bands obtained with the ammonium sulfate precipitation. The intensity of the signal was very strong in the concentrated sample, while no bands were present in the filtrate (Figure 18).



**Figure 18:** Concentration by ultrafiltration of PD-FcY from hrPD-FcY culture medium. WB analysis with an anti-IgY antibody of hrPD-FcY medium concentrated 10 fold by ultrafiltration. Samples were separated by 12% SDS-PAGE under non-reducing conditions. M: molecular mass marker; Med: induced HR culture medium (20  $\mu$ l); FT: ultrafiltration flow-through (20  $\mu$ l); Conc: concentrated medium (10  $\mu$ l).

Thereafter, PD-FcY was purified from the concentrated sample obtained by ultrafiltration using the Ligatrap IgY affinity chromatography method. WB analysis of the fractions eluted from the Ligatrap IgY column separated under reducing conditions showed only two bands migrating at about 50 and 30 kDa; the major one at 50 kDa corresponded to the intact form of PD-FcY, while the second was associated with a putative degradation product (Figure 19). No bands were detected in the column flow-through (FT), demonstrating the high binding efficiency of the resin for PD-FcY. To complete the process, the Ligatrap fractions were pooled together, dialysed and concentrated to about 0.25 mg/ml with centrifugal filters (Figure 19, Dia sample).



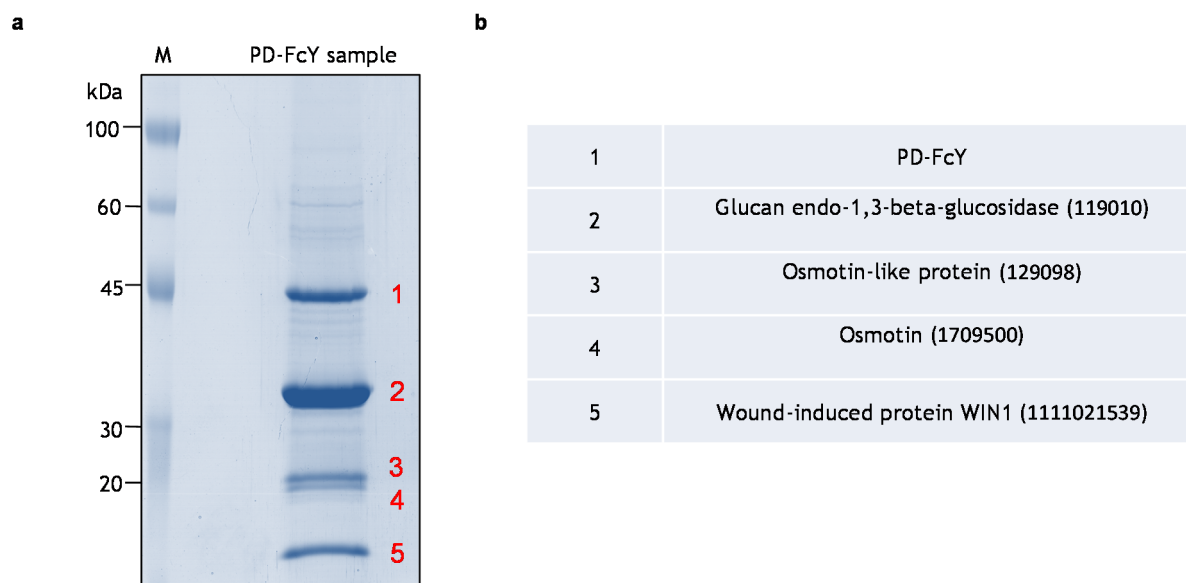
**Figure 19:** Purification of PD-FcY using the Ligatrap IgY affinity column

WB analysis with an anti-IgY antibody of the purified PD-FcY eluted from the Ligatrap IgY affinity column. Samples were separated by 12% SDS-PAGE under reducing conditions. M: molecular mass marker; Conc: concentrated medium (20  $\mu$ l); FT: flow through (20  $\mu$ l); EL1-2: fractions eluted from the Ligatrap IgY column (10  $\mu$ l); Dia: PBS dialysed and concentrated PD-FcY fractions (5  $\mu$ l).

### **Characterisation of the purified PD-FcY and removal of plant contaminants**

The purified PD-FcY was further characterized by SDS-PAGE performed under reducing conditions, which was followed by Coomassie staining (Figure 20a). This analysis evidenced the presence of 5 major bands. The identity of these bands was determined by proteomic analysis, which was performed as previously reported (Desiderio et al., 2019; Salzano et al., 2013); results confirmed that the band migrating at about 50 kDa corresponded to the intact PD-FcY, while the other bands with lower molecular mass values were plant-specific stress-related proteins (Figure 20b; Annex I). From a densitometric analysis of band intensities, PD-FcY represented 15% of the total purified proteins, corresponding to a final yield of about 0.8 mg/l of medium. Therefore, considering that the medium was replaced once after the induction, approximately 1.3 mg of purified PD-FcY were obtained per flask with an estimated purification yield of 20%.

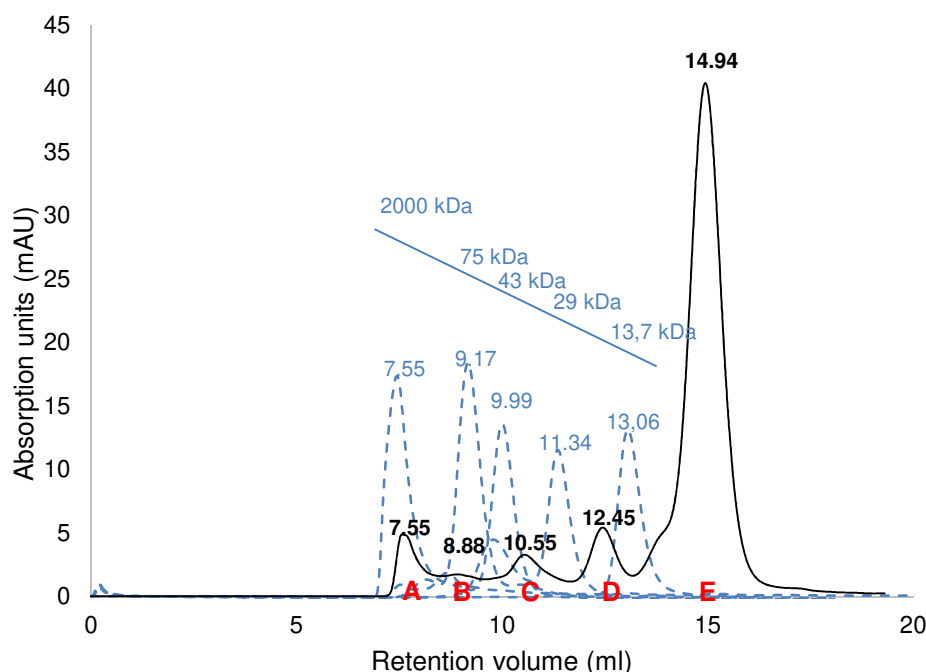




**Figure 20:** Characterization of purified PD-FcY protein sample

**(a)** Twelve % SDS-PAGE analysis under reducing conditions followed by Coomassie staining of the purified PD-FcY sample (20  $\mu$ l). M: molecular mass marker. **(b)** Protein identification of Coomassie G250-stained bands observed in the purified PD-FcY sample was performed by nLC-ESI-Q-Orbitrap MS/MS analysis, as previously reported (Desiderio et al., 2019). Numbers indicating the different identified proteins match those indicating the different bands in the reported SDS-PAGE gel. NCBI accession numbers for the identified proteins are reported in parenthesis.

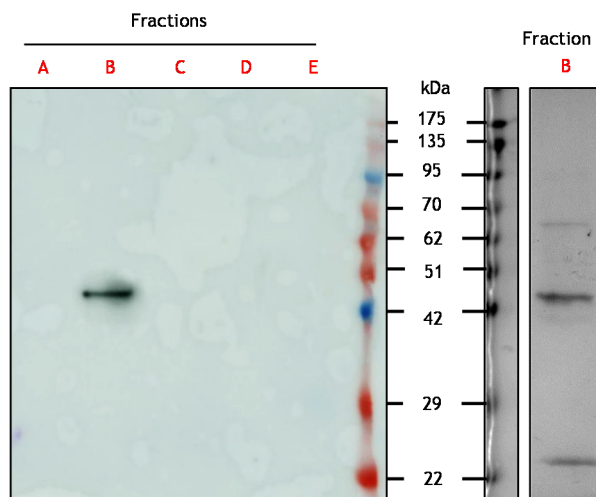
In order to separate PD-FcY from the plant protein contaminants present in the purified sample, SEC was performed using a Superdex™ 75 10/300 GL column. Five different peaks (A to E) were eluted, and peak B recovered at 8.88 ml corresponded to a component with a mass of 100 kDa matching the PD-FcY dimer (Figure 21).



**Figure 21:** Size-exclusion chromatography analysis of PD-FcY purified protein sample

Size exclusion chromatogram of purified PD-FcY sample performed using a calibrated Superdex™ 75 10/300 GL column. Protein standard retention volume: Blue dextran 2000 (7.55 ml), Conalbumin (9.17 ml), Ovalbumin (9.99 ml), Carbonic anhydrase (11.34 ml) and Ribonuclease A (13.60 ml). Eluted fractions from the SEC: A, B, C, D, and E.

The five eluted fractions further separated by SDS-PAGE under reducing conditions were analysed by WB; a specific 50 kDa band corresponding to the PD-FcY monomer was detected only in fraction B (Figure 22, left panel). The same sample was then separated by SDS-PAGE under reducing conditions followed by silver nitrate staining. In this case, two main bands were observed: a 50kDa major one, which was associated with the PD-FcY monomer, and a 24 kDa faint one probably corresponding to a residual carry-over of the osmotin plant protein contaminant (Figure 22, right panel).



**Figure 22:** Analysis of the SEC eluted fractions

WB analysis under reducing conditions with an anti-IgY antibody of the eluted fractions from the SEC (A, B, C, D, and E) (left panel). Fraction B was further separated by 12% SDS-PAGE and the gel was stained with silver nitrate (right panel).

### Glycosylation analysis

Glycosylation at the two expected Asn modification sites of PD-FcY was then determined by nLC-ESI-Q-Orbitrap MS/MS analysis of the endoprotease Asp-N digest of band 1, corresponding to the intact recombinant product (Figure 20a), which was followed by database search of the resulting mass spectrometry data with Byonic<sup>TM</sup> software (Annex II). Assignment to specific *N*-linked glycan structures was based on mass fragmentation data. This experiment validated the presence of two glycosylation sites Asn236 and Asn335, respectively present in the C<sub>v</sub><sub>2</sub> and C<sub>v</sub><sub>3</sub> of the Fc part of PD-FcY, similarly to what observed in PD-FcY transiently expressed in leaves (Rage et al. 2019). Estimation of relative abundance of the different glycoforms at the two modification sites was obtained by measuring the area of the extracted ions (XICs) of the identified glycopeptide species from nLC-ESI-MS chromatograms (Table I). For peptide SLSSRVNVSGT (Asn236), this analysis revealed the presence of plant-type *N*-linked glycostructures containing  $\alpha$ -1,3-fucose (F) and  $\beta$ -1,2-xylose (X) residues (Table I, upper panel), among which complex (GnXF) and paucimannose (core(-Man)XF) ones represented

approximately 70% of the identified glycoforms. For peptide EHFNGTYSASSAVPVSTQ (Asn335), this analysis also showed the presence of uncommon plant-type glycoforms with high-mannose moieties (Table I, lower panel), among which high mannose (M9) and high mannose precursor (M9Glc) species represented more than 70% of the identified glycoforms.

| Asn 236 - peptide (230-240) SLSSRVVMSGT        |                                |                               |                                       |                        |
|------------------------------------------------|--------------------------------|-------------------------------|---------------------------------------|------------------------|
| Glycan composition                             | Glycoform                      | Glycan modification mass (Da) | Observed mass (MH <sup>+</sup> ) (Da) | Relative abundance (%) |
| Non-glycosylated                               | -                              | -                             | -                                     | -                      |
| GlcNAc(2)Man(2)Fuc(1) Pent(1)                  | Paucimannose: core(-Man)XF     | 1008.36                       | 2114.95                               | 18.4±6.0               |
| GlcNAc(2)Man(3)Fuc(1)Pent(1)                   | Paucimannose: coreXF           | 1170.42                       | 2277.00                               | 27.7±3.2               |
| GlcNAc(3)Man(3)Pent(1)                         | Complex: GnX                   | 1227.44                       | 2333.66                               | 2.9±1.0                |
| GlcNAc(3)Man(3)Fuc(1)Pent(1)                   | Complex: GnXF                  | 1373.50                       | 2480.08                               | 43.1±5.7               |
| GlcNAc(4)Man(3)Fuc(1)Pent(1)                   | Complex: GnGnXF                | 1576.58                       | 2683.16                               | 5.6±1.3                |
| Asn 335 - peptide (332-349) EHFNGTYSASSAVPVSTQ |                                |                               |                                       |                        |
| Glycan composition                             | Glycoform                      | Glycan modification mass (Da) | Observed mass (MH <sup>+</sup> ) (Da) | Relative abundance (%) |
| Non-glycosylated, N335-deamidated              | -                              | -                             | 1882.86                               | 3.0±1.2                |
| GlcNAc(2)Man(7)                                | High Mannose M7                | 1540.53                       | 3422.39                               | 4.1±0.7                |
| GlcNAc(2)Man(8)                                | High Mannose: M8               | 1702.58                       | 3584.44                               | 19.0±1.8               |
| GlcNAc(2)Man(9)                                | High Mannose: M9               | 1864.63                       | 3746.51                               | 45.1±0.8               |
| GlcNAc(2)Man(9)Glc(1)                          | High Mannose precursor : M9Glc | 2026.69                       | 3908.56                               | 27.9±1.9               |

**Table 1:** Glycosylation analysis of the PD-FcY fusion protein

Data for the glycopeptides (230-240, upper part of the table) and (332-349, lower part of the table) are shown. Relative abundance of glycopeptides are reported as mean values from two replicates. Glycopeptide species presenting a percentage abundance value below 1% were not reported.

## Discussion

Expression of biopharmaceuticals in plant-based systems has been extensively investigated, and several production platforms are available, which are based either on whole plants or on *in vitro* culture of cells or tissues (Xu et al. 2012). Each platform presents some advantages and weaknesses that may vary depending on the biomolecule to be expressed and on its intended use (Schillberg et al. 2019). In this scenario, *in vitro* cultures have the advantage to be carried-out under contained sterile conditions with reduced biological contamination risks, and can be more easily adapted to current regulatory standards for biopharmaceuticals manufacturing. In the last years, HR cultures obtained by infecting monocot/eudicot tissue explants with *R. rhizogenes* have proved to be particularly attractive for heterologous protein expression, due to their rapid growth at room temperature in simple and low-cost culture mediums free of hormones and antibiotics, and for the possibility of scaling-up the cultures in bioreactors (Gao et al. 2015; Shams et al. 2019). Moreover, transformed HR cultures have proved to be genetically stable for years, and the recombinant protein can be accumulated in the culture medium facilitating recovery and purification (Häkkinen et al. 2014). The main bottleneck limiting the use of the above-mentioned platform is represented by the low yields that are generally achieved. However, it has been demonstrated that the addition of a nitrogen source (potassium nitrate) or the stabilisation of the secreted protein using polyvinylpyrrolidone (PVP) or gelatine may moderately enhance product accumulation (Pham et al. 2012; Madeira et al. 2016). Recently, very promising results have also been obtained by supplementing the medium with a very low concentration (0.1 mg/l) of the synthetic auxin 2,4-D, which resulted in a 28-fold increase of protein accumulation (Lonoce et al. 2019).

In a previous work, the IBDV chimeric antigen PD-FcY was successfully expressed by agroinfiltration in *N. benthamiana* leaves, but the recovered final product was shown to contain unwanted degradation products and plant protein contaminants (Rage et al. 2019). In the

attempt to improve the quality of the plant-produced chimeric antigen, *N. benthamiana* and *B. rapa* HR cultures were generated expressing the secretory version of PD-FcY. In fact, it has been previously shown that recombinant proteins accumulated in the culture medium represent a high percentage of TSP, thus facilitating the purification steps and increasing the quality of the final product (Lonoce et al. 2019). Treatment with 2,4-D of the best PD-FcY-expressing HR clones drastically changed the morphology of the roots, which visibly displayed the swelling of the tips and the expansion of the outer cell layers, probably corresponding to the cortex and the epidermis. In many cases, several days after hormone treatment, these outer cell layers appeared to be detached from the internal vascular cylinder (endodermis, pericycle and vascular tissues). In other studies, comparable changes, even if at a lesser extent, were also observed in *N. benthamiana* and *N. tabacum* HR induced with 1-naphthaleneacetic acid (NAA), in which the epidermis and the inner cortex cells were modified and appeared to be less packed (Häkkinen et al. 2014; Lonoce et al. 2016). It was also shown that auxin pressure may stimulate the formation of lateral roots primordia from pericycle cells (Du and Scheres 2018). In line with these evidences, 2,4-D treatment of PD-FcY-expressing HR stimulated the formation of hump-like structures emerging from the pericycle and resembling lateral root primordia. These structures are also similar to those identified in turnip (*B. rapa* subsp. *rapa*) HR induced with 2,4-D, defined as secondary root primordia ('rhizocalli'), and hypothesized to be unable to elongate and differentiate due to the high auxin concentration (Ele Ekouna et al. 2017). Another interesting finding was obtained in tobacco roots of hydroponically grown plants in which several cell wall remodelling proteins were modulated after treatment with auxins (Madeira et al. 2016). All these described morphological modifications were associated with the promotion of the secretion and accumulation of recombinant proteins in the culture medium for *N. benthamiana*. However, no secretion of PD-FcY was observed with *B. rapa* induced HR cultures even if many clones were generated, probably due to an incompatibility of the specie

to produce PD-FcY specifically, indeed many other proteins have already been secreted with this platform (Huet et al. 2014; Ele Ekouna et al. 2017; Cardon et al. 2019).

Previous works on the optimization of HR culture induction protocols have focused mainly on defining the physical cultivation parameters, the type of culture medium and the hormone concentrations that enable to maximize protein secretion (Sharp and Doran 2001a; Häkkinen et al. 2014; Lonoce et al. 2019). In all these studies, the cultures were typically induced twice and maintained for a maximum of 10 days after induction before collecting all the medium for protein analysis and purification. To further optimize currently used induction protocols, the evaluation of protein accumulation kinetics after just one treatment with a low concentration (0.1 mg/l) of 2,4-D was herein investigated. An HR clone expressing a secretory version of RFP revealed that secretion is constant at least up to 21 days after induction, and at this time point roots still appear healthy. This allowed to reach a concentration of the protein of about 400 mg/l, the highest accumulation in the medium ever obtained by HR culture secretions (Donini and Marusic 2018). It was also demonstrated that refreshing the culture medium without the addition of 2,4-D does not affect the HR secretion rate, which was still constant up to 42 days after induction, with an increase of about 18 mg/l of RFP per day. This protocol, which contemplates a total of 42 days of culture after a single hormonal induction, and medium substitution at day 21 for a final total volume recovery of 1600 ml, allowed obtaining 640 mg of RFP. A similar behaviour of this secretion kinetics was also observed for the HR PD-FcY clone but, in this case, the concentrations measured at day 21 and 42 were of about 4 mg/l, with a total final recovery of 6.4 mg.

When compared to RFP, the observed lower PD-FcY yields are not surprising as former is known to be highly expressed in plant systems (Huet et al. 2014; Mai et al. 2016). Nonetheless, this lower recovery may be also due to the protein degradation phenomena in the HR culture medium, which generally represent a major problem, as previously shown for antibodies

(Lonoce et al. 2016, 2019). However, PD-FcY (both extracted from HR or secreted) was shown to be mainly intact and efficiently assembled into dimers, differently from what observed when it was transiently expressed in *N. benthamiana* leaves, where it was partially degraded at its C-terminus (Rage et al. 2019). These results indicate that the chimeric polypeptide is not particularly susceptible to peptidases present in the culture medium, and that these are probably different from those present in the leaf extract (Donini et al. 2015; Schiermeyer 2020). This hypothesis is in line with previously published results in which lower proteolytic enzymes levels and complexity were observed in the rhizosecretome of *N. tabacum* hydroponic cultures, as compared to those present in leaf extracts (Drake et al. 2009). Interestingly, the profile of the secreted PD-FcY was very similar to that of PD-FcY in the intercellular fluids of *N. benthamiana* leaves possibly because these two environments lack some of the proteases that can be found intracellularly (Goulet et al. 2012; Rage et al. 2019).

The HR-derived PD-FcY showed a glycosylation pattern equivalent to that of the chimeric antigen produced in *N. benthamiana* leaves (Rage et al. 2019). In both cases, the Fc C<sub>v</sub>2 domain was characterised by the presence of typical plant complex sugars, while the glycosylation site present in the C<sub>v</sub>3 showed the occurrence of high mannose-type oligosaccharides. This particular pattern is similar to that observed in IgY present in the chicken serum, indirectly suggesting the correct folding of the Fc region. In fact, the C<sub>v</sub>3 domain undergoes a rapid folding in the nascent polypeptide chain of an IgY and becomes inaccessible to mannosidase enzymes present in the endoplasmic reticulum (Suzuki and Lee 2004; Sheng et al. 2017). The correct FcY structure is essential for binding to avian Fc receptors, and we previously demonstrated that the plant derived PD-FcY is able to bind to the CHIR-AB1 receptor expressed on immune cells, which is involved in the antibody-mediated activation of the immune response in chicken (Viertlboeck and Göbel 2011; Rage et al. 2019).

Downstream processing of HR secreted proteins is a crucial aspect to be also taken into



consideration, in particular when concentrations in the culture medium are low, with levels ranging from micrograms to few milligrams per litre (Sharp and Doran 2001b; Gurusamy et al. 2017). In these cases, at first it is fundamental to reduce the volume to be processed, concentrating the protein of interest. The two different methodologies used to this aim, *e.g.* ammonium sulfate precipitation and ultrafiltration, gave similar results in terms of final protein recovery, with only minor losses of PD-FcY. Although ammonium sulfate is widely used as a primary recovery step at lab-scale (Wilken and Nikolov 2012), it is not ideal in the case of large-scale productions, due to the reagent costs and to the necessity of centrifuging large sample volumes. For this reason, ultrafiltration, which is widely exploited in industrial processes as it allows to easily treat large volumes at lower costs, seems to be optimal for HR medium downstream concentration (Van Reis and Zydney 2001; Saraswat et al. 2013).

The concentrated sample was then processed in order to purify PD-FcY. The first step consisted in affinity chromatography using the Ligatrap resin. Purification of the chimeric antigen from agroinfiltrated leaves using this approach resulted in a final product contaminated by degradation products and different unwanted plant proteins that represented more than 80% of total proteins (Rage et al. 2019). This commercial resin, which is based on peptoid ligands that bind to the Fc region of IgY, was initially developed for immunoglobulin purification from the egg yolk, where IgY concentration is particularly high and protein composition is simple (Akita and Nakai 1993; Bordelon et al. 2019). For this reason, it is not surprising that its use for purifying a protein from leaf extracts also resulted in binding to unwanted plant proteins. The use of this method to purify PD-FcY from concentrated HR medium allowed to obtain a high recovery of the intact non-degraded chimeric antigen, but also in this case, evidenced the co-purification of unwanted proteins (85%). However, only four main protein contaminants were present and identified as stress-related proteins implicated in the plant response against pathogens. Interestingly, the most abundant one, namely glucan endo-1,3- $\beta$  glucosidase, was

previously demonstrated to exhibit immune-enhancing properties *in vitro* using the RAW 264.7 murine macrophage cell line (Li et al. 2019). Even though veterinary vaccines do not require the same purity level of human-grade biopharmaceuticals (Buyel et al. 2015), it was demonstrated that a final single size-exclusion chromatography step is sufficient to efficiently separate PD-FcY from most of these contaminants.

In conclusion, this study demonstrates that the PD-FcY fusion antigen can be successfully produced and secreted by *N. benthamiana* HR cultures, and this expression strategy may be regarded as a promising procedure for the cost-efficient production and purification of the IBDV subunit vaccine. Nevertheless, the accumulation of the protein could be further improved by optimizing the growth conditions, such as aeration and composition of the medium, and by up-scaling culture volumes using a bioreactor.

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**Annex I: Identification details from Mascot analysis of nLC-ESI-Q-Orbitrap MS/MS data from trypsin hydrolysis of protein bands from Comsatie-G250-stained 10% SDS-PAGE (Figure 20a)**

| BAND | prot_acc                       | prot_desc                        | prot_score | prot_mass | prot_matches | prot_sequences | prot_cover | pep_query | pep_rank | pep_isbold | pep_bunione | pep_exp_seq | pep_exp_mz | pep_calc_mz | pep_delta | pep_miss | pep_score | pep_expect | pep_res_before | pep_res_after | pep_var_mod              |                   |
|------|--------------------------------|----------------------------------|------------|-----------|--------------|----------------|------------|-----------|----------|------------|-------------|-------------|------------|-------------|-----------|----------|-----------|------------|----------------|---------------|--------------------------|-------------------|
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1195      | 1        | 0          | 0           | 523.7773    | 1045.54    | 2           | 1045.5345 | -0.0005  | 0         | 65.48      | 0.0006         | K             | HFLGSPNPK                | Q                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1195      | 1        | 0          | 0           | 375.5138    | 1123.5194  | 3           | 1123.5191 | -0.0003  | 0         | 64.54      | 0.0003         | K             | HFLGSPNPK                | W                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1194      | 1        | 0          | 0           | 375.5138    | 1123.5194  | 3           | 1123.5191 | 0.0002   | 0         | 62.06      | 0.0042         | K             | HFLGSPNPK                | W                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1195      | 1        | 0          | 0           | 375.5138    | 1123.5195  | 3           | 1123.5191 | -0.0003  | 0         | 60.35      | 0.0046         | K             | HFLGSPNPK                | W                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1196      | 1        | 0          | 0           | 375.5138    | 1123.5197  | 3           | 1123.5191 | 0.0004   | 0         | 60.01      | 0.0041         | K             | HFLGSPNPK                | W                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1198      | 1        | 0          | 0           | 375.5139    | 1123.5199  | 3           | 1123.5191 | 0.0007   | 0         | 60.26      | 0.0063         | K             | HFLGSPNPK                | W                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1858      | 1        | 1          | 0           | 578.8209    | 1155.6272  | 2           | 1155.6248 | 0.0024   | 0         | 62.84      | 0.0037         | K             | AFSEAGLGNK               | V                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1858      | 1        | 0          | 0           | 578.8209    | 1155.6272  | 2           | 1155.6248 | 0.0024   | 0         | 64.68      | 0.0033         | K             | AFSEAGLGNK               | V                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1876      | 1        | 0          | 0           | 579.8133    | 1156.612   | 2           | 1156.6088 | 0.0002   | 0         | 64.79      | 0.0028         | K             | AFSEAGLGNK               | V                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 1881      | 1        | 0          | 0           | 579.8053    | 1157.5961  | 2           | 1157.5928 | 0.0004   | 0         | 67.5       | 0.0037         | K             | AFSEAGLGNK               | V                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 2820      | 1        | 0          | 0           | 675.3693    | 1348.7241  | 3           | 1348.7211 | 0.0003   | 1         | 66.91      | 0.0019         | K             | NIJCHAEGR                | 2 Deamidated (NQ) |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3047      | 1        | 0          | 0           | 467.2567    | 1398.7484  | 3           | 1398.7408 | 0.0076   | 1         | 59.77      | 0.0092         | K             | HFLGSPNPK                | Y                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3064      | 1        | 0          | 0           | 700.8675    | 1599.7204  | 3           | 1599.7248 | -0.0044  | 1         | 66.01      | 0.0024         | K             | HFLGSPNPK                | Y                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3070      | 1        | 0          | 0           | 700.8717    | 1599.7209  | 3           | 1599.7248 | 0.0041   | 1         | 74.24      | 8.00027        | K             | HFLGSPNPK                | Y                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3222      | 1        | 0          | 0           | 720.3864    | 1438.7583  | 2           | 1438.7568 | 0.0015   | 0         | 82.93      | 4.20E-05       | R             | LYOPHISALQALK            | G                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3225      | 1        | 0          | 0           | 720.3872    | 1438.7599  | 2           | 1438.7568 | 0.003    | 0         | 79.07      | 9.80E-05       | R             | LYOPHISALQALK            | G                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3246      | 1        | 0          | 0           | 720.3872    | 1438.7599  | 2           | 1438.7568 | 0.0021   | 0         | 71.58      | 0.0005         | R             | LYOPHISALQALK            | G                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3233      | 1        | 0          | 0           | 720.388     | 1438.7615  | 2           | 1438.7568 | 0.0046   | 0         | 71.35      | 0.00059        | R             | LYOPHISALQALK            | G                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 3251      | 1        | 0          | 0           | 720.8799    | 1439.7453  | 2           | 1439.7408 | 0.0045   | 0         | 64.81      | 0.0039         | R             | LYOPHISALQALK            | G                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 4035      | 1        | 0          | 0           | 836.9342    | 1671.8338  | 3           | 1671.8502 | 0.0006   | 0         | 75.5       | 0.0002         | K             | GNIEVMGLPNSDVK           | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 4035      | 1        | 0          | 0           | 837.4259    | 1672.8373  | 3           | 1672.8342 | 0.0001   | 0         | 83.91      | 4.80E-05       | K             | GNIEVMGLPNSDVK           | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 4085      | 1        | 0          | 0           | 844.9288    | 1687.8431  | 3           | 1687.8451 | -0.0019  | 0         | 61.69      | 0.0085         | K             | GNIEVMGLPNSDVK           | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 4103      | 1        | 0          | 0           | 845.4246    | 1688.8347  | 3           | 1688.8291 | 0.0056   | 0         | 80.13      | 0.0002         | K             | GNIEVMGLPNSDVK           | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 4574      | 1        | 0          | 0           | 922.4501    | 1842.856   | 2           | 1842.8822 | 0.0004   | 0         | 113.7      | 4.90E-08       | R             | NIJCHAEGR                | S                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5880      | 1        | 0          | 0           | 1172.0684   | 2342.1222  | 2           | 2342.1213 | 0.0009   | 0         | 74.64      | 0.0045         | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5882      | 1        | 0          | 0           | 1172.0691   | 2342.1237  | 2           | 2342.1213 | 0.0024   | 0         | 88.81      | 1.70E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5883      | 1        | 0          | 0           | 1172.0694   | 2342.1243  | 2           | 2342.1213 | 0.003    | 0         | 88.26      | 1.90E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5885      | 1        | 0          | 0           | 1172.0696   | 2342.1246  | 2           | 2342.1213 | 0.0003   | 0         | 91.14      | 1.00E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5893      | 1        | 0          | 0           | 1172.0715   | 2342.1285  | 2           | 2342.1213 | 0.0072   | 0         | 82.23      | 7.80E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5893      | 1        | 0          | 0           | 781.7171    | 2342.1285  | 2           | 2342.1213 | 0.0061   | 0         | 86.91      | 2.70E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5897      | 2        | 0          | 0           | 782.045     | 2343.1132  | 3           | 2343.1103 | 0.0079   | 0         | 80.81      | 0.0001         | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5937      | 1        | 0          | 0           | 1180.0267   | 2358.1201  | 2           | 2358.1162 | 0.0039   | 0         | 70.14      | 0.0022         | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5942      | 1        | 0          | 0           | 1180.0267   | 2358.1202  | 2           | 2358.1162 | 0.0007   | 0         | 71.7       | 0.0004         | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5942      | 1        | 0          | 0           | 1180.0268   | 2358.1222  | 2           | 2358.1162 | 0.006    | 0         | 91.52      | 8.80E-06       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5943      | 1        | 0          | 0           | 1180.0268   | 2358.1225  | 2           | 2358.1162 | 0.0063   | 0         | 98.76      | 1.70E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 5947      | 1        | 0          | 0           | 1180.0299   | 2359.1229  | 2           | 2359.1202 | 0.0027   | 0         | 87.87      | 2.10E-05       | K             | VSTVDMTILGKPPFGSGSR      | N                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6745      | 1        | 0          | 0           | 942.1347    | 2823.3822  | 3           | 2823.3789 | 0.0003   | 1         | 80.91      | 0.0001         | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6748      | 1        | 0          | 0           | 942.1358    | 2823.3855  | 3           | 2823.3789 | 0.0066   | 1         | 83.05      | 6.70E-05       | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6748      | 1        | 0          | 0           | 706.9342    | 2823.3854  | 3           | 2823.3789 | 0.0009   | 0         | 70.04      | 0.0001         | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6751      | 1        | 0          | 0           | 706.9354    | 2823.3923  | 3           | 2823.3789 | 0.0134   | 1         | 61.33      | 0.0008         | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6753      | 1        | 0          | 0           | 942.4685    | 2824.3686  | 3           | 2824.3629 | 0.0057   | 1         | 85.5       | 3.80E-05       | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6797      | 1        | 0          | 0           | 943.9502    | 2829.3738  | 3           | 2829.3738 | 0.0062   | 0         | 69.82      | 0.0001         | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 6798      | 1        | 0          | 0           | 947.4678    | 2839.3815  | 3           | 2839.3738 | 0.0007   | 1         | 73.19      | 0.0006         | R             | KPHPIETFAAMFENNNFLK      | H                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 7308      | 1        | 0          | 0           | 1083.8992   | 3248.6757  | 3           | 3248.6679 | 0.0078   | 0         | 96.2       | 2.30E-05       | K             | YUAGNEISPTGTYSLTTPAMNYYK | A                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 7310      | 1        | 0          | 0           | 1083.8992   | 3248.6757  | 3           | 3248.6679 | 0.0078   | 0         | 96.2       | 2.30E-05       | K             | YUAGNEISPTGTYSLTTPAMNYYK | A                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 7330      | 1        | 0          | 0           | 1089.2338   | 3264.6765  | 3           | 3264.6628 | 0.0137   | 0         | 77.57      | 0.0002         | K             | YUAGNEISPTGTYSLTTPAMNYYK | A                 |
| 2    | #1101010 p P23546.1 E1R  TOBAC | Glucan endo-1,3-beta-glucosidase | 900        | 40426     | 46           | 11             | 45.1       | 7330      | 1        | 0          | 0           | 1089.2338   | 3264.6765  | 3           | 3264.6628 | 0.0137   | 0         | 77.57      | 0.0002         | K             | YUAGNEISPTGTYSLTTPAMNYYK | A                 |
| 2    | #120908 p P2587.1 OLPA  TOBAC  | Osmotin-like protein             | 399        | 28546     | 16           | 5              | 37.8       | 880       | 1        | 1          | 0           | 556.2327    | 1110.4508  | 2           | 1110.4513 | -0.0005  | 0         | 61.07      | 0.0034         | R             | TNCFNPGSGR               | G                 |
| 2    | #120908 p P2587.1 OLPA  TOBAC  | Osmotin-like protein             | 399        | 28546     | 16           | 5              | 37.8       | 880       | 1        | 1          | 0           | 556.2327    | 1110.4508  | 2           | 1110.4513 | -0.0005  | 0         | 61.07      | 0.0034         | R             | TNCFNPGSGR               | G                 |
| 2    | #120908 p P2587.1 OLPA  TOBAC  | Osmotin-like protein             | 399        | 28546     | 16           | 5              | 37.8       | 886       | 1        | 1          | 0           | 556.2344    | 1111.4542  | 2           | 1111.4532 | 0.001    | 0         | 59.03      | 0.0032         | R             | TNCFNPGSGR               | G                 |
| 2    | #120908 p P2587.1 OLPA  TOBAC  | Osmotin-like protein             | 399        | 28546</   |              |                |            |           |          |            |             |             |            |             |           |          |           |            |                |               |                          |                   |



| Protein Rank | Peptide                            | Glycans                      | Modification Type(s) | Observed m/z | z | Observed (M+H) | Calc. mass (M+H) | Mass error (ppm) | Score | Comment                                       | Scan # | Scan Time |
|--------------|------------------------------------|------------------------------|----------------------|--------------|---|----------------|------------------|------------------|-------|-----------------------------------------------|--------|-----------|
| 1            | Y.SLSSRVN[+203.079]VSGT.D          | HexNAc(1)                    | N[+203]              | 655.335      | 2 | 1309.663       | 1309.659         | 2.9              | 345.5 | PDFcY-HairyRoots-1.6908.6908.2                | 6908   | 33.80     |
| 1            | Y.SLSSRVN[+203.079]VSGT.D          | HexNAc(1)                    | N[+203]              | 655.332      | 2 | 1309.656       | 1309.659         | -2.5             | 201.8 | PDFcY-HairyRoots-1_20190527214957.6325.6325.2 | 6325   | 33.69     |
| 1            | Y.SLSSRVN[+1576.576]VSGT.D         | HexNAc(4)Hex(3)Fuc(1)Pent(1) | N[+1577]             | 895.059      | 3 | 2683.163       | 2683.156         | 2.4              | 170.8 | PDFcY-HairyRoots-1.6695.6695.3                | 6695   | 33.26     |
| 1            | Y.SLSSRVN[+1535.550]VSGT.D         | HexNAc(3)Hex(4)Fuc(1)Pent(1) | N[+1536]             | 1321.573     | 2 | 2642.139       | 2642.130         | 3.4              | 215.5 | PDFcY-HairyRoots-1.6707.6707.2                | 6707   | 33.29     |
| 1            | Y.SLSSRVN[+1373.497]VSGT.D         | HexNAc(3)Hex(3)Fuc(1)Pent(1) | N[+1373]             | 827.365      | 3 | 2480.081       | 2480.077         | 1.9              | 225.5 | PDFcY-HairyRoots-1.6683.6683.3                | 6683   | 33.23     |
| 1            | Y.SLSSRVN[+1373.497]VSGT.D         | HexNAc(3)Hex(3)Fuc(1)Pent(1) | N[+1373]             | 1240.545     | 2 | 2480.083       | 2480.077         | 2.6              | 223.3 | PDFcY-HairyRoots-1.6690.6690.2                | 6690   | 33.25     |
| 1            | Y.SLSSRVN[+1373.497]VSGT.D         | HexNAc(3)Hex(3)Fuc(1)Pent(1) | N[+1373]             | 827.365      | 3 | 2480.079       | 2480.077         | 1.1              | 188.1 | PDFcY-HairyRoots-1.6817.6817.3                | 6817   | 33.57     |
| 1            | Y.SLSSRVN[+1227.439]VSGT.D         | HexNAc(3)Hex(3)Pent(1)       | N[+1227]             | 1167.515     | 2 | 2334.022       | 2334.019         | 1.3              | 165.1 | PDFcY-HairyRoots-1.6767.6767.2                | 6767   | 33.44     |
| 1            | Y.SLSSRVN[+1170.417]VSGT.D         | HexNAc(2)Hex(3)Fuc(1)Pent(1) | N[+1170]             | 1139.004     | 2 | 2277.001       | 2276.997         | 1.7              | 372.1 | PDFcY-HairyRoots-1.6751.6751.2                | 6751   | 33.40     |
| 1            | Y.SLSSRVN[+1170.417]VSGT.D         | HexNAc(2)Hex(3)Fuc(1)Pent(1) | N[+1170]             | 759.671      | 3 | 2277.000       | 2276.997         | 1.0              | 269.5 | PDFcY-HairyRoots-1.6718.6718.3                | 6718   | 33.32     |
| 1            | Y.SLSSRVN[+1038.375]VSGT.D         | HexNAc(2)Hex(3)Fuc(1)        | N[+1038]             | 715.660      | 3 | 2144.964       | 2144.955         | 4.0              | 202.6 | PDFcY-HairyRoots-1.6765.6765.3                | 6765   | 33.44     |
| 1            | Y.SLSSRVN[+1024.359]VSGT.D         | HexNAc(2)Hex(3)Pent(1)       | N[+1024]             | 1065.977     | 2 | 2130.947       | 2130.940         | 3.5              | 198.2 | PDFcY-HairyRoots-1.6770.6770.2                | 6770   | 33.45     |
| 1            | Y.SLSSRVN[+1008.365]VSGT.D         | HexNAc(2)Hex(2)Fuc(1)Pent(1) | N[+1008]             | 705.656      | 3 | 2114.953       | 2114.945         | 3.8              | 282.0 | PDFcY-HairyRoots-1.6745.6745.3                | 6745   | 33.39     |
| 1            | Y.SLSSRVN[+1008.365]VSGT.D         | HexNAc(2)Hex(2)Fuc(1)Pent(1) | N[+1008]             | 1057.980     | 2 | 2114.952       | 2114.945         | 3.6              | 155.0 | PDFcY-HairyRoots-1.6730.6730.2                | 6730   | 33.35     |
| 1            | Q.EHFN[+203.079]GTYSSASSAVPVSTQ.D  | HexNAc(1)                    | N[+203]              | 1042.978     | 2 | 2084.948       | 2084.941         | 3.6              | 185.2 | PDFcY-HairyRoots-1.9214.9214.2                | 9214   | 39.61     |
| 1            | Q.EHFN[+2026.687]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(10)             | N[+2027]             | 1303.519     | 3 | 3908.542       | 3908.548         | -1.7             | 352.0 | PDFcY-HairyRoots-1_20190527231140.7854.7854.3 | 7854   | 38.40     |
| 1            | Q.EHFN[+2026.687]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(10)             | N[+2027]             | 1303.519     | 3 | 3908.542       | 3908.548         | -1.5             | 279.6 | PDFcY-HairyRoots-1_20190527214957.8052.8052.3 | 8052   | 38.41     |
| 1            | Q.EHFN[+2026.687]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(10)             | N[+2027]             | 1303.523     | 3 | 3908.553       | 3908.548         | 1.3              | 290.9 | PDFcY-HairyRoots-1.8741.8741.3                | 8741   | 38.42     |
| 1            | Q.EHFN[+2026.687]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(10)             | N[+2027]             | 977.895      | 4 | 3908.557       | 3908.548         | 2.2              | 171.6 | PDFcY-HairyRoots-1.8724.8724.4                | 8724   | 38.38     |
| 1            | Q.EHFN[+1864.634]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(9)              | N[+1865]             | 1249.501     | 3 | 3746.489       | 3746.496         | -1.7             | 313.4 | PDFcY-HairyRoots-1_20190527231140.7882.7882.3 | 7882   | 38.47     |
| 1            | Q.EHFN[+1864.634]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(9)              | N[+1865]             | 1249.503     | 3 | 3746.493       | 3746.496         | -0.6             | 380.1 | PDFcY-HairyRoots-1_20190527214957.8061.8061.3 | 8061   | 38.43     |
| 1            | Q.EHFN[+1864.634]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(9)              | N[+1865]             | 1249.505     | 3 | 3746.502       | 3746.496         | 1.7              | 310.8 | PDFcY-HairyRoots-1.8759.8759.3                | 8759   | 38.47     |
| 1            | Q.EHFN[+1702.581]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(8)              | N[+1703]             | 1195.485     | 3 | 3584.441       | 3584.443         | -0.5             | 349.2 | PDFcY-HairyRoots-1_20190527231140.7902.7902.3 | 7902   | 38.52     |
| 1            | Q.EHFN[+1702.581]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(8)              | N[+1703]             | 1195.485     | 3 | 3584.441       | 3584.443         | -0.5             | 212.2 | PDFcY-HairyRoots-1_20190527214957.8106.8106.3 | 8106   | 38.54     |
| 1            | Q.EHFN[+1702.581]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(8)              | N[+1703]             | 1195.487     | 3 | 3584.447       | 3584.443         | 1.2              | 366.9 | PDFcY-HairyRoots-1.8748.8748.3                | 8748   | 38.44     |
| 1            | Q.EHFN[+1540.529]GTYSSASSAVPVSTQ.D | HexNAc(2)Hex(7)              | N[+1541]             | 1141.465     | 3 | 3422.380       | 3422.390         | -2.9             | 186.4 | PDFcY-HairyRoots-1_20190527214957.8088.8088.3 | 8088   | 38.50     |
| 1            | Q.EHFN[+0.984]GTYSSASSAVPVSTQ.D    |                              | N[+1]                | 941.926      | 2 | 1882.845       | 1882.845         | 0.0              | 583.1 | PDFcY-HairyRoots-1.9927.9927.2                | 9927   | 41.41     |
| 1            | Q.EHFN[+0.984]GTYSSASSAVPVSTQ.D    |                              | N[+1]                | 628.288      | 3 | 1882.850       | 1882.845         | 2.6              | 393.7 | PDFcY-HairyRoots-1.9950.9950.3                | 9950   | 41.46     |

**Annex II:** Details of mass spectrometry identification of glycosylated peptides (230-240) SLSSRVNVSGT (Asn236) and (332-349) EHFNGTYSASSAVPVSTQ (Asn335) from intact PD-FcY, as obtained through Byonic search of nLC-ESI-Q-Orbitrap MS/MS data of the endoprotease Asp-N digest.



## **Chapter 4:**

**Design and production in plants of immunogens based on the Fusion protein  
of Newcastle disease virus**

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**Abstract**

Newcastle Disease Virus (NDV) 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 different formulations of innovative subunit vaccines constructions based on the fusion protein (F, the membrane glycoprotein involved in the virulence of the virus and target of the immune responses) of NDV. In particular, the F1 subunit of the F protein was transiently expressed in *Nicotiana benthamiana* plant platforms: i) as fusion to the constant region of avian immunoglobulin Y (FcY), ii) as fusion to the coat protein (CP) of Potato virus X (PVX) in order to obtain chimeric virus particles (CVPs); iii) as single protein with the aim of purifying from plant tissues Heat Shock Proteins 70 (HSP70) chaperoning its peptides. In general, the expression of the protein in plants was difficult to achieve for all the strategies employed, nevertheless, purification of low antigen amounts for some of the formulations was performed. Preliminary immunization studies were set up, but no activation of anti-NDV immune responses in chickens was observed. However, this could be ascribed to the fact that the delivered doses were too low as the antigen content in the preparations could not be determined due to the lack of appropriate reagents. Further experiments will be necessary for production scale up and immunogen quantification in order to test the efficacy of the proposed formulations in NDV prevention.

## Introduction

Newcastle disease (ND) is one of the major viral-induced poultry diseases that affects avian breeding and production worldwide with tremendous economic losses (Mayers et al. 2017). The severity of the symptoms depends on the aggressiveness of the infecting Newcastle Disease virus (NDV) strain (asymptomatic, lentogenic, mesogenic and velogenic neurotropic or viscerotropic) (Alexander 2000) but also on the age and type of host. These range from mild respiratory symptoms to head twisting, spasms, paralysis, tremors, prostration, diarrhoea which can lead to a mortality rate close to 100% (Lamb and Parks 2007). A major problem for disease spreading is the wide diversity of avian species, both domestic and wild, that may be infected with asymptomatic lentogenic isolates and that represents reservoirs of the virus that can be transmitted by shedding, air exhalation or through faeces. Furthermore, the persistence of these asymptomatic viral strains in poultry contributes to maintain the virus in the farm environment and increases the risk of emergence of new viral mutants (Gould et al. 2001).

NDV is a spherical pleomorphic enveloped virus, member of the genus *Avulavirus*, belonging to the family *Paramyxoviridae*. The viral genome is a non-segmented, single-stranded negative sense (ss(-)) RNA of 15.2 kilobases (kb) (Dortmans et al. 2011). It contains six genes, five of which are monocistronic and encode: the nucleocapsid protein (N), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase protein (HN), and the large protein (L) that is the RNA dependent RNA polymerase (RdRp). The sixth gene can be transcribed by RNA editing into three different mRNAs encoding the structural phosphoprotein (P) and the non-structural V and W proteins (Ganar et al. 2014). The HN and F glycoproteins are anchored at the outer surface of the viral envelope and are involved in the elicitation of neutralizing antibodies (Kumar et al. 2011). They are both required for infection, and the F protein is specifically involved in the fusion of the virus envelope with the host cell plasma membrane. It assembles in homotrimers at the surface of the virion in a precursor form identified as F0 that

is cleaved by the proteases of the infected host into two subunits, F1 and F2, that are linked together via a disulfide bond (Swanson et al. 2010).

There are no specific therapeutic treatments for NDV infection, and the disease diffusion is mostly controlled with vaccination programs based on inactivated or live-attenuated viruses (Mayers et al. 2017). Inactivated vaccines need to be injected several times together with adjuvants, contributing to increase labour-intensity and costs, while attenuated vaccines, generally based on lentogenic strains such as Hitchner B1 and La Sota, may induce post-vaccination reactions, such as mild respiratory symptoms, and can be ineffective in controlling the spread of virulent strains, presenting the risk of propagating to healthy animals and of reversion to virulence (Bello et al. 2018). In order to overcome these issues, novel recombinant subunit vaccines are under development and offer several advantages among which the most important ones are the intrinsic safety and the possibility to differentiate infected from vaccinated animals (DIVA) (Capua et al. 2003). Recombinant NDV subunit vaccines are commonly based on the production of the major immunogenic proteins F and HN in different expression systems, such as *Escherichia coli* (Wong et al. 2009), insect cells through baculovirus (Lee et al. 2008; Ge et al. 2016) and yeasts (*Pichia pastoris*) (Kang et al. 2016).

Plant expression systems have been extensively used for the production of recombinant antigens for veterinary applications mainly because of their safety and potentially low manufacturing costs (Topp et al. 2016). The first plant-made biopharmaceutical licensed was a vaccine against NDV based on HN produced in cultured plant cells (Vermij and Waltz 2006). Different platforms, such as transgenic rice and maize, have been used to produce F (Guerrero-Andrade et al. 2006; Yang et al. 2007). In these studies, the expression of the full-size protein was verified as well as its immunogenicity and also when orally delivered to chickens demonstrated efficient in inducing protection from NDV challenge (Guerrero-Andrade et al. 2006). Neutralizing epitopes of the F protein were also transiently expressed in *Nicotiana*



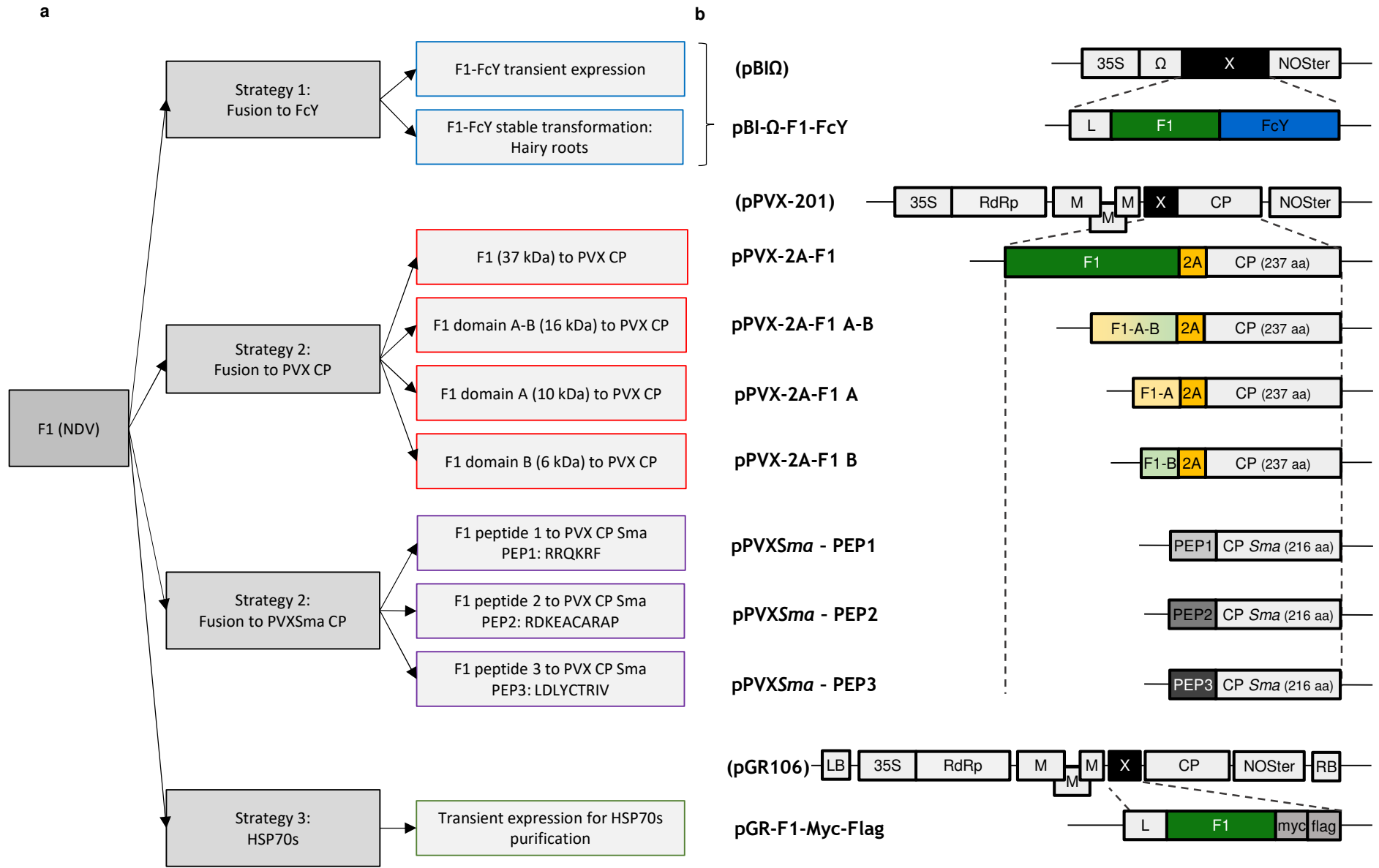
*benthamiana* as a fusion to neutralizing epitopes of the HN protein (Shahriari et al. 2016) or to the capsid protein of Cucumber Mosaic Virus (CMV) (Natilla and Nemchinov 2008).

In the present work, the F1 subunit of the F protein was produced in *N. benthamiana* and *Brassica rapa* subsp. *rapa* expression platforms with the aim of improving immunogenicity but at the same time to simplify purification. F1 was selected instead of the whole protein because it contains important neutralizing epitopes and allows to reduce the dimensions and structural complexity of the expressed polypeptide (Toyoda et al. 1989; Chen et al. 2001b). To further reduce factors that may affect protein accumulation/recovery, the sequences encoding the N-terminal fusion peptide, responsible for promoting the protein insertion into the target membrane that leads to the fusion process and the C-terminal transmembrane domain of F1 were removed.

Three main expression strategies were adopted (Figure 1). The first strategy was based on the fusion of F1 to the fragment crystallizable of avian IgY immunoglobulins (FcY) in order to improve its targeting and uptake by antigen-presenting cells (APCs) through receptors such as FcRY and CHIR-AB1 (He and Bjorkman 2011; Viertlboeck and Göbel 2011; Rage et al. 2019). The second strategy was based on the fusion of F1 or its domains/peptides (Figure 2) to the coat protein (CP) of potato virus X (PVX) (with or without the 2A linker of the Foot and Mouth Disease Virus, FDMV). This strategy may allow to obtain high yields of easily purifiable chimeric virus particles (CVPs) displaying the antigen and endowed with the ability to induce the activation of both antibody and cell-mediated immune responses without adjuvant (Marusic et al. 2001; Lico et al. 2009; Lico et al. 2015). The last strategy was based on the transient expression of F1 in leaves and on the purification from plant tissues of plant Heat Shock Proteins 70 (HSP70) (Rosenzweig et al. 2019). Indeed, it has been demonstrated that these molecular chaperones, involved in protein folding, carry polypeptides that are the fingerprinting of the proteins expressed into a cell and may be used as vehicles for the delivery of these

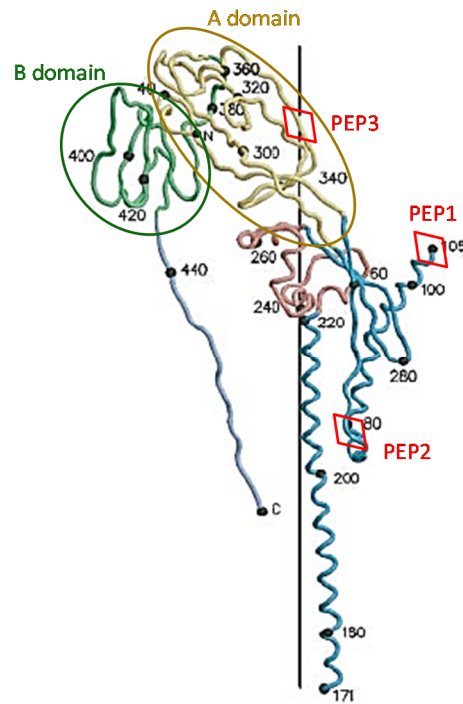
polypeptides that by binding to receptors expressed on APCs can activate specific immune responses (Buriani et al. 2012).

Overall, the obtained results indicate that F1 is a protein difficult to express, probably due to intrinsic features and difficult to analyse experimentally, due to the lack of appropriate reagents. Further experiments will be necessary to scale up production and recover quantities of the antigen sufficient to test immunogenic properties.



**Figure 1:** Schematic representation of the designed F1-based immunogens and of the vectors used for their expression in plants

(a) Schematic representation of the designed F1-based immunogens. In Strategy 1, F1 was fused to the fragment crystallizable region of a chicken IgY antibody (FcY). In Strategy 2, to obtain chimeric PVX particles the F1 protein or its domains (F1 A-B, F1 A and F1 B) were fused to the coat protein (CP) of wild type (w.t.) PVX through the FMDV-derived 2A peptide, while F1 peptides were fused to the CP of PVXSma mutant (Lico et al. 2006). In Strategy 3, F1 fused to the c-Myc and Flag tags was transiently expressed in leaves and plant HSP70 purified. (b) Schematic representation of the constructs used to express the different F-based immunogens in plants. To express the F1-FcY fusion, the encoding nucleotide sequence was inserted into the pBI- $\Omega$  vector to obtain the pBI- $\Omega$ -F1-FcY construct. To produce PVX chimeric particles, the pPVX201 and pPVXSma viral expression vectors, carrying the complete cDNA genome of w.t. PVX and PVXSma mutant respectively, were used to obtain the pPVX-2A-F1, pPVX-2A-F1 A, pPVX-2A-F1 B, pPVX-2A-F1 A-B, pPVXSma-PEP1, pPVXSma-PEP2, and pPVXSma-PEP3 constructs. To produce plant HSP70, the nucleotide sequence encoding F1 fused to c-Myc and Flag tags was inserted as additional open reading frame into the pGR106 vector, obtaining the pGR-F1-Myc-Flag construct. In all the constructs, gene expression was under the control of the Cauliflower Mosaic Virus 35S promoter (35S), the translation enhancer sequence ( $\Omega$ ) of Tobacco Mosaic Virus, and the terminator sequence of the *nopaline synthase* gene (NOSter) of *Agrobacterium tumefaciens*. L: sequence encoding the secretion signal peptide from the heavy chain of an embryonic mouse immunoglobulin; 2A: sequence encoding the 2A peptide of Foot and Mouth disease virus; RdRp: RNA dependent RNA polymerase; M: movement proteins; CP: coat protein; LB and RB: Left and Right Border sequences.



**Figure 2:** Schematic localisation onto the stereo backbone structure of the monomeric NDV-F protein (Chen et al. 2001b) of the F1 domains and peptides.

The F1 protein expressed as fusion with FcY goes from amino acid (aa) 143 to aa 500.

## Strategy 1: F1 fusion to the fragment crystallizable of avian IgY immunoglobulins

### Materials and methods

#### Plant-expression constructs

The nucleotide sequence encoding F1, and derived from an Egyptian velogenic NDV strain isolated in 2013, was kindly provided by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe).

The *f1-fcy* gene was synthetically constructed by fusing the nucleotides encoding the amino acid sequence 143-500 of the NDV fusion protein (GenBank Accession Number AYN07325.1), corresponding to the NDV F1 protein deprived of the fusion peptide and of the transmembrane domain (*f1* gene), with those encoding the amino acid sequence 167-504 of a chicken IgY (GenBank Accession Number S00390) corresponding to the fragment crystallizable domain (FcY) (Figure 1a,b) (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 a similarly digested pGEM-NOS plasmid (Lombardi et al. 2009) to be then transferred, together with the terminator of *Agrobacterium tumefaciens nopaline synthase* gene (NOSter), into the binary vector pBI-Ω (Marusic et al. 2007) using *Bam*HI/*Eco*RI restriction sites, obtaining pBI-Ω-F1-FcY. In this vector, gene expression is under the control of the constitutive 35S promoter of Cauliflower Mosaic Virus (35SCaMV), the Ω translational enhancer sequence from Tobacco Mosaic virus (Gallie 2002) and the NOSter sequence.

### **Transient expression in *N. benthamiana* plants**

*A. tumefaciens* (LBA4404) (Thermo Fisher Scientific, Rockford, IL, USA) harbouring either the plant expression vector pBI- $\Omega$ -F1-FcY or the p35:AMCV-P19 construct (carrying the p19 silencing suppressor gene from the Artichoke Mottled Crinkle Virus, AMCV) (Lombardi et al. 2009) were grown overnight (o.n.) at 28 °C separately in 500 ml of Luria-Bertani (LB) medium supplemented with 50 mg/l Kanamycin (Kan), then centrifuged at 4,000 x g for 10 min and each pellet resuspended in infiltration buffer [10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM MgCl<sub>2</sub>, pH 5.8] to obtain a final optical density at 600 nm (OD<sub>600</sub>) of 0.8. Equal volumes of each suspension were then mixed together (final OD<sub>600</sub>= 0.4 each) and used to infiltrate *N. benthamiana* plants. Small scale production was obtained by infiltrating the leaves with the bacterial suspension using a needle-less syringe, while large-scale production was obtained by submerging 6 weeks-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 achieve the penetration into the leaves. Leaves were collected 4 to 10 days post infiltration (d.p.i.) for the validation of F1-FcY expression (small scale) or at 6 d.p.i. for purification (large scale). The negative control was obtained by infiltrating plants only with *A. tumefaciens* carrying the p19 construct.

### **F1-FcY protein extraction**

For the validation of F1-FcY expression, protein extracts from agroinfiltrated leaves were obtained by grinding 200 mg of tissue in liquid nitrogen and then homogenizing with Ultraturrax T25 in phosphate-buffered saline (PBS) pH 7.5 supplemented with a protease inhibitor cocktail (cOmplete<sup>TM</sup>, Roche, Mannheim, Germany) in a 1:2 weight/volume (w/v) ratio. The extracts were centrifuged at 4 °C, 20,000 x g, 20 min and the supernatants recovered for further analysis.

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 x *g* for 10 min to obtain clear fluids corresponding to IF.

For F1-FcY clarification experiments, leaves were ground in liquid nitrogen in the presence of 4% weight/weight (w/w) polyvinylpolypyrrolidone (PVPP), 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 x *g* for 20 min, the sample was added with 4% (w/v) PVPP, incubated 2 h at room temperature (r.t.) and then centrifuged at 10,000 x *g* for 10 min. The sample was adjusted to pH 7.5, centrifuged again at 20,000 x *g* for 20 min and the clarified supernatant recovered.

For F1-FcY large scale extraction, 20 g of leaves were ground in liquid nitrogen, mixed 1:2 (w/v) with extraction buffer (20 mM sodium phosphate, pH 7.5) supplemented with cOmplete™, and homogenized using Ultraturrax T 25. The plant extract was then filtered with Miracloth paper and centrifuged at 20,000 x *g* for 20 min. A first precipitation step was performed by adding to the supernatant ammonium sulfate up to 10% saturation, at 4 °C, for 1 h, with gentle agitation. The solution was then centrifuged at 10,000 x *g* for 20 min, and the supernatant added with ammonium sulfate up to 30% saturation for the second precipitation step and gently stirred o.n. at 4 °C. After further centrifugation at 10,000 x *g* for 20 min, the pellet was resuspended in extraction buffer (20 mM sodium phosphate, pH 7.5). Extracts were then filtered through a 0.45 μM polyethersulfone (PES) membrane before purification.



**F1-FcY purification**

In the case of affinity chromatography based on thiophilic adsorption (HiTrap IgY HP, GE Healthcare, Uppsala, Sweden), the extract was supplemented with potassium sulfate (to a final concentration of 0.5 M) and passed through the column following manufacturer's instructions. The F1-FcY fusion protein was eluted from the column using 20 mM sodium phosphate buffer pH 7.5, collecting 1.5 ml fractions.

Alternatively, the extract was also passed through the Ligatrap IgY column based on peptoid ligands that specifically bind IgY (Ligatrap<sup>TM</sup> Technologies, Raleigh, NC, USA) and elution from the column (0.4 ml fractions) was performed using 0.1 M sodium acetate pH 4.0. Fractions were then neutralized to pH 7.0 using 3 M Trizma base pH 11.0.

In the case of the GenScript IgY-based purification method (GenScript, Piscataway, NJ, USA), the extract was incubated with the resin at r.t. for 30 min. The resin was recovered by centrifugation at 5,000 x g for 1 min and, after three washes with PBS, proteins were eluted by boiling the resin 5 min in SDS-PAGE loading buffer (50 mM Tris HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.1% (w/v) bromophenol blue) supplemented with 3%  $\beta$ -mercaptoethanol. The supernatant containing the purified product was recovered after centrifugation at 5,000 x g for 30 s.

**Hairy roots generation**

The pBI- $\Omega$ -F1-FcY construct was electroporated into *Rhizobium rhizogenes* (A4) (ATCC<sup>®</sup> 43057<sup>TM</sup>, Manassas, VA, USA). The transformed bacteria were grown in 50 ml of Yeast Extract Beef (YEB) medium supplemented with appropriate antibiotics (50 mg/l Kanamycin) at 28°C to be then pelleted at 3,000 x g, 10 min and resuspended to a final OD<sub>600</sub> of 1 in Murashige and Skoog medium (MS) pH 5.8, containing 100  $\mu$ M 3',5'-Dimethoxy-4'-hydroxyacetophenone (acetosyringone) and 3% (w/v) sucrose.

*N. benthamiana* leaves were sterilized by 10 min incubation in 0.5 % sodium hypochlorite, washed 4 times in sterile water, cut in segments of 1x1 cm, wounded on the abaxial side with a needle tip and immersed into the *R. rhizogenes* suspension for 30 min swirling in the dark. The fragments were then dried and positioned with the abaxial side upwards on Petri dishes containing MS agar medium supplemented with 2 mM 2-(N-morpholino) ethanesulfonic acid (MES), 3% (w/v) sucrose, 300 mg/l Kanamycin and cefotaxime (MS/agar/Kan/Cef), to be transferred onto fresh substrate every two weeks.

*B. rapa subsp. rapa* seeds were sterilized by 10 min incubation in 70% volume/volume (v/v) ethanol followed by 2 min incubation in 5% (w/v) sodium hypochlorite before 2 washes of 10 min in sterile water. Seeds were grown on solid medium ( $\frac{1}{2}$  MS, 1% (w/v) sucrose and 2 mM MES, pH 5.7) for 10 days at 25 °C with 16 h light and 8 h dark. Plant stems were then wounded with a sterile needle previously submerged in the *R. rhizogenes* suspension and incubated in the same conditions for 1 week.

Hairy roots (HR) clones eventually emerging from the leaves fragments (*N. benthamiana*) or from hypocotyls (*B. rapa*) were excised and transferred individually onto fresh Petri dishes containing MS agar (*N. benthamiana*) or  $\frac{1}{2}$  MS agar (*B. rapa subsp. rapa*) medium, supplemented with 3% (w/v) sucrose, 2 mM MES, pH 5.7. The growing HR clones were then transferred every 2 weeks for *R. rhizogenes* eradication onto plates with decreased concentrations of cefotaxime (250, 125 and 50 mg/l) until no antibiotic was added.

### **SDS-PAGE and Western Blot (WB) analysis**

Proteins were separated by 10-12% SDS-PAGE under non-reducing and reducing conditions, the latter obtained by adding 3%  $\beta$ -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 Polyvinylidene Difluoride (PVDF) membrane (Amersham<sup>TM</sup> Hybond® P 0.45

$\mu$ M, GE Healthcare, Freiburg, Germany) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare) for 70 min, at  $0.8 \text{ mA/cm}^2$  and 30 V. Membranes were incubated o.n. at  $4^\circ \text{C}$  in PBS containing 5% (w/v) skimmed milk (5% milk-PBS, blocking solution). PiNK plus or BLUelf (GeneDireX, Las Vegas City, NV, USA) were used as pre-stained protein molecular weight (MW) markers. Purified chicken IgY (AC146, Millipore, Temecula, CA, USA) was used as a positive control. The detection was performed with an anti-IgY horseradish peroxidase (HRP)-conjugated antibody (A9046, Sigma-Aldrich, Saint-Louis, MO, USA) diluted 1:2,000 in 2% milk-PBS. The ECL SuperSignal® West Pico (Thermo Fisher Scientific, Rockford, IL, USA) and the ImageQuant™ LAS 500 system (GE Healthcare) were used for chemiluminescence signal detection.

### **Proteomic and glycosylation analysis**

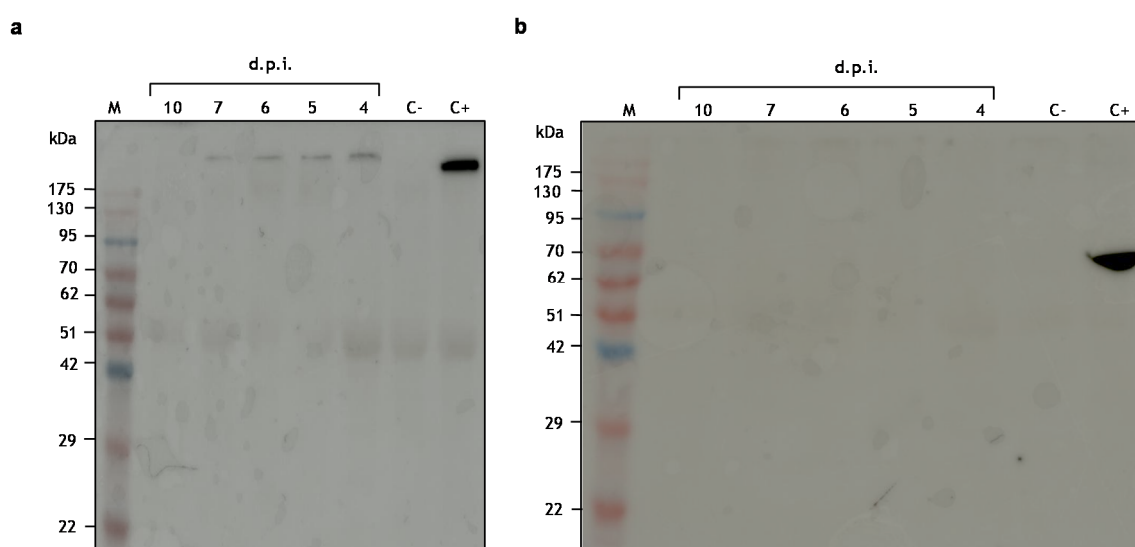
Proteins were separated on a reducing 10% SDS-PAGE, Coomassie-stained and bands were excised, triturated, *in-gel* reduced and S-alkylated and finally digested with endoprotease Asp-N (Roche, Mannheim, Germany) (Salzano et al. 2013). Peptides were extracted from the gel particles using 5% formic acid/acetonitrile (1:1 (v/v)), and digest solutions were concentrated and desalted before mass spectrometry analysis by using  $\mu$ ZipTipC18 pipette tips (Millipore). Protein digests were analysed by nanoLC-ESI-Q-Orbitrap MS/MS using an 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  $\times$  75  $\mu$ m internal diameter, 2- $\mu$ m particles, 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 F1-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, 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.

## Results

### F1-FcY transient expression in leaves

*N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* LBA4404 strains carrying pBI- $\Omega$ -F1-FcY and p35:AMCV-P19 constructs to produce a secretory version of F1-FcY recombinant fusion protein (Figure 1a,b). The p19 silencing suppressor protein was used to enhance recombinant protein accumulation by reducing gene silencing plant defence response. Leaves were collected at 4, 5, 6, 7 and 10 d.p.i., and the presence of F1-FcY in crude extracts was assessed by SDS-PAGE followed by WB analysis using an anti-IgY antibody (Figure 3). Extracts from leaves infiltrated only with *A. tumefaciens* carrying the p19 construct were used as negative control (C-), while purified IgY was used as positive control and, as expected, migrated with an apparent molecular mass of about 180 kDa in non-reducing conditions (Figure 3a), and of about 70 kDa in reducing conditions (Figure 3b). The analysis of the samples under non-reducing conditions demonstrated the expression of F1-FcY into the leaves with decreasing yields from 4 to 7 d.p.i. evidencing the presence of a band with high molecular mass value (>170 kDa), possibly indicating the formation of F1-FcY multimers/aggregates (Figure 3a). At 10 d.p.i. no band could be detected possibly indicating low stability of the fusion protein.



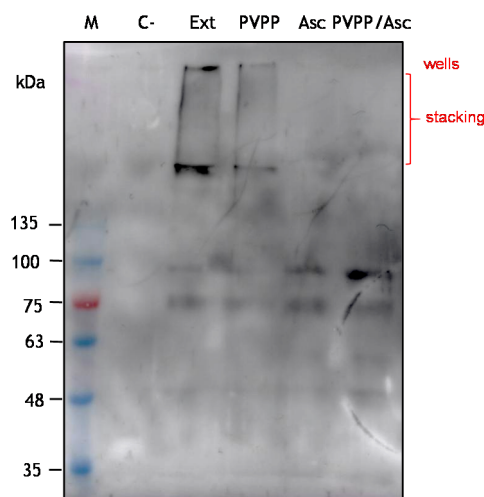
**Figure 3:** Analysis of F1-FcY expression in *N. benthamiana* leaves under non-reducing and reducing conditions

WB analysis with an anti-IgY antibody of normalized protein extracts prepared from leaves infiltrated with *A. tumefaciens* strains harbouring pBI- $\Omega$ -F1-FcY and p35:AMCV-P19 at different d.p.i., and separated by 10% SDS-PAGE under (a) non-reducing or (b) reducing conditions. M: molecular weight marker; C-: leaves infiltrated only with *A. tumefaciens* harbouring p35:AMCV-P19; C+: purified chicken IgY (100 ng).

Under reducing conditions, the presence of F1-FcY was not detected and no further information could be obtained about the molecular mass of the accumulated F1-FcY protein. To verify the secretion and correct processing of the fusion protein, intercellular fluids (IF) of leaves at 6 d.p.i. were also assayed by SDS-PAGE performed under non-reducing conditions followed by WB analysis but also in this case the protein could not be detected (data not shown).

### **F1-FcY extraction and purification**

The extraction/purification protocol of F1-FcY was set-up using batches of 20 g of vacuum-agroinfiltrated *N. benthamiana* leaves at 6 d.p.i.. In the attempt to remove undesired plant compounds (e.g. phenols/pigments) and proteins, similarly to what obtained with PD-FcY (see Chapter 2), the extraction was performed using an acidic buffer containing PVPP and ascorbic acid but, by using this approach, F1-FcY was significantly lost during extraction. In particular, the signal was reduced of more than fifty percent after PVPP treatment and the totality of the multimeric forms disappeared after the treatment with ascorbic acid, alone or in combination with PVPP (Figure 4).



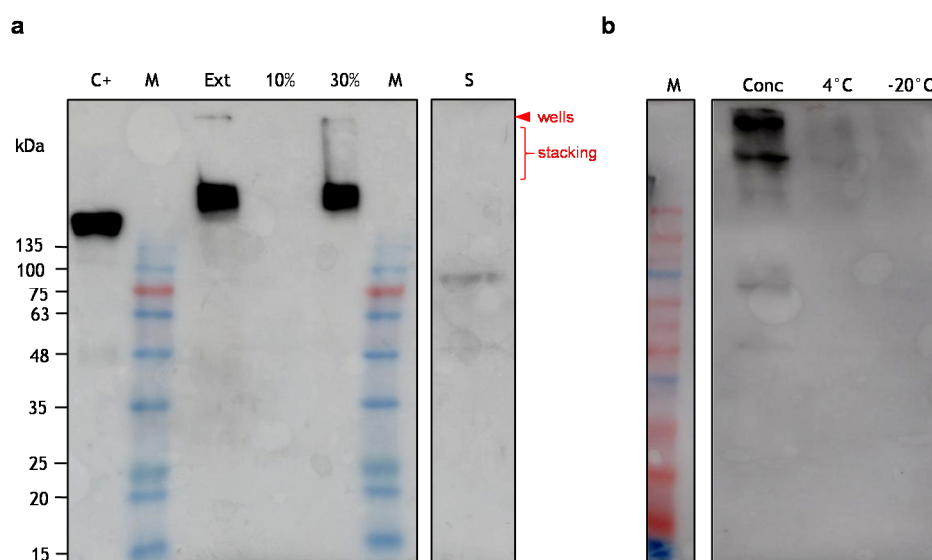
**Figure 4:** Analysis of the effects of the clarification of crude plant extract on F1-FcY

WB analysis with an anti-IgY antibody of clarified F1-FcY plant protein extracts separated by 10% SDS-PAGE under non-reducing conditions. Four different extraction protocols were compared in which leaves were: i) ground in liquid nitrogen and homogenized in the presence of extraction buffer (20 mM sodium phosphate) (Ext); ii) ground in liquid nitrogen in the presence of 4% (w/w) PVPP and further processed by adding extraction buffer supplemented 4% (w/v) PVPP iii) ground in liquid nitrogen and further processed by adding extraction buffer supplemented with 1% (w/v) ascorbic acid and then adjusted to pH 7.5 (Asc) iv) ground in liquid nitrogen in the presence of 4% (w/w) (PVPP), and further processed by adding extraction buffer supplemented with 1% (w/v) ascorbic acid and 4% (w/v) PVPP and then adjusted to pH 7.5 (PVPP/Asc). All extracts were then clarified by centrifugation before further analysis. Twenty  $\mu$ L of extract was loaded in each well. M: molecular weight marker; C-: protein extract from leaves infiltrated only with *A. tumefaciens* harbouring the p19 construct.

A second approach based on protein extraction with 20 mM sodium phosphate and precipitation with ammonium sulfate was then carried out. In the attempt to remove part of the unwanted proteins, ammonium sulfate was added to the extract to reach 10% saturation and the sample centrifuged to pellet the precipitated proteins. The saturation in the supernatant was then increased to 30% by supplementation with ammonium sulfate before a second centrifugation step. The pellets obtained after each centrifugation were resuspended in a 5-10-fold lower volume of extraction buffer, respectively, and the last supernatant was recovered. The analysis of an aliquot of these samples by SDS-PAGE performed in non-reducing conditions followed

by WB with the anti-IgY antibody confirmed the presence of one main band with high molecular mass ( $>170$  kDa) in the pellet obtained after the second centrifugation corresponding to the proteins precipitated between 10 and 30% of ammonium sulfate saturation (Figure 5a) while no signal was observed in the pellet of the proteins precipitated between 0 and 10% ammonium sulfate saturation. A faint band with molecular mass of about 70 kDa was evidenced in the supernatant obtained after the second centrifugation. The presence of bands into the wells and in the stacking gel was also revealed both in the crude extract and in the concentrated sample, probably indicating that the fusion protein tends to precipitate.

Considering the very faint intensity of the band observed in the supernatant and the absence of a signal in the first pellet, the precipitation of the proteins did not result in appreciable F1-FcY losses. A reduction of the complexity in plant compounds content was also observed in the concentrated sample as previously observed for PD-FcY (see Chapter 2, Figure 4a). However, once concentrated, F1-FcY was not stable and the protein was rapidly lost just by storage for about 4 hours or more at 4 °C or by performing a freeze/thaw cycle at -20 °C. (Figure 5b).



**Figure 5:** Analysis of the precipitation of F1-FcY from plant extract with ammonium sulfate.

(a) WB analysis with an anti-IgY antibody of F1-FcY protein extracts after separation by 10% SDS-PAGE under



non-reducing conditions. Two-fold concentration was performed by ammonium sulfate precipitation. M: molecular weight marker; C+: purified chicken IgY (100 ng); Ext: F1-FcY crude plant extract; 10%: proteins detected in the pellet obtained by 10% ammonium sulfate saturation of the F1-FcY plant extract (10 µl); 30%: proteins detected in the pellet obtained by 10% to 30 % ammonium sulfate saturation of the F1-FcY plant extract (10 µl); S: proteins detected in the supernatant obtained by 30% ammonium sulfate saturation of the F1-FcY plant extract (20 µl). **(b)** WB analysis with an anti-IgY antibody of freshly prepared F1-FcY concentrated protein extract (Conc) or after storage at 4 °C or -20 °C separated by 10% SDS-PAGE under non-reducing conditions.

F1-FcY was purified from the concentrated protein sample obtained through ammonium sulfate precipitation using three affinity-chromatography methods: HiTrap IgY, Ligatrap IgY and GenScript IgY.

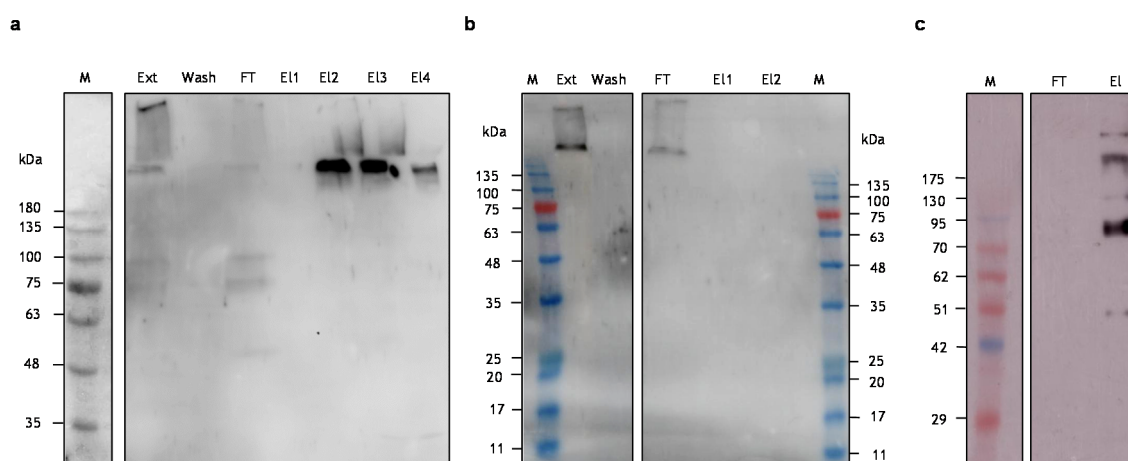
WB analysis of the fractions eluted from the HiTrap IgY column separated under non-reducing conditions (El1-4) revealed the presence of one band migrating at high molecular mass (>170 kDa) mainly in the fractions 2 and 3 (Figure 6a). Bands at about 100 and 75 kDa were also observed in the extract (Ext) and in the flow-through (FT) but not in the eluted fractions. The low intensity of the signal observed in the flow-through compared to the extract demonstrates the binding efficiency of the resin. Furthermore, the band detected in the eluted fractions and migrating at high molecular mass (above 180 kDa) were more intense as compared to the equivalent bands observed in the concentrated extracts, while the other bands were absent, demonstrating that the resin binds preferentially the multimeric F1-FcY form. However, also the purified F1-FcY was not stable and rapidly degraded (data not shown). Moreover, due to the impossibility of adopting the protocol developed to clarify the plant extract before purification using PVPP and ascorbic acid, the sample was rich in plant compounds that bound with high affinity and permanently to the resin, reducing its durability and its efficiency and preventing its further use.

The purification adopting the Ligatrap IgY column demonstrated that this resin do not bind to F1-FcY, as samples separation under non-reducing conditions and analysed by WB evidenced

the presence of bands only in the extract and in the flow-through (Figure 6b).

WB analysis of the sample eluted from the GenScript IgY resin (EI; Figure 6c) showed a complex pattern of bands. No bands were detected in the column FT indicating the high binding efficiency of the resin. Unfortunately, the release of F1-FcY can be obtained only by boiling the resin beads in SDS-PAGE sample loading buffer, thus making the final product unsuitable for functional immunization purposes.

Based on these results, the GenScript-purified F1-FcY was only used for further mass spectrometry characterization experiment.



**Figure 6:** Purification of F1-FcY using different affinity chromatography methods

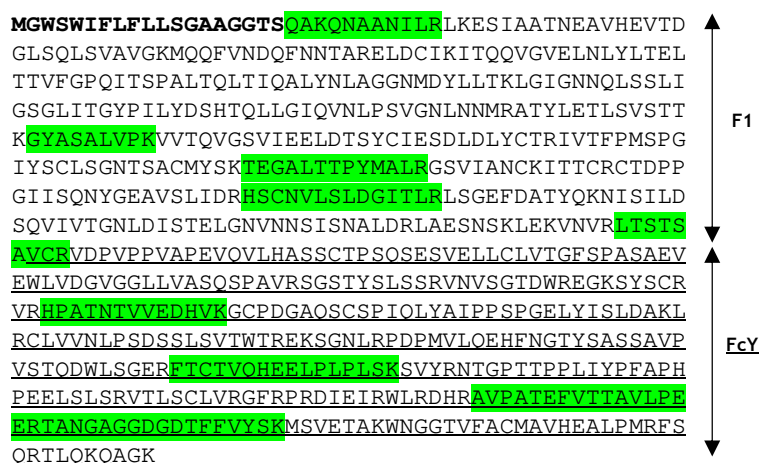
WB analysis with an anti-IgY antibody of the fractions eluted from the **(a)** HiTrap IgY resin, the **(b)** Ligatrap IgY resin and the **(c)** GenScript IgY resin. The samples were separated by 12% SDS-PAGE under reducing conditions. M: molecular mass marker; Ext: concentrated plant extract (20  $\mu$ l); Wash: resin wash with equilibration buffer (20  $\mu$ l); FT: flow-through (20  $\mu$ l); EI1-4: eluted fractions (5  $\mu$ l for HiTrap IgY, 20  $\mu$ l for Ligatrap IgY and 2  $\mu$ l for GenScript).

### F1-FcY proteomic analysis

The GenScript-purified F1-FcY was separated on SDS-PAGE gel and stained with Coomassie but because of the low concentration of proteins in the sample, no bands specific for F1-FcY

were visible (data not shown). Fragment corresponding to the lane of the gel was therefore excised and analysed by nanoLC-ESI-Q-Orbitrap MS/MS in order to have indications about the F1-FcY protein present in the purified sample. The proteomic analysis based on peptide mapping confirmed the presence of F1-FcY in the sample by identifying peptides covering all the protein sequence, except the last 38 aa at the C-terminus (Figure 7).

Due to the very low quantity of F1-FcY recovered, the glycosylation profile of the fusion protein could not be determined.



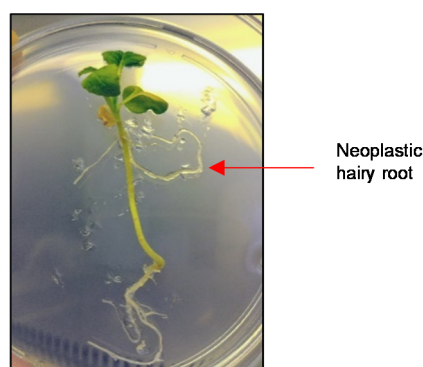
**Figure 7:** Proteomic characterization of the F1-FcY protein purified with the GenScript IgY resin

Distribution on the F1-FcY aminoacidic sequence of the peptides identified (highlighted in green) with nLC-ESI-Q-Orbitrap MS/MS and Byonic search in the protein sample eluted from the gel. Before the proteomic analysis the sample was digested with the endoprotease Asp-N. The signal peptide is indicated in bold and FcY domain is underlined.

### Generation of HR expressing F1-FcY

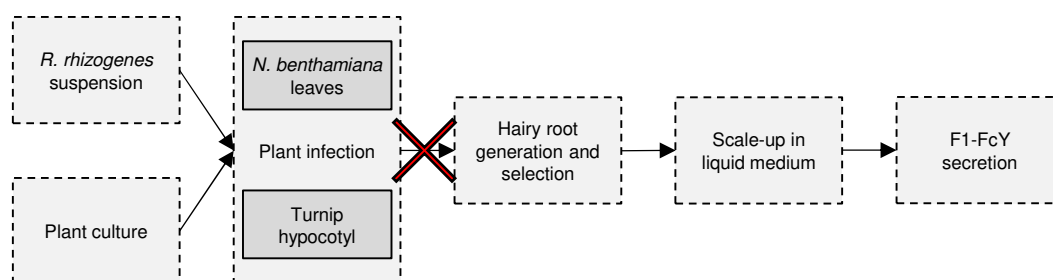
*N. benthamiana* leaf fragments or *B. rapa subsp. rapa* plantlets cultivated *in vitro* were inoculated with *R. rhizogenes* A4 strain carrying the pBI-Ω-F1-FcY construct to generate HR stably expressing a secretory version of the F1-FcY recombinant protein (Figure 1a,b).

HR were expected to emerge from the wounding sites of *N. benthamiana* leaf fragments and *B. rapa subsp. rapa* hypocotyls about 3 weeks and 1 week after the infection, respectively, as previously observed for PD-FcY (see Chapter 3). However, no HR were generated from *N. benthamiana* infected leaf fragments while a total of about 150 HR clones were excised from *B. rapa subsp. rapa* hypocotyls (Figure 8).



**Figure 8:** Example of HR emerging from turnip (*B. rapa subsp. rapa*) hypocotyl after infection with *R. rhizogenes* carrying pBI- $\Omega$ -F1-FcY.

HR clones were cultured separately on solid medium but no growth was observed 1 month later whereas one week is generally sufficient to see elongation/proliferation (see Chapter 3). For this reason, all the clones were eliminated and the HR approach discontinued (Figure 9).



**Figure 9:** Schematic illustration of the different steps representing the strategy to produce F1-FcY fusion protein from HR.

## Strategy 2: F1, F1 domains and F peptides fusion to the coat protein of PVX

### Materials and methods

#### Plant-expression constructs

The pPVX-2A-CP vector (Marconi et al. 2006) derived from the pPVX201 plasmid (Baulcombe et al. 1995), harbours the whole PVX genome cDNA with the Foot and Mouth disease virus (FMDV) 2A sequence (Donnelly et al. 2001) inserted at the 5'-end of the *cp* gene. In this vector, the genome expression is under the control of the constitutive 35SCaMV promoter and the NOSTer sequence. (Figure 1a, b). The *fl-2a-cp* gene was obtained by inserting the *fl* gene into the pPVX-2A-CP plasmid as fusion to the 5'-end of the *2a-cp* gene. The DNA fragment obtained by Polymerase chain reaction (PCR) amplification of the *fl* gene (described in Strategy 1) with the *PfuTurbo* DNA polymerase using oligonucleotides 5'Not F1 and 3'F1 Sal (Table 1) was digested with the restriction enzymes *NotI* and *SalI* and then inserted into the similarly digested pPVX-2A-CP vector obtaining the pPVX-2A-F1 vector. The *fl(a-b)-2a-cp*, *fl(a)-2a-cp*, *fl(b)-2a-cp* genes were similarly constructed; to this aim DNA fragments were obtained by PCR amplification (*PfuTurbo* DNA polymerase) of the *fl* gene with the oligonucleotides 5'NotF1A and 3'F1BSal, or 5'NotF1A and 3'F1ASal, or 5'NotF1B and 3'F1BSal, respectively (Table 1). The obtained DNA fragments were digested with the restriction enzymes *NotI* and *SalI* and then inserted into the similarly digested pPVX-2A-CP obtaining the pPVX-2A-F1 A-B, pPVX-2A-F1 A and pPVX-2A-F1 B vectors, respectively. The *fl(a-b)*, *fl(a)* and *fl(b)* genes encode the amino acid sequences 288-437 (F1 A-B); 288-381 (F1 A) and 382-437 (F1 B) of the NDV fusion protein (GenBank Accession Number AYN07325.1) corresponding to exposed domains of the NDV F1 protein (Figure 2). *pep1-cp*, *pep2-cp* and *pep3-cp* genes were synthetically constructed by fusing the sequence encoding each peptide in the pPVXSma vector (Lico et al. 2006) to the N-terminus of the mutated PVX CP gene (*pvxSma*) (Figure 1a, b). To this aim, DNA fragments were obtained by annealing a pair of the

sense and anti-sense oligonucleotides 5'NheISacIPVXPep1XmaI and 3'PVXPep1, or 5'NheIPVXPep2SacIXmaI and 3'PVXPep2, or 5'NheISacIPVXPep3XmaI and 3'PVXPep3 (Table 1). The oligonucleotides were designed to generate a fragment with 5'-*NheI* and 3'-*SmaI* sticky ends and were directly ligated into the properly digested pPVXSma plasmid obtaining the plasmids pPVXSma-PEP1, pPVXSma-PEP2 and pPVXSma-PEP3, respectively. PEP1 (RRQKRF), PEP2 (RDKEACARAP) and PEP3 (LDLYCTRIV) immunogenic peptides are positioned on the entire NDV F protein (Figure 2).

| Primers                 |                                                                        |
|-------------------------|------------------------------------------------------------------------|
| 5'F1ClaI                | 5' attccatcgatgcaggctaagcagaatgctgc 3'                                 |
| 3'F1cMylinker           | 5' tccaagatcttcttcagaaataagctttgttctgatgtagatgtcaacc 3'                |
| 3'cMylinkerFlagstopSalI | 5' gccgacgtcgacctattacttatcatcatcatccttataatctcctggccaagatcttcttcag 3' |
| 5'Not F1                | 5' ataagaatgcggccgcgcagctaagcagaatgctgc 3'                             |
| 3'F1 Sal                | 5' gccgacgtcgactgatgtagatgtcaacc 3'                                    |
| 5'NotF1A                | 5' ataagaatgcggccgcgcgaaccttctcagtgaggaaatttg 3'                       |
| 3'F1ASal                | 5' gccgacgtcgacagtagtcaatgcaccctctg 3'                                 |
| 5'NotF1B                | 5' ataagaatgcggccgcgcatacatggcattgagagg 3'                             |
| 3'F1BSal                | 5' gccgacgtcgacagaaagtctaagtgaattcc 3'                                 |
| PVX forward             | 5' ctggggaatcaatcacagtgttg 3'                                          |
| 5'NheISacIPVXPep1XmaI   | 5' ctaggagctcatgtctactgatagaagacagaagagatttctgaagatc 3'                |
| 3'PVXPep1               | 5' ccgggatcttcagaaaatcttctgtcttctatcagtagacatgagctcg 3'                |
| 5'NheIPVXPep2SacIXmaI   | 5' ctagcatgtctagagataaggaagcttgctagagctc 3'                            |
| 3'PVXPep2               | 5' ccgggagctctagcacaagcttcttctctagacatg 3'                             |
| 5'NheISacIPVXPep3XmaI   | 5' ctaggagctcatgtctctggatctgtattgtactagaattgttc 3'                     |
| 3'PVXPep3               | 5' ccgggaacaattctagtacaatacagatccagagacatgagctcg 3'                    |
| PVX reverse             | 5' gcatcagcagagctagact 3'                                              |

**Table 1:** List of the oligonucleotides used for cloning, PCR and sequencing analysis

### PVX infection

Four weeks old *N. benthamiana* plants were inoculated with the different recombinant PVX-expression vectors (pPVX-2A-F1, pPVX-2A-F1 A-B, pPVX-2A-F1 A, pPVX-2A-F1 B, pPVXSma-PEP1, pPVXSma-PEP2, pPVXSma-PEP3) as described previously (Marusic et al. 2001). Briefly, inoculation was performed by abrading the upper side of two leaves (3<sup>rd</sup> and 4<sup>th</sup> leaves)/plant with carborundum (Silicon Carbide, VWR International, West Chester, PA, USA) and then distributing 20 µg of plasmid DNA in 50 µl per leaf. Approximately two weeks later, plant tissues exhibiting typical symptoms of viral systemic infection (upper leaves showing a chlorotic mosaic) were collected. Plants were inoculated in parallel with control vectors carrying the wild type (w.t.) viral genome (pPVX201) or the viral genome with the mutated CP (pPVXSma).

The genetic stability and infectivity of the viral particles generated by each construct was verified by repeated cycles of infection performed as described previously (Lico et al. 2006). Briefly, the leaves collected from infected plants were homogenized in PBS (pH 7.5) in a 1:2 (w/v) ratio and the extracts centrifuged at 4 °C, 20,000 x g, 3 min. Fifty µl of the supernatant were then used to infect the leaves of a new set of plants. At least 2 cycles of reinfection were performed for each construct (3 infections in total). Total RNA was extracted from the leaves using the RNeasy plant mini kit (QIAGEN, Germantown, MD, USA) following the manufacturer instructions. Reverse Transcription (RT)-PCR was then performed using the QuantiTect Reverse Transcription Kit (QIAGEN), following manufacturer instructions, with the PVXforward and PVXreverse oligonucleotides (Table 1) to amplify the N-terminal region of the *cp* gene. The amplified fragments were purified using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer instructions and analysed by sequencing (BMR Genomics, Padova, IT).

**Transmission electron microscopy**

Transmission Electron microscopy (TEM) analysis was performed at the IZS-Ve. Briefly, crude extracts from infected systemic leaves were distributed on carbon-coated grids, stained with a 2% sodium phosphotungstate solution in dH<sub>2</sub>O pH 6.8 and observed on a transmission electron microscope (Philips 208F).

**Fractionated extraction of proteins from leaves**

The fractionated extraction of proteins from leaves was performed as previously described (Donald et al. 1993). Briefly, 200 mg of leaves were ground in liquid nitrogen using a mortar and pestle and then homogenized with Ultraturrax T25 (IKA-Werke, Staufen, Germany) in an extraction buffer (GB buffer: 400 mM sucrose, 100 mM Tris-HCl pH 8.1, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM  $\beta$ -mercaptoethanol) supplemented with protease inhibitor cocktail (cOmplete<sup>TM</sup>; Roche, Mannheim, Germany) in a 1:1 (w/v) ratio. Extracts were centrifuged at 4 °C, 1,000 x g, 10 min obtaining a low-speed pellet fraction enriched in nuclei, chloroplast fragments, and a small proportion of mitochondria (P1). The supernatant was centrifuged at 4 °C, 30,000 x g, 30 min to obtain a high-speed pellet fraction, containing the remaining mitochondria and membranes derived from the endoplasmic reticulum and disrupted organelles (P30), and a supernatant fraction enriched in cytosolic and soluble proteins (S). P1 and P30 were resuspended in TEPT buffer (3.2 M urea, 2% SDS, 62.5 mM Tris-HCl pH 6.8, 5% (v/v)  $\beta$ -mercaptoethanol), boiled 10 min and centrifuged at 4 °C, 1,000 x g, 10 min and supernatants recovered for WB analysis.

**PVX purification**

Chimeric PVX particles were purified from infected plant tissues following the protocol described in (Lee et al. 2014) with slight modifications. Briefly, leaves showing the symptoms



of viral infection were grinded in liquid nitrogen and homogenized with Ultraturrax T25 in extraction buffer (0.1 M Na<sub>3</sub>PO<sub>4</sub>, 0.2% β-mercaptoethanol, 10% volume/volume (v/v) ethanol, pH 7.5) supplemented with cOmplete™, in a 1:2 (w/v) ratio. The plant extract was filtrated with Miracloth paper, centrifuged at 4 °C, 7,800 x g, 20 min and the obtained supernatant recovered, supplemented with 1% (v/v) of Triton X-100 working solution (20% (v/v) in water, pre-autoclaved for better solubility) and incubated at 4 °C for 1 h with gentle stirring before centrifugation at 4 °C, 5,500 x g, 20 min. The sample was then supplemented with 1/5 volume of 1M NaCl, 20% (w/v) PEG, incubated at 4 °C o.n., and then centrifuged at 4 °C, 7,800 x g, 10 min. The pellet was resuspended in 0.05 M Na<sub>3</sub>PO<sub>4</sub> buffer pH 8.0, 1% (v/v) Triton X-100 working solution, centrifuged at 4 °C, 7,800 x g, 30 min and the obtained supernatant was layered on a 30% sucrose cushion and centrifuged in a Sorvall™ WX Ultra 100 ultracentrifuge (Thermo Fischer Scientific) with a Sorvall™ AH-629 rotor at 4 °C, 72500 x g, 150 min. The pellet, containing the virus particles, was resuspended in 0.01 M sodium phosphate buffer pH 7.5 and PVX concentration was measured by Bradford protein assay using as standard different concentrations of bovine serum albumin (BSA).

### **SDS-PAGE and WB analysis**

SDS-PAGE and WB analyses were performed as described in Strategy 1 using purified PVX particles (available in the lab; Lico et al. 2009) as positive control. The detection was performed with a rabbit anti-PVX antibody (Bioreba, n° 1104, Reinach, Switzerland) diluted 1:1000 in 2% milk-PBS followed by incubation with a sheep anti-rabbit HRP-conjugated antibody (Thermo Fischer Scientific) diluted 1:2500 in 2% milk-PBS. Proteins migrating in SDS-PAGE were stained with silver nitrate at r.t.. In detail, to fix the proteins, the gel was immersed in fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) of formaldehyde stock solution (37%)) and incubated with agitation for 30 min. The gel was then washed twice for 10

min with agitation in 50% (v/v) ethanol and then immersed for 1 min in pre-treatment solution (200 mg/l  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ). After two rapid rinses in water, the gel was then immersed in staining solution (2.5 g/l  $\text{AgNO}_3$ , 0.075% (v/v) of formaldehyde stock solution (37%)) for 10 min, washed twice with water and then immersed in development solution (60 g/l  $\text{Na}_2\text{CO}_3$ , 0.05% (v/v) of formaldehyde stock solution (37%), 14 mg/l  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) until protein bands are sufficiently visible. The development reaction was blocked by dipping the gel in a solution containing 50% (v/v) methanol and 12% (v/v) acetic acid. The coloured gel was finally washed with water and stored in 10% (v/v) glycerol.

### **Enzyme-linked immunosorbent assay (ELISA)**

Direct ELISA was performed by coating the wells of ELISA MaxiSorp1 plates (NUNC, Roskilde, Denmark) with the antigens o.n., at 4 °C. Purified w.t. PVX (available in the lab) and NDV (kindly provided by the IZSVe) particles were used as positive controls. The wells were then blocked with 5% milk-PBS for 2 h, at 37 °C, washed 4 times with PBS-0.1% Tween and 2 times with PBS and to be then incubated for 2 h, at 37 °C either with a rabbit anti-PVX antibody (Bioreba, n° 1104) diluted 1:1000 in 2% milk-PBS or with a rabbit anti-NDV serum (kindly provided by the IZSVe) diluted at 1:500 in the same solution. Wells were then washed and incubated for 1 h, at 37 °C, with a secondary sheep anti-rabbit HRP-conjugated antibody (Thermo Fischer Scientific) diluted 1:2500 in 2% milk-PBS. 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).

### Animal studies

Animal studies were performed by IZS-Ve. All animals, Specific Pathogen-Free (SPF) chickens, were kept at a constant temperature range (20-25 °C) and 30-40% relative humidity, with free access to food and water. These chickens are characterized by the absence of Maternal Derived Antibodies (MDA), antibodies that may interfere with the immunization experiment and falsely raise specific antibody titers. In the case of PVX-2A-F1 A-B immunization experiments, 9 SPF chickens were randomly divided into 3 groups of 3 animals. One group of animals was intramuscularly injected with 165 µg of purified chimeric PVX particles per dose emulsified in Montanide ISA 71 VG (Seppic, France). The other two groups of animals were used as positive controls and injected intramuscularly with 8.5 log<sub>10</sub> EID<sub>50</sub> (50% embryo infective dose) of inactivated NDV virus per dose alone or emulsified in Montanide ISA 71 VG (Seppic, France). All the groups were injected 2 times at 6 and 9 weeks post-hatch before challenge at 12 weeks of age. In the case of PVX*Sma*-PEP2 immunization experiments, 9 SPF chickens were randomly divided into 3 groups of 3 animals. Two groups of animals were immunized intramuscularly with 50 µg of purified chimeric PVX particles per dose alone or emulsified in Montanide ISA 71 VG (Seppic, France), while the animals in the third group were used as negative control and injected intramuscularly with 50 µg of PVX*Sma* particles per dose emulsified in Montanide ISA 71 VG (Seppic, France). All groups were injected 4 times weekly starting at 4 weeks post-hatch before challenge at 10 weeks of age.

Blood samples were collected from the wing vein the day before each immunization and before the challenge. The presence of NDV specific antibodies in the sera of immunized chickens was determined by IZS-Ve using a commercial ELISA kit (ID Screen® Newcastle Disease Competition kit, ID.Vet, Grabels, France) following manufacturer's instructions, in the case of PVX-2A-F1 A-B immunization, and with a microneutralisation assay in the case of PVX*Sma*-PEP2 immunization. Briefly, in the microneutralisation assay, serum samples were mixed with

the NDV virus, incubated 1h at 37 °C and the serum-virus mixture added to DF1 chicken fibroblast cells (4<sup>th</sup> passage). If neutralizing antibodies are present in the serum they bind to the virus, preventing the infection of the cells and multiplication of the virus. ELISA measurement with an anti-NDV antibody results in low absorbance values while, in the absence of neutralizing antibodies in the serum, infection of the cells occurs and the virus multiplies resulting in high absorbance values. The presence of PVX specific antibodies in the sera of immunized chickens was determined per ELISA, the wells of MaxiSorp1 plates (NUNC) were first coated with purified w.t. PVX particles (available in the lab) o.n. at 4 °C. The wells were then blocked with 5% milk-PBS for 2 h, at 37 °C, washed and then incubated o.n. at 4 °C with sera of the immunized chickens diluted 1:50 in 2% milk-PBS. Wells were then washed and incubated with rabbit anti-IgY HRP-conjugated antibody (A9046, Sigma-Aldrich, Saint-Louis, MO, USA) diluted 1:2000 in 2% milk-PBS before revelation as previously described.

All chickens were challenged via the oculo-nasal route with a velogenic NDV strain Hertz and the titer of the NDV inoculum was  $10^7$  EID<sub>50</sub>. The post-challenge clinical signs and mortality were recorded daily.

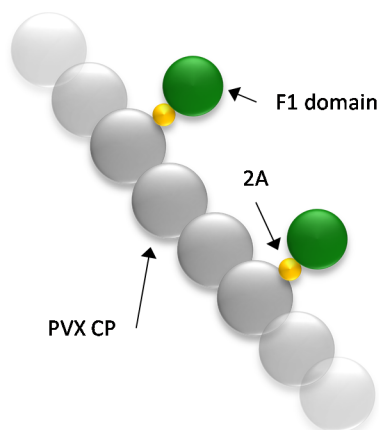
### **Statistical analysis**

Statistical analysis was performed using Student's t-test on R Software (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](http://www.graphpad.com).

## **Results**

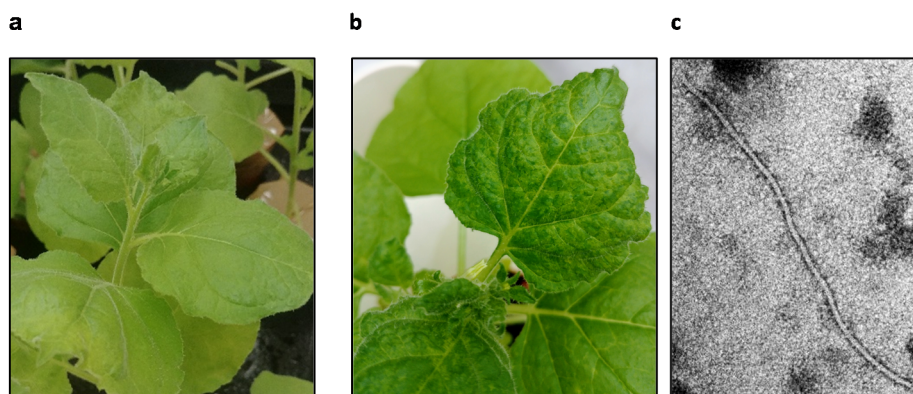
### **Production in leaves of PVX CVPs displaying F1 or its domains**

To produce PVX CVPs, the viral genome within the vector pPVX-2A-CP was modified by inserting as fusion with the 5'-end of the nucleotide sequence encoding the 2A peptide those encoding either the F1 protein deprived of the fusion peptide and of the transmembrane domain (37 kDa) or those of its domains A (10 kDa), B (6 kDa) or A-B (16 kDa), generating the vectors pPVX-2A-F1, pPVX-2A-F1 A, pPVX-2A-F1 B and pPVX-2A-F1 A-B respectively (Figure 1a,b). Because of the large size of the F1 protein (37 kDa) compared to the fusion strategies tested in PVX with the 2A peptide (only one example with a larger protein, 46 kDa), (O'Brien et al. 2000) smaller domains of the fusion protein were also tested to be fused to PVX with a lower risk of steric hindrance and deletion of the inserted sequence (Shukla et al. 2014). These domains were selected for their location in the head region of the F1 protein in the post-conformation state (Figure 2) with potentially enhanced exposure and immunogenicity. The 16 amino acids long 2A peptide of FDMV, fused to the N-terminus of PVX CP, induces a ribosomal skip during the translation of the protein and results in self-cleavage (with an efficiency of about 20%) and production of an heterogeneous population of CP, composed of both w.t. and chimeric units. Both types of CP are involved in viral assembly and this allows to reduce steric hindrance due to the display of the antigen at the N-terminus of the CP and therefore allow to obtain proper assembly of the CVPs and the movement throughout plants (Figure 10) (Lico et al. 2015).



**Figure 10:** Schematic representation of a PVX particle obtained with the 2A fusion strategy. Only some of the CP are chimeric and fused to the antigen while the others are unmodified.

All the constructs were used to inoculate *N. benthamiana* leaves to verify their ability to promote the formation of infective viral particles. No symptoms of PVX infection were observed in the plants inoculated with the construct encoding the fusion of the CP to the whole F1 protein (Figure 11a), while the constructs encoding the fusion to shorter F1 sequences (A, B and A-B) induced the appearance of symptoms of infection throughout the plants in about 2 weeks (Figure 11b). The proper assembly of the filamentous and flexible virus particles was verified by TEM for all constructs (Figure 11c). The genomic stability (genomic portions deletions, rearrangement or mutations) of the genetically chimeric viruses infecting the plants was demonstrated by RT-PCR and sequence analysis in three consecutive infection cycles.



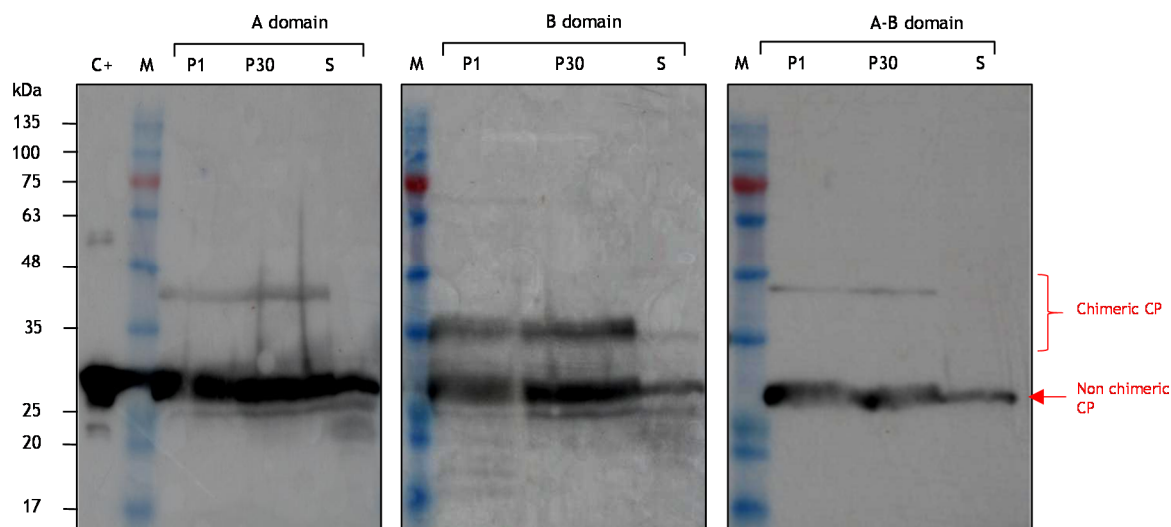
**Figure 11:** Analyses of the PVX infections in plants.

(a) *N. benthamiana* plants 14 d.p.i. with a viral vector unable to result in infection onset. (b) *N. benthamiana* plants 14 d.p.i with typical PVX infection symptoms. (c) TEM acquired image illustrating the virus particles infecting plant tissues.

### Fractionated protein extraction from infected leaves

The fractionated extraction of proteins from the infected leaf tissues allows to analyse separately soluble and insoluble protein fractions in order to determine the distribution and localization of the chimeric and non-chimeric CP in the plant cell. The presence of the viral CP in the different fractions was assessed by SDS-PAGE in reducing conditions followed by WB analysis using an anti-PVX antibody. Purified w.t. PVX particles were used as positive control with the CP migrating, as expected, with an apparent molecular mass of about 25 kDa. In the extracts obtained from the plants infected with the chimeric constructs, two populations of CP were observed, the non-chimeric CP migrating at about 25 kDa and the chimeric CP migrating at 35 (PVX-2A-F1 A), 31 (PVX-2A-F1 B) or 42 (PVX-2A-F1 A-B) kDa (Figure 12). The non-chimeric CP was observed in all the fractions but with higher intensity in those enriched in proteins associated to membranes of organelles (P1 and P30). The intensities of the bands corresponding to the chimeric CP were lower compared to those of the non-chimeric CP and were evidenced only in P1 and P30 fractions with the exception of the CP fused to the B domain of F1. The chimeric CP was therefore synthesized but appeared to be mainly blocked in the

membranes and for this reason probably poorly or not incorporated into the virus particles present in the cytoplasm and circulating in the plant.



**Figure 12:** Analysis of the fractionated protein extracts from infected leaves

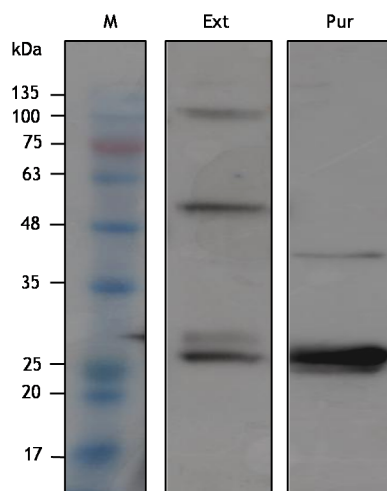
WB analysis with an anti-PVX antibody of fractionated protein extracts prepared from the leaves of the plants infected with PVX-2A-F1 A (left panel), PVX-2A-F1 B (central panel) and PVX-2A-F1 A-B (right panel). Twenty  $\mu$ l samples were separated by 12% SDS-PAGE under reducing conditions. C+: purified w.t. PVX particles (1  $\mu$ g); M: molecular mass marker; P1: low-speed pellet fraction; P30: high-speed pellet fraction; S: soluble fraction.

### PVX-2A-F1 A-B CVPs purification and chickens immunization

The soluble fractions obtained from the leaves infected with each chimeric constructs are poorly concentrated in chimeric CPs carrying the F1 domains, for this reason crude plant extracts are not optimized for immunization. Purification and concentration of virus particles was thus performed in order to enrich in chimeric CP the formulation and focusing on the pPVX-2A-F1 A-B construct that encodes the larger F1 domain. PVX was purified by ultracentrifugation on a 30% sucrose cushion and an aliquot of the obtained sample was separated by SDS-PAGE in reducing conditions followed by WB analysis using an anti-PVX antibody. By this approach, both the w.t. and the putatively chimeric CP could be observed and at a higher concentration



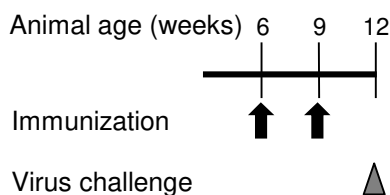
compared to that observed in the crude extract (Figure 13).



**Figure 13:** Analysis of the purified PVX-F1 A-B CVPs.

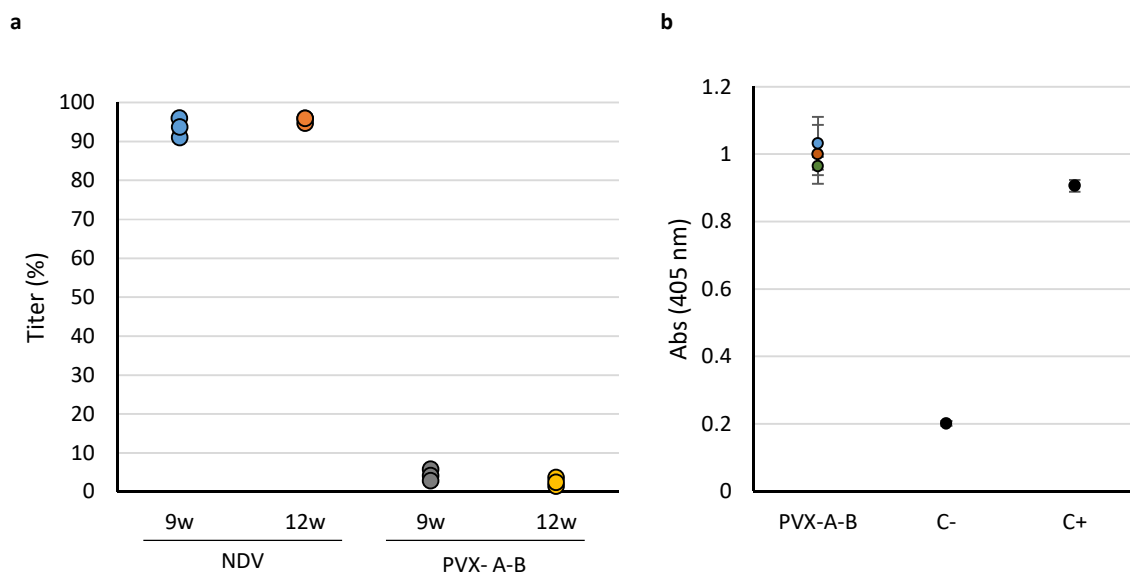
WB analysis with an anti-PVX antibody of the virus particles purified from the plants inoculated with pPVX-F1 A-B after separation by 12% SDS-PAGE under reducing conditions. M: molecular mass marker; Ext: plant extract (20 $\mu$ l); Pur: purified PVX-2A-F1 A-B particles (1 $\mu$ l containing 2  $\mu$ g).

The immunogenicity of the purified particles, that were quantified by Bradford protein assay to 2  $\mu$ g/ $\mu$ l, was then evaluated by intra-muscular administration to SPF chickens. The animals were immunized at week 6 and 9 after hatching with 165  $\mu$ g of PVX plus adjuvant and challenged with velogenic NDV at week 12 after hatching (PVX A-B group) (Figure 14). Two groups of chickens were injected following the same immunization schedule with the commercial inactivated NDV vaccine with (positive control - NDV group) or without (positive control – *ndv* group) adjuvant. Serum samples were collected one day before each immunization and before the viral challenge.



**Figure 14:** Schematic representation of the chicken immunization schedule.

Individual responses were evaluated using a commercial ELISA kit, which measures antibodies specific to NDV in the serum. The results indicated that chickens belonging to the PVX A-B group were unable to rise NDV-specific antibodies (values under the threshold indicated in the kit) in contrast with those immunized with the vaccine plus the adjuvant (NDV group) where antibody titers close to 100% were observed in all animals (Figure 15a). The delivery of the vaccine without the adjuvant (*ndv* group) was less efficient in inducing the production of antibodies after the first immunization (40% instead of 95%) but was fully efficient after the second delivery. All the chickens of the NDV and *ndv* groups were still alive 7 days after the viral challenge while all the chickens of the PVX A-B group died. An ELISA was set up in order to verify the presence in the serum of the animals belonging to this group of antibodies specific for PVX CP. The result of this experiment clearly demonstrated that, at week 9, PVX-specific antibodies were present (Figure 15b).



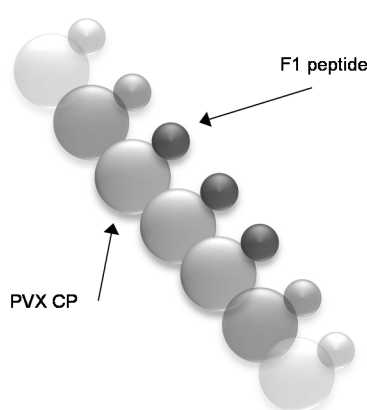
**Figure 15:** Evaluation of the presence of anti-NDV and anti-PVX antibodies in the sera of immunized chickens.

Animals were immunized intramuscularly with PVX particles (emulsified in adjuvant) (PVX A-B group) or inactivated NDV emulsified (NDV group) or not (*ndv* group; not shown) in adjuvant at 6 and 9 weeks (w) of age and sera were collected one day before the second immunization and just before the viral challenge at 12 weeks of age. **(a)** Anti-NDV antibody titers in each immunized chicken at 9 or 12 weeks. **(b)** Anti-PVX antibodies before challenge. Wells were coated with purified w.t. PVX (1 $\mu$ g) and then incubated with sera (1:50) of the chickens immunized with PVX (PVX A-B), with anti-PVX antibody (C+) or with sera (1:50) collected from non-immunized chicken (kindly provided by IZSve) (C-). Results represent the means of the absorbance values ( $OD_{405} \pm SD$ ) of triplicate wells for each animal.

### Production in leaves of PVX CVPs displaying F peptides

To produce PVX CVPs displaying F-derived peptides, the viral genome within the vector pPVXSma was modified by inserting the synthetic sequence encoding either peptide 1 (PEP1), peptide 2 (PEP2) or peptide 3 (PEP3) as fusion to the 5'-end of the *cpSma* gene, thus generating the vectors pPVXSma-PEP1, pPVXSma-PEP2, pPVXSma-PEP3 (Figure 1a,b). When used to infect *N. benthamiana* plants these vectors are expected to induce the synthesis of a homogenous population of chimeric CPs displaying the peptide at their N-terminal end (Figure

16). Because an epitope mapping of the F protein has never been performed, PEP2 and PEP3 peptides were selected for their presence in the portions of the protein F involved in virus inhibition and neutralization (Toyoda et al. 1989; Chen et al. 2001b) while PEP1 was selected because it is involved in the determination of NDV virulence that depends on the amino acid composition (velogenic strain sequence) (Manoharan et al. 2018). The choice of the peptides was validated by *in silico* analysis of the F protein by bioinformatics tools (hosted on the website <http://www.iedb.org/>) that perform the prediction of the immunogenic peptides (epitopes) of a protein and their antigenicity and accessibility, based on a database of experiments demonstrating recognition of immune epitopes by adaptive immune receptors (Vita et al. 2019). The amino acidic sequences of the peptides were optimized to comply with plant virus genetic stability and infectivity rules (described in Lico et al. 2006) by the addition to the N-terminus of the residues Ser (PEP1, PEP2, PEP3), Thr and Asp (PEP1) and to the peptide C-terminus of the residues Ser, Glu and Asp (PEP1). The objective was to obtain proper CVPs assembly and movement throughout plants, minimizing the risk of occurrence of undesired mutations.



**Figure 16:** Schematic representation of the CVPs obtained with the peptide fusion strategy.

All the CP units are chimeric and fused to the antigenic peptide.

Systemic symptoms were observed 1 week after *N. benthamiana* plants inoculation with pPVXSma-PEP2 and after 3 weeks in the case of pPVXSma-PEP1 and pPVXSma-PEP3. Genomic stability of the chimeric viruses was verified by RT-PCR and sequencing analysis on leaves collected during three successive infections. The PVXSma-PEP2 *cp* gene demonstrated to be stable over-time, while mutations occurred for PVXSma-PEP3 virus since the first infection. PVXSma-PEP1 was also demonstrated to be unstable over-time with a large deletion involving the sequence encoding the peptide and the *cp* gene. These sequence modifications generally restore a more efficient CP structure necessary for virus replication, assembly and movement throughout the plant (Table 2).

|                           | PEP1                                                                              | PEP2                                       | PEP3                                      |
|---------------------------|-----------------------------------------------------------------------------------|--------------------------------------------|-------------------------------------------|
| Plasmid                   | <u>MST</u> <b>DRRQKR</b> <u>F</u> SED–CP <i>Sma</i>                               | <u>MS</u> <b>RDKEACARAP</b> –CP <i>Sma</i> | <u>MS</u> <b>LDLYCTRIV</b> –CP <i>Sma</i> |
| 1 <sup>st</sup> infection | <u>MST</u> <b>DRRQKR</b> <u>F</u> SED–CP <i>Sma</i>                               | <u>MS</u> <b>RDKEACARAP</b> –CP <i>Sma</i> | <u>MS</u> <b>LDLHCTKIV</b> –CP <i>Sma</i> |
| 3 <sup>rd</sup> infection | <u>MST</u> <b>DR</b> <span style="background-color: red;">I</span> –CP <i>Sma</i> | <u>MS</u> <b>RDKEACARAP</b> –CP <i>Sma</i> | <u>MS</u> <b>MDKHCKKIV</b> –CP <i>Sma</i> |

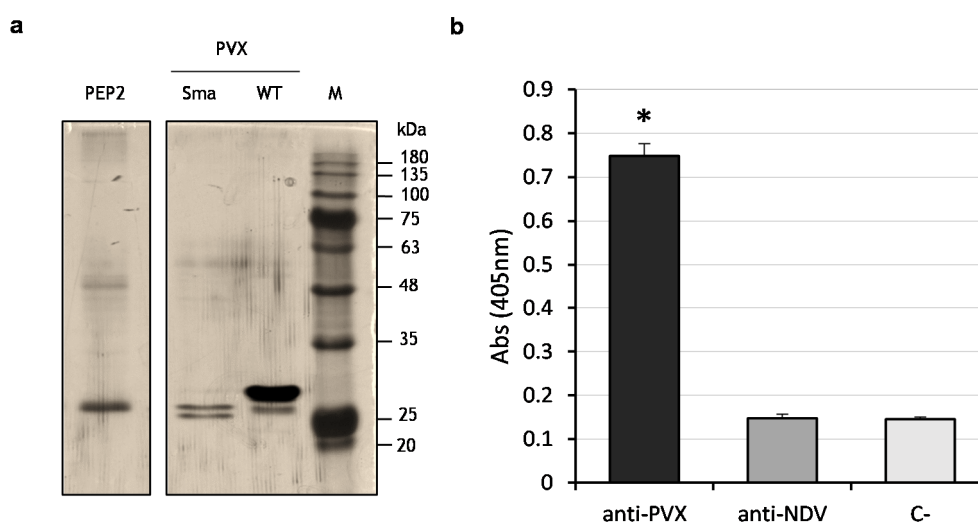
**Table 2:** Analysis of the sequences encoding the different peptides fused to PVXSma CP.

Sequences were obtained for three successive infections in *N. benthamiana* plant. The amino acids of the F1 peptides are in bold while the residues added to functionally optimize the sequence are underlined. The mutations are highlighted in red.

### Purification of PVXSma-PEP2 CVPs

PVXSma-PEP2 CVPs were extracted from the infected leaf tissues, purified by ultracentrifugation on a 30% sucrose cushion and an aliquot separated by SDS-PAGE in reducing conditions followed by silver staining. The chimeric PVXSma-PEP2 CP was observed in the purified sample with a band at about 25 kDa, similarly to what observed in the unmodified PVXSma used as control and no other bands were evidenced validating the efficiency of the purification process that guarantees a very high purity and concentration (Figure 17a).

The purified sample was analysed by an ELISA with an anti-NDV serum (kindly provided by IZSVe) and a commercial anti-PVX antibody. The particles were specifically recognized by the anti-PVX antibody (Figure 17b) confirming the identity of the protein observed on the gel but were not recognized by the anti-NDV serum raising the possibility the PEP2 is not immunogenic.



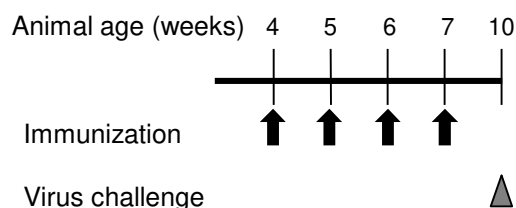
**Figure 17:** Analysis of the purified PVXSma-PEP2 chimeric particles.

(a) Silver staining of the proteins present in the purified PVXSma-PEP2 sample (PEP2, 1  $\mu$ g) separated by 12% SDS-PAGE under reducing conditions. Sma: purified PVXSma particles (1  $\mu$ g); WT: purified w.t. PVX particles (1  $\mu$ g). (b) ELISA analysis of the proteins present in the purified PVXSma-PEP2 sample. One  $\mu$ g of the sample was used to coat the wells and then incubated with an anti-NDV serum, an anti-PVX antibody or serum from non-immunized chicken (kindly provided by IZSVe). Results are shown as mean absorbance values (OD<sub>405</sub>)  $\pm$  SD of triplicate wells.

### Chicken immunization with PVXSma-PEP2 CVPs

The immunogenicity of the purified PVXSma-PEP2 CVPs was evaluated by intra-muscular administration to SPF chickens. Chickens were immunized at week 4, 5, 6 and 7 after hatching with 50  $\mu$ g of the CVPs with (PEP2+adj group) or without (PEP2 group) adjuvant and

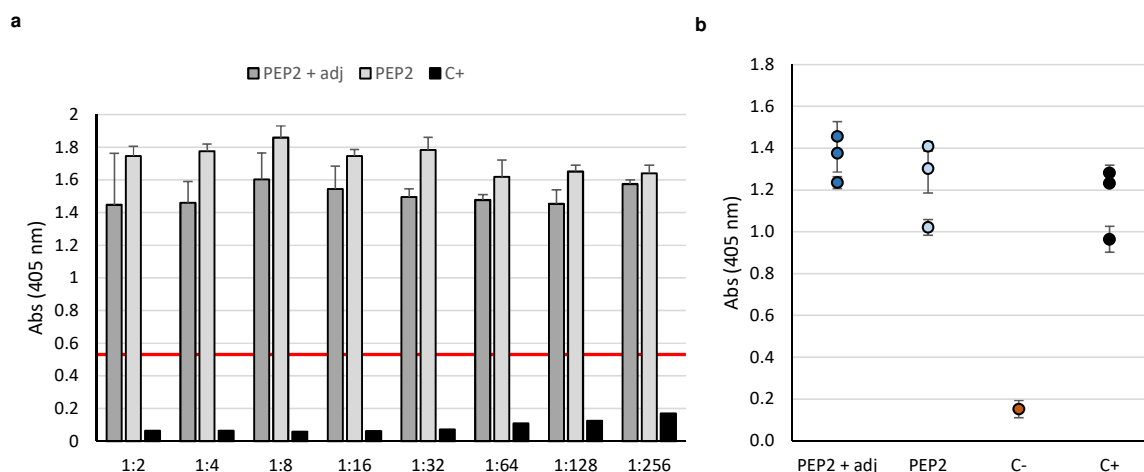
challenged with velogenic NDV at week 10 (Figure 18). One group of chickens was injected following the same immunization schedule with 50 µg of unmodified PVX*Sma* particles with adjuvant (negative control). Serum samples were collected from chickens one day before each immunization and before the viral challenge.



**Figure 18:** Schematic representation of the chicken immunization schedule.

All immunized chickens died indicating that no protection was provided by administration of the CVPs. The absence or presence of adjuvant did not result in a significant difference. Individual responses were evaluated by the NDV microneutralisation assay that allows to determine the last dilution of serum able to neutralize a set amount of virus. The presence of anti-NDV neutralizing antibodies in the serum prevents cell infection and virus multiplication that results in the ELISA as low absorbance values. All the collected sera, independently from the dilution, presented absorbance values above the threshold indicated in red (only sera collected before the challenge are represented in Figure 19a), in contrast to what observed for the serum considered as an internal positive control.

Therefore, no cell protection has been observed with the sera collected from chicken immunized with PVX*Sma*-PEP2 CVPs), indicating that anti-NDV neutralizing antibody were not induced. The absence or presence of adjuvant did not result in any significant difference. An ELISA, set up in order to verify the presence in the serum of the animals immunized with the CVPs of antibodies specific for PVX CP, demonstrated that, at week 10, PVX-specific antibodies are present (Figure 19b).



**Figure 19:** Analysis of the sera of immunized chickens.

Animals were immunized intramuscularly with PVX*Sma*-PEP2 chimeric particles alone (PEP2) or with adjuvant (PEP2+adj) at 4, 5, 6 and 7 weeks of age and sera were collected just before the viral challenge at 10 weeks of age.

(a) Evaluation of the presence of anti-NDV neutralizing antibodies in chicken sera before viral challenge determined by microneutralisation assay. The serum from chickens immunized with NDV (kindly provided by IZS<sup>Ve</sup>) was used as positive control (C+). Serial dilutions of the sera (1:2 to 1:256) were set up in order to determine the last dilution able to neutralize the fixed amount of virus. The red line indicates the threshold of the experiment. (b) Evaluation of the presence of anti-PVX antibodies in chicken sera collected before challenge by direct ELISA. Wells were coated with purified w.t. PVX (1µg). PEP2 + adj: sera (1:50) of the chickens immunized with PVX*Sma*-PEP2 particles plus adjuvant; PEP2: sera (1:50) of the chickens immunized with PVX*Sma*-PEP2 particles; C+: sera (1:50) of the chickens immunized with unmodified PVX*Sma* particles; C-: sera of non-immunized chicken (kindly provided by IZS<sup>Ve</sup>). Results are shown as mean absorbance values ( $OD_{405} \pm SD$ ) of triplicate wells for each animal sera.



### Strategy 3: F1 peptides complexes with plant HSP70

#### Materials and methods

##### **Constructs for F1 expression in plants**

The *fl-myc-flag* gene was constructed by fusing the *fl* gene (described in Strategy 1) to the sequence encoding the c-Myc tag peptide (EQKLISEEDL, Kawarasaki et al. 2003) followed by the sequence encoding the FLAG tag peptide (Figure 1a,b) (DYKDDDDK, Chiang and Roeder 1993). The sequences encoding the two tags were separated by a sequence encoding a linker peptide made of three amino acids (Glycine-Proline-Glycine; GPG). The fusion was obtained by two sequential PCR amplifications of the *fl* gene with the *PfuTurbo* DNA polymerase. In the first reaction (PCR1) the gene was amplified with the oligonucleotides 5'F1ClaI and 3'F1cMylinker (Table 1). The DNA fragment obtained through PCR1 was then amplified in the second PCR (PCR2) with the oligonucleotides 5'F1ClaI and 3'cMylinkerFlagstopSalI (Table 1). The PCR2 product, digested with the restriction enzymes *ClaI* and *SalI*, was inserted into the similarly digested binary vector pGR106 that carries between the left and right borders the cDNA sequence corresponding to the whole PVX genome (Lu et al. 2003), obtaining the vector pGR-F1-Myc-Flag.

##### **Transient expression in *N. benthamiana* plants**

The procedure described in the Strategy 1, was also adopted to transiently express the F1-Myc-Flag recombinant protein. Leaves were agroinfiltrated with *A. tumefaciens* (GV3101, pSOUP) harbouring the plasmid pGR-F1-Myc-Flag and *A. tumefaciens* (C58C1, pCH32) harbouring the binary vector p35S:p19 carrying the p19 silencing suppressor gene of Tomato Bushy Stunt Virus, TBSV (Voinnet et al. 2003). Leaves were collected 5 to 7 d.p.i. for the validation of F1-Myc-Tag and plant HSP70 expression or at 7 d.p.i. for plant HSP70 purification. The negative control was obtained by infiltrating plants with *A. tumefaciens* carrying the p19 construct and *A. tumefaciens* harbouring the empty plant expression vector (pGR106).

**F1-Myc-Flag protein extraction**

For the validation of F1-Myc-Flag and plant HSP70 expression, protein extracts from agroinfiltrated leaves were obtained by grinding 200 mg of tissue in liquid nitrogen and then homogenizing with Ultraturrax T25 in phosphate-buffered saline (PBS) pH 7.5 supplemented with a protease inhibitor cocktail (cOmplete™, Roche, Mannheim, Germany) in a 1:2 (w/v) ratio. The extracts were centrifuged at 4 °C, 20,000 x g, 20 min and the supernatants recovered for further analysis.

**Plant HSP70 purification**

Plant HSP70 bound to naturally chaperoned peptides were extracted and purified from leaves by adenosine diphosphate (ADP)-affinity chromatography as previously described (Buriani et al. 2011). Briefly, agroinfiltrated leaves were ground in liquid nitrogen and homogenised with Ultraturrax T25 in a 1:6 (w/v) ratio of cold buffer B (20 mM Tris-acetate, 20 mM NaCl, 15 mM  $\beta$ -mercaptoethanol, 3 mM  $MgCl_2$ , pH 7.5) supplemented with a protease inhibitor cocktail (cOmplete™). The plant extract was filtered with Miracloth paper, centrifuged at 4 °C, 20,000 x g, 35 min and the supernatant loaded on a column packed with ADP-agarose resin (Sigma Aldrich, A2810) pre-equilibrated with buffer B. The resin was washed with 10 column volumes of buffer B, then with 10 column volumes of buffer B supplemented with 0.5 M NaCl and finally with 10 column volumes of buffer B. The column was then added with 1/3 column volume of buffer B supplemented with 3 mM ADP (Sigma, A2754) and incubated for 30 min at r.t.. Elution was performed with 5 column volumes of buffer B supplemented with 3 mM ADP collecting fractions of 1/3 column volume each. Selected fractions were then pooled, dialysed and concentrated in PBS buffer pH 7.5, using PES membrane, 50,000 molecular weight cut-off concentrators (Vivaspin 20, Sartorius, Stonehouse, UK) following the manufacturer's instructions. Plant HSP70 concentration in the samples was measured by

Bradford analysis using as standard different concentrations of BSA or by ELISA using as calibration curve different concentrations of purified human HSP70 (Sigma Aldrich, H7283).

### **ELISA analysis**

Direct ELISA was performed similarly to what described in Strategy 2, but using purified human HSP (Sigma Aldrich, H7283) as positive control and as primary antibody either a murine anti-HSP70 antibody (Sigma Aldrich, H5147) diluted 1:1000, or a rabbit anti-c-Myc antibody (Thermo Fischer Scientific) diluted 1:2000. The binding of the primary antibodies was revealed with a goat anti-mouse HRP-conjugated antibody (KPL, 074-1807) diluted 1:2000 in 2% milk-PBS or a sheep anti-rabbit HRP-conjugated antibody (Thermo Fischer Scientific) diluted 1:2500 in 2% milk-PBS. In order to detect the Flag peptide, the incubation was performed with a mouse anti-Flag HRP-conjugated antibody (Sigma Aldrich, A8592) diluted 1:4000 in 2% milk-PBS for 2 h at 37 °C (single incubation).

### **SDS-PAGE and WB analysis**

SDS-PAGE and WB analysis were performed as described in Strategy 1 but using purified human HSP as positive control (Sigma Aldrich, H7283) and performing detection with a mouse anti-HSP antibody (Sigma Aldrich, H5147) diluted 1:1000 in 2% milk-PBS followed by incubation with a secondary goat anti-mouse HRP-conjugated antibody (KPL, 074-1807) diluted 1:2000 in 2% milk-PBS.

Proteins migrating 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.

**Animal studies**

Animal studies were performed by IZS Ve. All animals were kept at a constant temperature range (20-25 °C) and 30-40% relative humidity, with free access to food and water. In details, 10 SPF chickens were randomly divided into 2 groups of 5 animals. The first group was immunized intramuscularly with 35 µg of purified plant HSP70-F1 per dose emulsified in Montanide ISA 71 VG (Seppic, France). The second group, used as negative control, was injected intramuscularly with 35 µg of plant HSP70-NP (Buriani et al. 2012) per dose emulsified in Montanide ISA 71 VG. Both groups were injected 3 times at 42, 52 and 66 days post-hatch. Blood samples were collected from the wing vein the day before each immunization and 14 days after the last one. The presence of NDV and NP-specific antibodies in the sera of immunized chickens was determined using commercial ELISA kits (IDEXX NDV F protein (Westbrook, ME, USA) and ID.Vet competitive ELISA NP (ID.Vet, Grabels, France), respectively) following the manufacturer's instructions.

**Statistical analysis**

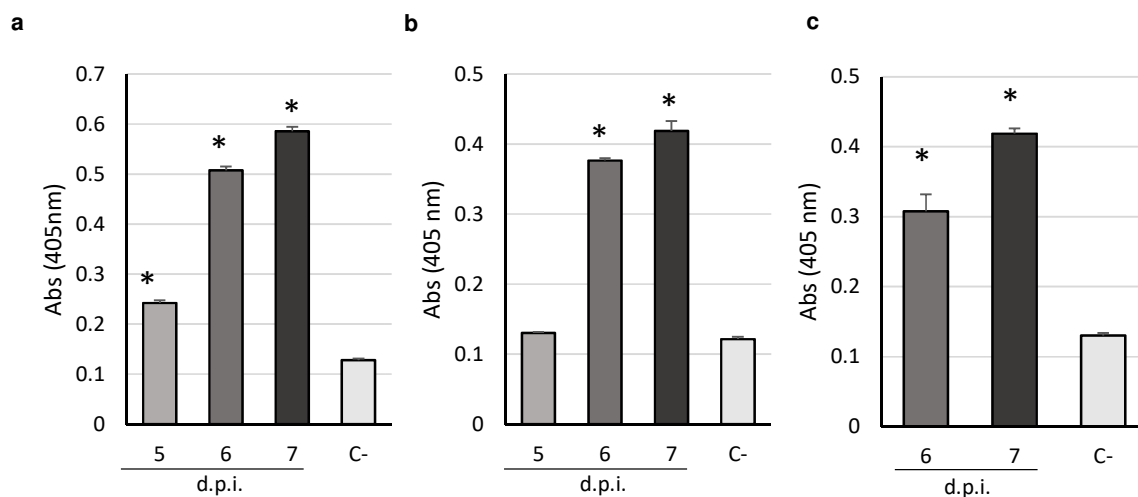
Statistical analysis was performed as described in Strategy 2.

## **Results**

### **Transient expression of F1-Myc-Flag in *N. benthamiana* leaves**

The transient expression of F1-Myc-Flag in leaves had the objective of recovering plant HSP70 carrying peptides deriving from the proteins expressed in the plant cells, including F1-derived peptides. To this purpose, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying the constructs pGR-F1-Myc-Flag and p35S:p19 (Figure 1a,b). The p19 silencing suppressor protein was used to reduce the natural plant gene silencing response, thereby enhancing the accumulation of the recombinant protein. Leaves were collected at 5, 6 and 7 d.p.i., and the presence of F1-Myc-Flag in crude extracts was assessed by ELISA, coating the wells with the plant-derived samples and revealing the presence of the heterologous protein using either anti-c-Myc or an anti-Flag antibodies. Leaves collection was stopped before 8 d.p.i. because strong signs of leaf degeneration were observed, dramatically reducing tissues quality and quantity (data not shown). Extracts from leaves infiltrated only with *A. tumefaciens* strains carrying the unmodified pGR106 plasmid and the p19 construct were used as negative controls (C-). The analysis of the samples with both the anti-c-Myc (Figure 20a) and the anti-Flag (Figure 20b) antibodies demonstrated that the F1-Myc-Flag protein was expressed and that accumulation increased over-time reaching maximum levels at 7 d.p.i..

HSP70 expression in crude extracts was also tested by ELISA with an anti-HSP70 antibody and demonstrated to similarly increase over-time reaching the highest accumulation levels 7 d.p.i (Figure 20c) similarly to what previously demonstrated (Buriani et al. 2011).

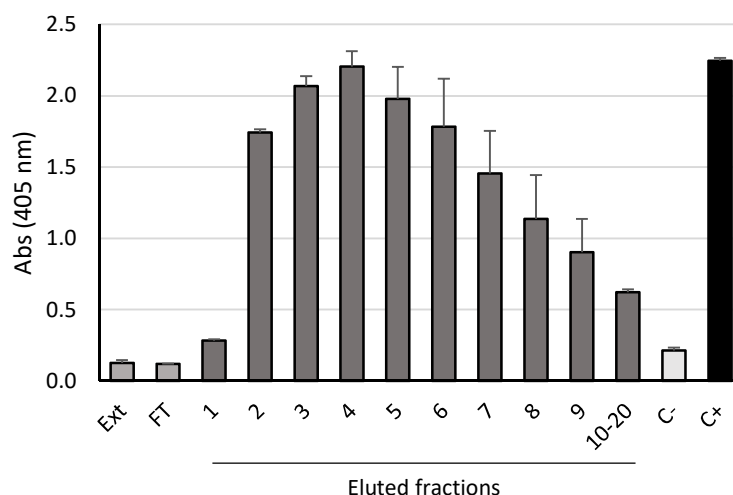


**Figure 20:** Analysis of the expression of F1-Myc-Flag and plant HSP70 in the leaves of agroinfiltrated *N.benthamiana* plants.

Analysis of F1-Myc-Flag and HSP70 expression in protein extracts of agroinfiltrated leaves by direct ELISA using (a) anti-c-Myc, or (b) anti-Flag, or (c) anti-HSP antibodies. Wells were coated with 100  $\mu$ l of the plant extract. d.p.i.: days post infiltration; C-: Protein extract prepared from leaves agroinfiltrated with unmodified pGR106 vector and collected at 7 d.p.i.; PBS in panel (c).

### Purification of plant HSP70 complex

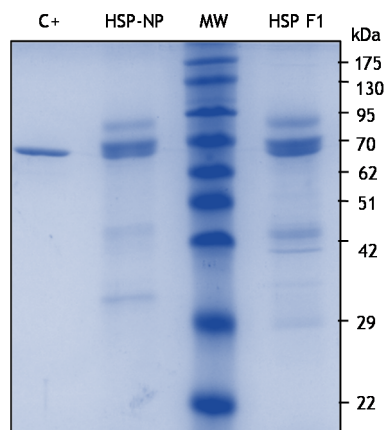
The extraction of plant HSP70 complexed to chaperoned polypeptides from leaves collected 7 d.p.i. was performed in buffer B supplemented with cOmplete™ and the proteins were purified by ADP-affinity chromatography, a method allowing to recover specifically the HSP70 bound to the naturally chaperoned peptides. The eluted fractions were analysed by ELISA using an anti-HSP antibody (Figure 21) and those containing the highest concentrations of the protein (eluted fractions 2 to 9) were pooled together, dialysed and concentrated by using centrifugal concentrators.



**Figure 21:** Analysis of the plant HSP70 content in the fractions eluted from ADP-agarose chromatography column

Analysis by direct ELISA of plant HSP70 content in the fractions (1 to 20) eluted from the ADP-agarose chromatography column loaded with the extracts of leaves transiently expressing F1-Myc-Flag using anti-HSP antibody. The wells were coated with 20  $\mu$ l of the first 9 fractions (1 to 9) or with a pool of the fractions 10 to 20 (10-20). Ext: crude plant extract; FT: flow-through; C-: PBS; C+: purified human HSP70 (1  $\mu$ g).

The sample was then separated under reducing conditions on SDS-PAGE and stained with Coomassie, revealing a complex pattern of bands (Figure 22): two major bands at the expected size of about 70 kDa and some less intense bands at about 90 and 40 kDa. In a previous study, the band with low molecular mass was identified by mass spectrometry as a degradation product of plant HSP70, while the two bands with molecular mass of about 70 kDa were both identified as cytosolic plant HSP70 (Buriani et al. 2011). These results confirmed the high binding selectivity of the ADP-agarose resin.

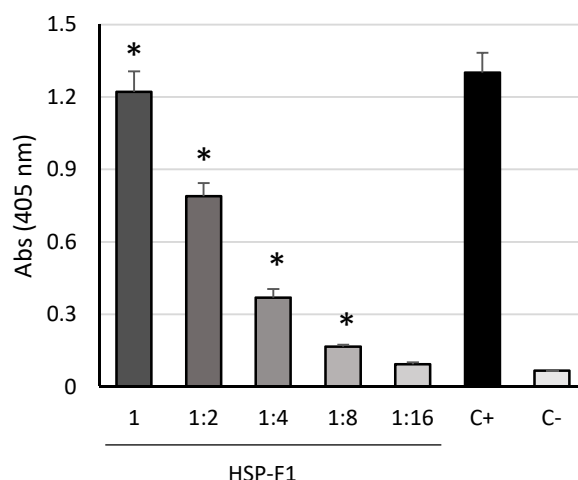


**Figure 22:** Coomassie staining of purified and dialyzed HSP70

Proteins eluted from ADP-affinity chromatography, dialysed and concentrated were separated by 12% SDS-PAGE under reducing conditions and Coomassie stained. One  $\mu$ l out of 1 ml of the purified and concentrated sample was loaded on gel. C+: purified human HSP70.

The quantification of HSP70 was performed by analysing serial dilutions of the sample by ELISA in parallel to a calibration curve of a purified human HSP70 (Figure 23) or by densitometric analysis of bands in Coomassie stained SDS-PAGE in parallel to a BSA calibration curve (data not shown). By both analysis, the final HSP70 concentration was determined to be of 550  $\mu$ g/ml corresponding to a recovery of approximately 15  $\mu$ g of HSP70 per gram of agroinfiltrated fresh leaves.



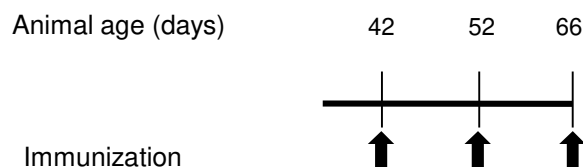


**Figure 23:** Quantification by ELISA of the purified plant HSP70 sample.

Direct ELISA of the purified plant HSP70 obtained from F1-Myc-Flag agroinfiltrated leaves using anti-HSP antibody. One  $\mu$ l of the purified HSP70 (1) and serial dilutions (1:2 to 1:16) were coated on wells. C-: PBS; C+: purified human HSP70 (500 ng).

### **Immunization of chickens with plant HSP70 chaperoning peptides, extracted from plants transiently expressing the F1-Myc-Flag protein**

SPF chickens were immunized intra-muscularly at days 42, 52 and 66 after hatching with 35  $\mu$ g of the purified HSP70-peptides complexes together with adjuvant (HSP70-F1 group) (Figure 24). One group of chickens was injected following the same immunization schedule with 35  $\mu$ g of plant HSP70-peptides complexes extracted from leaves transiently expressing the nucleoprotein (NP) of the influenza A virus (Buriani et al. 2012) together with adjuvant (negative control, HSP70-NP group). Serum samples were collected from chickens one day before each immunization and 14 days after the last one.



**Figure 24:** Schematic representation of the immunization schedule.

Sera were collected one day before immunization at 42, 52 and 66 days after hatching and 14 days after the last one

Individual responses were evaluated using a commercial ELISA kit developed to measure antibodies specific to NDV in the serum. The results indicated that the immunization with the purified HSP70-peptide complexes purified from the leaves transiently expressing the F1-Myc-Flag protein (HSP70-F1 group) was unable to raise detectable levels of NDV-specific antibodies (values under the threshold indicated in the kit). The same was true for anti-NP antibodies in the group immunized with HSP70-NP (data not shown). These last results are in contrast with previous data obtained by immunizing mice with the same dose of these HSP70-peptides complexes without adjuvant demonstrating the ability of this immunogen to induce the production of anti-NP specific antibodies production (Buriani et al. 2012).

## Discussion

ND is one of the major viral avian diseases causing extremely severe economic losses and affecting poultry breeding and production worldwide. It is also a threat for other avian species such as pigeons and wild birds, and this increases the risk of uncontrolled spread of the disease. Conventional protection strategies against NDV are based on vaccination with inactivated and live attenuated viruses (Mayers et al. 2017). The latter is the preferred solution in poultry farms, due to the high immunogenicity and suitability for large-scale application. Nonetheless, this type of vaccines pose some safety concerns due to the possibility of reverting to virulence causing the disease. In addition, live attenuated vaccines are efficient to protect poultry from clinical symptoms but are ineffective in preventing the shedding of the virus, thus causing its persistence in the farm environment (Bello et al. 2018). Efforts are being made to develop innovative recombinant subunit vaccines that provide greater safety and the possibility of easily discriminating vaccinated from infected animals (Hasan et al. 2016). However, recombinant subunit vaccines have some disadvantages compared to the conventional ones, mainly represented by low immunogenicity and protective efficacy if delivery is not supplemented with adjuvants (Karch and Burkhard 2016).

The aim of the present work was to develop NDV immunization strategies based on recombinant antigen constructions produced in plants as a cost-effective platform and designed to induce both mucosal and systemic immunity. The selected antigen was the F1 subunit of the F fusion protein as it plays a major role in the virulence of NDV strains and carries major neutralizing epitopes (Kattenbelt et al. 2006).

A first approach consisted in fusing F1, depleted of the fusion and transmembrane domains, to the fragment crystallisable (Fc) domain of a chicken IgY (FcY). Indeed, it has been demonstrated that chimeric proteins composed of antigens fused to the Fc of IgG, the mammalian functional homolog of IgY, are effectively targeted to APCs through the binding to

Fc receptors expressed on the membrane of these cells, and activate specific immune responses also when delivered without adjuvants (Yang et al. 2017). Recently, it was demonstrated that also FcY fusions are able to stimulate the activation of avian immune cells by increasing recombinant antigen uptake and processing by chicken macrophages that ultimately lead to an enhancement of the immune response (Dong et al. 2016; Wang et al. 2017). The Fc domain, by stabilizing the recombinant antigen may also increase expression levels in plants and simplify the purification of the chimeric antigen avoiding the fusion to protein tags that may alter the immunogenicity and safety of the final product (Czajkowsky et al. 2012). In this study, a secretory version of the F1-FcY protein was transiently expressed in *N. benthamiana*. The analysis of the accumulation of the protein in the leaves showed that the expression was much less efficient if compared to that of a similar protein, *i.e.* PD-FcY, consisting in the fusion of the FcY domain to the projection domain (PD) of the VP2 protein, the major antigenic component of the Infectious Bursal Disease virus (IBDV) (Rage et al. 2019). F1-FcY was present as aggregates unable to enter in SDS-PAGE gels (a fraction of the protein remained both inside the wells and in the stacking) or as high molecular mass multimers. This behaviour may be due to the formation of disulfide bonds between FcY domains or to the natural tendency of the F protein to form trimers (Chen et al. 2001a). Furthermore, the secretion in the apoplast of F1-FcY could not be validated because the presence of the chimeric protein in the plant intercellular fluids could not be demonstrated. In addition, F1-FcY in crude plant extracts was very unstable and rapidly lost. This was in contrast to previously published data demonstrating that F is stable when expressed through stable transformation approaches in maize or rice seeds and able to induce protection from NDV challenge in chickens when orally delivered with transgenic maize kernels (Guerrero-Andrade et al. 2006; Yang et al. 2007). In spite of the low expression levels, different methods were tested in order to improve F1-FcY recovery and stability and to minimize the contamination by unwanted plant products. Different extraction

conditions were tested to clarify the crude plant extract such as heating and acidification (data not shown) or PVPP and ascorbic addition but the chimeric protein was partially or totally lost also under these conditions. The purification directly from the extract of the fusion protein, exploiting the FcY domain, was also attempted using affinity chromatography methods alternative to those based on protein A or G resins traditionally adopted to purify mammalian immunoglobulin but that do not bind avian immunoglobulin Y. The Ligatrap resin, that binds to FcY through peptoid ligands, that was very efficient in purifying PD-FcY and may be used up to 20 times (see Chapter 2 and 3) was not successful in purifying F1-FcY probably because too many contaminants were present in the sample and these somehow damaged the resin and/or interfered with the protein binding. The thiophilic adsorption-based purification method exploited by the HiTrap IgY resin and currently adopted to purify IgY from the egg yolk (Belew et al. 1987) allowed to selectively purify the high molecular weight forms of F1-FcY but the final product was particularly rich in phenolic compounds and the chimeric protein was lost after storage for about 4 hours at +4 or -20 °C. Moreover, also in this case the resin permanently bound to plant compounds present in the crude plant extract and lost its binding efficacy after a single purification trial. The most selective purification method tested was based on immunoprecipitation of the fusion protein using a resin coupled with antibodies specific for the Fc region of IgY (GenScript IgY). In this case, F1-FcY was very selectively bound by the resin, but efficiently released only under harsh conditions (*e.g.* boiling in SDS-PAGE sample buffer) that did not allow the use of the final recovered product for immunization purposes due to the toxic components content. Nonetheless, the material purified by this method was useful to characterize the plant-expressed F1-FcY protein by mass spectrometry analysis. The obtained results confirmed that in the fusion protein the F1antigenic moiety was intact while a minor degradation was revealed at the C-terminus of the FcY domain. However, this degradation should not interfere with the binding to the Fc receptors that engage the C $\nu$ 3-C $\nu$ 4 and C $\nu$ 2-C $\nu$ 3

inter-domains (Zhang et al. 2017). In conclusion, the transient expression and purification of F1-FcY were successful, but the chimeric protein demonstrated to be highly unstable and its purification possible only adopting a method that results in a final product not compatible with the *in vivo* experiments, necessary to verify the immunological properties of the suggested fusion strategy.

In the attempt to improve the yield of F1-FcY and facilitate its recovery, the developed construct was used to generate HR from *N. benthamiana* and *B. rapa* subsp. *rapa* secreting the fusion protein into the liquid culture medium. However, HR could not be obtained in *N. benthamiana* and, while those of *Brassica rapa* subsp *rapa* were unable to proliferate.

Based on the comparison of these data with those previously obtained with the PD-FcY fusion protein, some aspects need to be taken into consideration in the attempt to explain the difficulties encountered to express and purify F1-FcY. The first observation is that F1 is more than 2 times larger than PD (358 aa and 37 kDa vs. 139 aa and 16 kDa, respectively) thus it is possible that the fusion to FcY of such a large polypeptide interferes with correct folding. Second, F is a membrane protein that undergoes significant structural modifications during the fusion process of the viral envelope to the cell membrane and it is possible that the expression of the isolated F1 subunit in a soluble version, deprived of the transmembrane domain, interferes with correct processing and folding of the chimeric antigen (Swanson et al. 2010). In fact, it has been observed that the recombinant F protein, the precursor of F1, produced in a heterologous expression system forms rosette-like aggregates after *in vitro* cleavage with trypsin (Chen et al. 2001a; Connolly et al. 2006). These characteristics may be related to the low stability and solubility of the transiently expressed F1-FcY and/or to the toxic effects in plant cells that prevent the development of HR in contrast with what observed with PD-FcY (see Chapter 3). Interestingly, difficulties in expressing the F protein in *E.coli* were also

previously pointed out (Kang et al. 2016) such as a reduction in the number of kernels in transformed maize plants that were also described to produce abnormal cobs suggesting the possible interference of F protein expression with maize fertility (Guerrero-Andrade et al. 2006). On the other side, the F protein has been successfully expressed in *Pichia pastoris*, under the control of a methanol inducible promoter, and in potato and rice plants showing good immunogenicity in mice (Berinstein et al. 2005; Yang et al. 2007; Kang et al. 2016).

The second strategy adopted to produce an immunopotentiated version of the F1 protein, was based on its fusion to the CP of PVX, selected because previously demonstrated to be a good scaffold for the display of foreign polypeptides and for their delivery in immunization experiments (Lico et al. 2015). The immunization of mice with PVX chimeric particles is able to induce the activation of immune responses specific for the displayed antigen also without adjuvant, probably because the particulate structure of the virus and the presence of the genomic RNA are able to stimulate Toll-Like Receptors (Jobsri et al. 2015) and activate innate immunity (Lico et al. 2009). Moreover, this expression strategy usually allows to obtain large quantities of easily purifiable CVPs. In this study, the attempt to produce in *N. benthamiana* plants chimeric PVX particles displaying the whole F1 or different domains/peptides of the protein as fusion to the CP was carried out. Because the whole F1 protein is too large to be compatible with the correct assembly of the virions, the first fusion strategy consisted in inserting the F1 coding sequence into a viral expression vector that allows to produce a mixed population of CP made both of unmodified and chimeric versions of the protein. Nonetheless, inoculation of the plants with this viral expression vector pPVX-2A-F1 did not determined the appearance of the symptoms typical of PVX infection. With the hypothesis that the reason of the failure may be related to the dimension of the fused heterologous sequence, too big if compared to those previously expressed by this approach (Cruz et al. 1996; Dickmeis et al. 2015), the same vector

was used to fuse to the *cp* gene the nucleotide sequences encoding smaller F1 domains . Because the epitope mapping of this protein is not available, these domains were selected on the basis of the analysis of the crystal structure of F focusing the attention on regions that appear to be located on the solvent-exposed part of the protein. In these cases, the obtained modified viral expression vectors were able to induce the assembly of infectious virus particles. However, the yields were lower than expected probably because, as revealed by the fractionated extraction of the cellular proteins, a large fraction of the CPs (most of the chimeric type) remained blocked in the membranes of the cell compartments. This was unexpected as the domains of the protein involved in membrane anchoring (transmembrane domain) and fusion of viral and cellular membranes (fusion peptide) were removed from the expressed sequence. Even if no antibodies specific for the F protein are available, and those specific for NDV were unable to confirm the presence of the F1-derived domains as fusion to the CP and to allow to define which proportion of the CP was chimeric, the particles expected to display the larger F1 domain (F1 A-B, 16 kDa) were produced on large scale, purified and used to immunize chickens (two doses of 165 µg). However, the immunized chickens were unable to produce anti-NDV antibodies and were not protected against NDV challenge, while in the sera of these animals anti-PVX antibodies were present. These results may indicate that the quantity of the A-B domain delivered through the chimeric PVX was not sufficient or that this domain is not effective in eliciting protective immune response and/or not correctly folded (Cox and Plemper 2017). Even though the results were not satisfactory, this was the first attempt to produce entire domains of the F protein (6, 10, 16 kDa) in a heterologous expression system and as fusion to a plant virus. In parallel, a different strategy was adopted in the attempt to produce CVPs in which all the CP units were chimeric. To this aim, peptides of the F protein, 6 to 10 amino acids in length, were selected on the basis of their supposed immunogenicity and of their role in the virulence of NDV and their coding sequences inserted as fusion with the 5'-end of the *cp* gene in the expression vector



pPVXSma, that carries the genome of a viral mutant isolated and optimized for this type of applications in the ENEA laboratories (Lico et al. 2006). The sequences of the PEP2 and PEP3 peptides were derived from the most immunogenic regions of F that are involved in inhibition and neutralization (Toyoda et al. 1989; Chen et al. 2001b) while the sequence of PEP1 was selected because it is involved in the determination of NDV virulence (velogenic strain sequence) (Alexander 2000; Manoharan et al. 2018). The aminoacidic sequences were optimized to comply with plant virus genetic stability and infectivity rules by the addition of amino acids at the N and C-termini. Only the viral vector encoding the CP fusion with PEP2 (RDKEACARAP) was able to infect *N. benthamiana* plants producing particles that were genetically stable through several successive infections, while the particles displaying PEP1 (RRQKRF) and PEP3 (LDLYCTRIV) were not genetically stable. Similar results have been previously obtained trying to produce that as reported for a chimeric Cucumber mosaic virus (CMV) particles displaying F peptides that since the first viral infection were reported to carry several sequence deletions (Zhao and Hammond 2005). Intrinsic F traits may therefore be the cause of the instability of the viral particles fused to F peptides. The stable CVPs displaying PEP2 were produced on large scale, purified and used to vaccinate chickens. Even if anti-PVX antibodies were present in the serum of the immunized animals, no anti-NDV neutralizing antibodies could be detected and no protection from virus challenge was observed. A possible reason for this failure may be related to the inability of the peptide to elicit the activation of the immune system as this peptide was never clearly demonstrated to be endowed of this property. In a previous work, an F peptide comprising a sequence close to PEP2 with an almost two times larger size (17 aa), was successfully expressed in fusion with the CMV CP recognized and by an anti-F antibody but did not show any immunogenic potential (Natilla and Nemchinov 2008). The same peptide was also produced as fusion with a peptide of the HN protein of NDV and demonstrated able to induce protection against viral challenge in chickens but it was not

determined if both peptides were immunogenic (Shahriari et al. 2019).

The third and last strategy adopted in the attempt to produce in plants an F1-based immunogen against NDV relied on the transient expression of the protein in *N. benthamiana* leaves and on the use of the agroinfiltrated tissues to extract and purify plant HSP70 complexed to the peptides they naturally chaperoned. It has indeed been demonstrated that peptides associated to HSP represent a sort of fingerprinting of the proteins expresses inside cells (Li et al. 2002). These complexes specifically bound to receptors expressed on the membrane of APCs and their binding allows uptake and processing resulting in the activation of immune responses specific for the chaperoned peptides, also by cross-presentation through MHC class I molecules, without adjuvants (Brauns et al. 2015). Moreover, they are able to induce the production and release of cytokines and chemokines and to stimulate dendritic cells maturation activating the innate immune system (Joly et al. 2010). An interesting aspect of this approach is that HSP70 complexed to chaperoned peptides can be very easily, quickly and efficiently purified also from plant tissues (Buriani et al. 2012) through affinity chromatography based on ADP-agarose resin (Ménoret 2004). The F1 antigen was successfully expressed transiently in *N.benthamiana* leaves reaching the highest accumulation levels at 7 d.p.i. similarly to what observed for pHSP70 (Buriani et al. 2011). Plant HSP70-peptide complexes (expected to carry also F1 derived peptides) were successfully purified from agroinfiltrated leaves and used to immunize chickens. Unfortunately, as with the previous immunization strategies, the analysis of the sera did not evidence the presence of anti-NDV antibodies. In this case, again the possible explanation could be that the delivered dose was too low. In a previous work, by using a vaccination strategy based on HSP70, authors demonstrated the possibility to protect chickens against the challenge with H9N2 avian influenza virus (Dabaghian et al. 2014) but about 6 µg of the influenza antigen were injected alone or as fusion to the C-terminus of HSP70. In our case, it was not possible to estimate the exact dose of F1 peptides injected, but the dose of

HSP70 complexes and the immunization schedule were the same to that previously used in mice. In that case, HSP70-based immunogen against influenza raised the production in sera of anti-NP antibodies and the activation of cytotoxic T lymphocyte mediated responses (Buriani et al. 2012). Considering the difference in the body weight (about 20 g for the mouse vs. about 300 g for the chicken) it is probable that a higher dose of this type of antigen preparation must be injected in chickens to induce the activation of protective immunity.

In conclusion, the presented results illustrate the expression in plants and purification of different versions of the NDV F1 protein. The aim was to develop novel antigen formulations. It is evident that the production of F1 present problems in terms of expression efficiency and stability. Moreover, the lack of specific reagents is a major obstacle to the characterization and quantification of the different versions of F1 produced in plant tissues. Furthermore, the absence of a commercial ELISA kit specific for the recognition of anti-F antibodies also limited the analysis of the sera of the immunised animals. Further studies will be required to demonstrate the potential of the suggested innovative fusion strategies for the development of plant-made low-cost anti NDV veterinary vaccines.

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- Rage E, Drissi Touzani C, Marusic C, Lico C, Göbel T, Bortolami A, Bonfante F, Salzano AM, Scaloni A, Fellahi S, El Houadfi M, Donini M, Baschieri S (2019) Functional characterization of a plant-produced infectious bursal disease virus antigen fused to the constant region of avian IgY immunoglobulins. *Applied Microbiology and Biotechnology*. September 2019, Volume 103, Issue 18, pp 7491–7504. <https://doi.org/10.1007/s00253-019-09992-9>. (Published)
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