UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Nuevas metodologías para la producción de anticuerpos recombinantes en plantas

Estefania Huet Trujillo

IBMCP

Valencia, July 2017

Director

Dr. Diego Orzáez Calatayud

Co-director

Prof Antonio Granell Richart

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El **Dr. Diego Orzáez Calatayud**, Científico Titular del Consejo Superior de Investigaciones Científicas, y el **Dr. Antonio Granell Richart**, Profesor de Investigación del Consejo Superior de Investigaciones Científicas, ambos pertenecientes al instituto de Biología Molecular y Celular de Pantas,

CERTIFICAN que la Licenciada en Bioquímica y Máster en Biotecnología Molecular y Celular de Plantas **Estefania Huet Trujillo**, ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas el trabajo que lleva por título ***Nuevas metodologías para la producción de anticuerpos recombinants en plantas***, y autorizan su presentación para optar al grado de Doctor en Biotecnología.

Y para que así conste, expiden y firman el presente certificado en Valencia, a 18 de Mayo de 2017.

Dr. Diego Orzáez Calatayud

Dr. Antonio Granell Richart

Als meus pares,

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RESUMEN

La ingeniería genética ha permitido el diseño y la producción de anticuerpos recombinantes (rmAbs) en plantas. Hoy en día, los rmAbs se utilizan en el tratamiento de un amplio rango de patologías como enfermedades infecciosas, enfermedades inflamatorias y cáncer, convirtiéndose en un importante grupo de biomoléculas dentro de la industria farmacéutica y biotecnológica.

Hasta la fecha de este estudio, en plantas se ha producido mayoritariamente la inmunoglobulina del tipo G (IgG). Gracias al desarrollo de la ingeniería del ADN recombinante y de la ingeniería de anticuerpos, es posible diseñar y producir nuevos formatos de rmAbs. Sin embargo, apenas existen estudios comparativos donde se demuestre si el formato de anticuerpo elegido es el idóneo en términos de rendimiento y capacidad neutralizante. Por tanto, el punto de partida del primer Capítulo de esta tesis consistió en la realización de un estudio comparativo de la expresión en plantas de cinco formatos distintos de un mismo rmAb comercial (Infliximab) frente a la citoquina humana Tumor Necrosis Factor (TNF-α). Los resultados obtenidos en el Capítulo 1 demuestran que tanto el isotipo como la estructura del rmAb elegido influye en los niveles de rendimiento y en la capacidad neutralizante del rmAb.

La expresión de nuevos formatos de anticuerpos no solo afecta al isotipo o a la estructura de las regiones constantes, sino que también se puede incluir en este término la expresión conjunta de distintos idiotipos de anticuerpos recombinantes, dando lugar a anticuerpos policlonales u oligoclonales recombinantes. Por tanto en esta tesis se planteó la posibilidad de co-expresar simultáneamente distintos anticuerpos monoclonales en plantas formando un cóctel oligoclonal. En el segundo Capítulo de esta tesis se diseñaron tres rmAbs frente a la glicoproteína de la cubierta del virus del Ébola. Los tres rmAbs se expresaron transitoriamente en *N. benthamiana* de manera individual mediante el establecimiento de líneas paralelas de producción y también se co-expresaron los tres rmAbs simultáneamente en una misma línea de producción. Los resultados obtenidos en este Capítulo demostraron que la expresión de los rmAbs de manera individual es factible. Sin embargo, cuando se co-expresan los tres rmAbs se

observa una drástica disminución en la unión del anticuerpo al antígeno debido al barajado de cadenas, fenómeno por el cual cada cadena pesada (HC) se puede unir con cualquier cadena ligera (LC) distinta de su acompañante, dando lugar a un anticuerpo con una baja actividad.

Finalmente, con el objetivo de desarrollar un método que permita co-expresar en una misma línea de producción varios rmAbs de forma reproducible se propuso explotar el fenómeno de la exclusión viral, un característica propia de los virus de plantas. Los resultados mostrados en el Capítulo 3 demuestran que es posible la producción de un cóctel oligoclonal compuesto por 36 rmAbs en *N. benthamiana* aprovechando el fenómeno de la exclusión viral. Los datos obtenidos en este capítulo muestran que el cóctel oligoclonal producido de esta forma mantiene intactas las actividades de los anticuerpos individuales y es capaz de neutralizar las actividades tóxicas del veneno de la serpiente *Bothrops asper* en ensayos *in vitro* e *in vivo.*

Los resultados de esta tesis confirman y avalan el uso de las plantas como plataformas de expresión de formatos alternativos de anticuerpos.

RESUM

El desenvolupament de l'enginyeria genètica ha permès el disseny i la producció d'anticossos recombinants (rmAbs) en plantes. Hui en dia, els rmAbs s'utilitzen en el tractament d'un ampli rang de patologies com malalties infeccioses, malalties inflamatòries i càncer convertint-se en un important grup de biomolècules dins de les indústries farmacèutiques i biotecnològiques.

Fins a la data, s'han expressat majoritàriament la immunoglobulina del tipus G. Gràcies al desenvolupament de l'enginyeria de l'ADN recombinant i l'enginyeria dels anticossos s'han desenvolupat i expressat formats alternatius de rmAbs. Tanmateix, hi ha molts pocs estudis comparatius on es demostra si el format de l'anticòs elegit influeix en el rendiment i en la capacitat neutralitzant. Per tant, el punt de partida del primer Capítol d'esta Tesi és la realització d'un estudi comparatiu on s'expressen cinc formats diferents d'un mateix anticòs comercial (Infliximab) front a la citocina humana Tumor Necrosis Factor (TNF-α). Els resultats obtesos demostren que tant l'isotip com l'estructura del rmAb elegit influeix en el rendiment i en la capacitat neutralitzant del rmAb.

L'expressió de nous formats d'anticossos no sols afecta a l'isotip o a l'estructura del rmAb sinó que també pot incloure's dins d'aquest concepte l'expressió individual i l'expressió conjunta de diferents rmAbs. Partint d'aquesta hipòtesi, es va plantejar la possibilitat de co-expressar diferents rmAbs (còctel oligoclonal) en plantes. En el segon Capítol d'esta tesi es dissenyaren tres rmAbs front a la glicoproteïna del virus de l'Ébola. Els tres rmAbs s'expressaren transitòriament en *N. benthamiana* de manera individual mitjançant l'establiment de línies paral·leles de producció i també es co-expressaren els tres rmAbs en la mateixa línia de producció. Els resultats obtesos en este Capítol demostraren que l'expressió dels rmAbs de manera individual és factible. Tanmateix, quan es co-expressaren els tres rmAbs s'observà una dràstica disminució en la unió de l'anticòs a l'antigen com a conseqüència del *shuffling chain*, pel qual la cadena pesada (HC) s'uneix amb qualsevol cadena lleugera (LC) diferent a la seua acompanyant, formant un anticòs amb una baixa capacitat d'unió a l'antigen.

Amb l'objectiu de desenvolupar un mètode que permeta co-expressar, en una mateixa línia de producció, un còctel oligoclonal es proposà explotar el fenomen de l'exclusió viral. Els resultats obtesos en el Capítol 3 demostren que l'expressió d' un còctel oligoclonal format per 36 rmAbs en plantes és possible. Els resultats mostren que el nostre còctel oligoclonal es capaç de neutralitzar activitats tòxiques del verí de la serp *Bothrops asper* en assaigs *in vitro* i *in vivo*.

Els resultat obtesos en aquesta Tesi confirmen i avalen l'ús de les plantes com plataformes d'expressió de formats alternatius d'anticossos.

SUMMARY

Genetic engineering has allowed the design and production of recombinant antibodies (rmAbs) in plants. Nowadays, rmAbs are used in the treatment of a wide range of pathologies such as infectious diseases, inflammatory diseases and cancer, making rmAbs an important group of biomolecules within the pharmaceutical and biotechnology industry.

By the time this study was started, the immunoglobulin G (IgG) was the antibody isotype predominantly expressed in plants. In recent years Modular DNA cloning technology has facilitated antibody engineering, with the development and expression of new rmAbs formats. However, there is hardly any study where different antibody formats are produced and compared in terms of yield and neutralizing capacity. Therefore, the starting point of the first chapter of this thesis is a comparative study where five different formats of the same commercial rmAb (Infliximab) against the human cytokine Tumor Necrosis Factor (TNF-α) were expressed and compared. The results obtained in Chapter 1 demonstrate that both the isotype and the structure of the chosen rmAb influence the yield and the neutralizing capacity of rmAb.

The expression of new antibody formats not only refers to the antibody isotype or structure; the format also refers to the combination of antibody idiotypes, leading to the production of oligo or polyclonal antibodies. Therefore, the possibility of co-expressing different monoclonal antibodies simultaneously in plants (creating oligoclonal or polyclonal formats) was raised. In the second chapter of this thesis, the expression of three rmAbs against the Ebola virus glycoprotein was studied. The three rmAbs were transiently expressed in *N. benthamiana* individually, by establishing separated production lines; in parallel, all three rmAbs were also co-expressed simultaneously in the same production line. The results obtained in this chapter demonstrated that the individual expression of rmAbs is feasible. However, when all three rmAbs are co-expressed, a drastic decrease in the binding of the antibody to the antigen was observed due to chain shuffling, as each heavy chain (HC) can be bound to

any light chain (LC) other than its cognate chain, giving rise to an antibody cocktail with lower activity.

With the objective of developing a method that allows co-expression of several rmAb in a single production line, we next proposed to exploit the viral interference phenomenon (also known as superinfection exclusion, SE). The results shown in Chapter three demonstrate that the production of an oligoclonal cocktail composed of 36 rmAbs in plants was possible using a viral expression system showing SE. The data obtained in this chapter showed that the resulting oligoclonal cocktail was active and capable of neutralizing toxic activities of the venom of the snake *Bothrops asper in vitro* and *in vivo,* wich was used as a model for studying the efficacy of the oligoclonal antibodies produced.

The results of this thesis confirm and support the use of plants as platforms for the expression of alternative formats of antibodies.

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INTRODUCTION

1. Production of recombinant proteins in plants

Humans have always used plants as an important source of food and other natural compounds. In the last 30 years, technical developments in the area of the recombinant DNA technology and plant transformation have enabled the introduction of specific genes into plants, resulting in genetically modified plants. A promising area of plant biotechnology focuses on the use of transgenic plants for the production of valuable recombinant proteins. This field is also known as molecular farming (Franken et al., 1997).

Molecular farming represents an alternative to bacteria, yeast, baculovirus or mammalian cells as recombinant factories, since it offers several advantages, namely high yield, enhanced safety (plant production reduces risk of human and animal pathogen contamination) and easier scalability. Moreover, plants can also perform some of the post-translational modifications needed for biological activity in many proteins. However, the use of plants as recombinant production systems presents some disadvantages. The most notorious is the adverse public perception of the transgenic plants linked to ethical and intellectual property issues, that precludes the use of plants as an attractive expression system for therapeutic proteins (Ferrer-Miralles et al., 2009). Fortunately, recent studies reflect a higher acceptance of plant molecular farming compared to genetically modified food, since the society is more favorable to the application of plant biotechnology for medical purposes (Daniel Hell et al., 2015).

In 1986, Barta and colleagues made the first breakthrough in molecular farming when they achieved the expression of a gene encoding the human growth hormone in plants (Barta et al., 1986). Since then, proteins produced in plants for molecular farming purposes can be categorized into two broad areas: industrial proteins and biopharmaceutical proteins.

In 1997, the first plant-derived recombinant protein was commercialized. This protein was the chicken avidin produced in maize by ProdiGene for its use in diagnosis (Hood et al., 1999). As a non-pharmaceutical protein, the regulatory requirements for its commercialization were more straightforward than the ones that pharmaceutical products have to meet. The same year, Chong and colleagues achieved the expression

of recombinant human milk β-casein in potato plants. Other types of proteins such as amylases, phytases and hydrolases are also produced in transgenic plants and they are currently used in the industry (Horn et al., 2004).

One of the most recent breakthroughs in molecular farming was the production of ELELYSO® (taliglucerase alfa) in carrot cells, which is used in enzyme replacement therapy to treat adult patients with Gaucher disease (Protalix BioTherapeutics, Israel). The taliglucerase alfa is the first example of a plant-made therapeutic protein for human use approved by the FDA in 2012. Plant-derived-pharmaceutical products have attracted great interest over the past years due to its high demand for clinical applications increasing over the past years.

Vaccines have been produced in plants to treat viral and bacterial diseases for both human and veterinary applications. The first experimental plant-derived-vaccine reported in 1992, was the hepatitis B virus surface antigen (HBsAg) (Mason et al, 1992). Since then, plant-made-vaccines have been produced for mucosal and parenteral use. In 1995 the first proof-of-principle of edible plants vaccines was produced, the *E. coli* heat-labile enterotoxin (LT-B) produced in potatoes. These transgenic potatoes were fed to mice and proven to be orally immunogenic (Haq et al., 1995). In the last years, some of these plant-made-vaccines have entered human clinical trials (Daniel H et al., 2009, Takeyama, 2015). For example, in 2008 a vaccine for the treatment of non-Hodgkin's lymphoma was the first injectable plant-made vaccine produced in tobacco for human use to enter phase I clinical trials (McCornick et al, 2008).

Antibodies are another group of therapeutic proteins produced in plants. Their specificity for particular antigens allows their use in several fields that range from research to diagnostic and biological therapy. Thanks to their potential to bind virtually all type of molecules, antibodies are increasingly demanded as therapeutic drugs, particularly for cancer treatment (Nayeem et al., 2006). Since the first plant-made-antibody was produced in 1989, antibodies expression in plants has become an attractive target for plant expressions (Liao et al., 2006).

The topic of this thesis is the production of several antibodies in plants or also called plantibodies. I will focus on them during the rest of this study.

2. Plant-made antibodies

Plant production systems have been proposed as advantageous platforms to produce antibodies at a large scale (Sheshukova et al., 2016, Lomonossoff et al., 2016). As in other systems like baculovirus-infected cells (Palmberger et al., 2011) or mammalian cells (Wurm, 2004), the number of recombinant antibodies expressed in plants have increased since the production of the first plant-derived-antibody was reported in 1989 (Hiatt et al., 1989) (Table 1).

Table 1. Examples of pharmaceutical antibodies produced in transgenic plants. Adapted from Stoger et al., 2002.

Antibody format	Antigen specifities	Transgenic plant	Application	References
IgG and sIgA	*S. mutans* SAI/II	Tobacco	therapeutic	Planet Biotechnology
IgG	Herpes simplex virus	rice, soybean	therapeutic	EPIcyte
IgG	Human IgG	Alfalfa	diagnostic	
diabody	Carcinoembriogenic antigen	Tobacco	Diagnostic/therapeutic	EPIcyte
IgA	Non-Hodgkin's lymphoma	Ducweed	Therapeutic	Biolex Therapeutic
IgG	Sperm	Corn	Contraceptive	EPIcyte
IgG	Glycoprotein Ebola Virus	Tobacco	Therapeutic	Mapp Biopharmaceutical
IgG	TNF-α	Carrot cells	Therapeutic	Protalix
IgA	Rotavirus	Tomato	N/A	Juárez et al., 2012

During the last two decades, there were several unsuccessful attempts of commercialization of plant-derived-antibodies. Large Scale Biology's Corporation and Bayer Innovation conducted phase I clinical trials with antibodies for the treatment of

the non-Hodgkin's lymphoma (McCormick et al., 2008). Planet Biotechnology Inc, produced an antibody (CaroRx) against *Streptococus mutans* to prevent dental caries which was evaluated in phase I and II and it was registered as a medical device in Europe (Larrick et al., 1998). In recent years, more antibodies produced in plants have been presented as promising therapeutic treatments. For instance, LeafBio, the commercial arm of Mapp Biopharmaceutical Inc., announced in 2015 that the U.S. Food and Drug Administration (FDA) has granted ZMapp 'Fast Track' designation for the treatment of the Ebola virus disease. Fast track designation is granted to drugs that the FDA determines have the potential to address an unmet medical need, based on non-clinical or clinical data (Largent EA, 2016)

From a commercial point of view, the use of plants for the production of recombinant antibodies is attractive because they offer significant advantages over others expression systems. For example, up-scaling production can be achieved more easily because there is not requirement of expensive equipment and media compared with the mammalian cell culture expression system. Moreover, the plant approach is especially interesting when high amounts of antibodies are required. Competitive yields, in comparison with Chinese hamster ovary (CHO) cells, have been reported in a laboratory scale and in also in prototype industrial setups (Vézina et al., 2009). Nowadays, the costs of the plant-derived-antibodies can not be estimated because they are not yet commercialized. However, Planet Biotechnology Inc. has calculated that the costs of an IgA produced in plants are only 1-10% compared with those in mammalian cells（Frenzel et al.,2013).

3. Expression systems for plant-made-antibodies

Host species, expression systems and expression strategies are decisive for a successful antibody production. Each plant species has different characteristics that affect expression, quality, storage and downstream processes of the final antibody product. A number of expression systems have been developed, which can be classified in stable or transient attending to the localization of the transgene. The two types of strategies differ greatly in speed, cost, scalability, and host range (Dugdale et al., 2013).

3.1. Stable gene transformation

The stable transformation is defined as cells that have integrated the foreign DNA into their genome. The integration usually produces a permanent expression of the transgen, and the offspring of transgenic plant will express the protein encoded in the foreign gene. Stable transformation is achieved either through the use of *Agrobacterium tumefaciens* or particle bombardment depending on the species (Schillberg et al., 2003). The stable transformation process demands the simultaneous occurrence of two independent biological events: on one hand, an insertion of a transgene into the plant genome; and on the other hand, a regeneration of the transformed cells. The need of both events occurring in the same cell makes it a limitation for the transformation efficiency (Gelvin, 2003). Scalability is the most important advantage of the stable strategy because the cost of recombinant protein produced in transgenic plants is inversely proportional to the production scale.

The principal disadvantage of stable transformation is that is a time-consuming process as tissue culture procedures taking a minimum of three to nine months are needed, depending on the plant species required for stable transformation. There are several remarkable examples of recombinant protein production achieved with stable plant transformation. Planet Biotechnology (USA) reported the production a secretory immunoglobulin called *CaroRX™* against *Streptococcus mutans* in stably transformed tobacco plants where the levels of productions were up to 0.5mg/g of fresh weight (FW) (Wycoff, 2005).

The stable transformation strategy can be also applied to plant cell cultures that are directly used as production platforms. Plant cell cultures are *in vitro* systems that can be used for recombinant antibody production (Doran, 2013, Huang et al., 2012). In this case, the process can be speeded up because cell cultures do not require the complete regeneration of the plant. Plant cell cultures are often derived from calli cultivated in a solidified medium. The transfer of callus to a liquid medium and its agitation on shakers or in fermenters, results in cultures of single cells or small aggregates around 10 to 20 cells (Hellwing et al., 2004). A great advantage of plant-cell-suspension cultures is that recombinant proteins can be produced under conditions certified Good Manufacturing

Practice ('cGMP') conditions (Fisher et al., 2012). The plant-cell suspension cultures permit a high level of accumulation (yields up around 25mg/L of recombinant antibody), with simpler and cheaper purification and biosafety profiles compared to intact plants (Schillberg et al., 2013). Suspension cells from tobacco, maize and rice have been used for the production of recombinant antibodies, for instance a recombinant human IgG antibody M12 is produced in tobacco cell cultures with a yield of 20mg/L (Madeira et al., 2015).

3.2. Transient gene expression

Transient transformation is defined as cells that have not integrated the introduced DNA into their genome. This approach has become an attractive manufacturing system because it is fast when generating recombinant proteins and permits the scale up for commercial manufacture; as a drawback, the recombinant protein will be expressed for a short period of time (Sainsbury et al., 2014).

Agroinfiltration is the most commonly used method for transient expression of recombinant proteins, where the foreign gene inserted in a T-DNA is moved into the host cell nucleus by bacterial proteins (Figure 1a). Agroinfiltration in plants can be performed by syringe or vacuum infiltration. Vacuum infiltration is more convenient for large scale production because all the parts of the leaf can be infected by *Agrobacterium*, increasing the yield and reducing the manufacturing cost (Kapila et al., 1997, Bechtold, 1998). Using the agroinfiltration method, it is easy to produce a full length antibody by transforming the light and heavy chain simultaneously in the host plant (Vaquero et al., 1999). However, using a standard integrative plant expression vector the level of transgen expression is often low as it is limited by post-transcriptional gene silencing (PTGS) (Johansen et al., 2001). The co-expression with a viral-encoded suppressor of gene silencing prevents the PTGS in the infiltrated leaves. The use of a suppressor of gene silencing as the p19 protein of tomato bushy stunt virus (TBSV) allows high levels of the recombinant antibody, reported expression levels of 1.5g/kg FW (25% of total soluble protein) (Voinet et al., 2003, Vézina et al., 2009, Sainsbury et al., 2009)

The development of virus-based vectors has allowed rapid and high-level transient expression of recombinant proteins in plants, due to the ability of viruses to amplify gene copy numbers and, with it, increase the accumulation of the recombinant protein (Gleba et al., 2008, Lico et al., 2008). Nowadays, two main approaches based on viral vectors are used, namely the independent-virus and the minimal-virus strategies. Independent virus vectors are inoculated as a viral RNA or as a viral particle on leaves, generally by a mechanical inoculation. The virus moves cell to cell and systemically, extending the expression of the foreign gene throughout the plant (Pogue et al., 1998). In this case, the protein of interest is expressed as a fusion to the coat protein (CP) or from a duplicated viral promoter. The limitation of this approach is that only small proteins could be effectively expressed as fusion partners, and inserts larger than 1kb cannot be well-expressed (Gleba et al., 2007) (Figure 1b).

In the minimal virus strategy, *Agrobacterium* carries the T-DNA encoding DNA or RNA replicons for initiating infections. The ability to simultaneously deliver two or more T-DNA to the majority of the cells implies that it was no longer essential that the virus retains the ability to move systemically from the initial infected cells. This led to the creation of 'deconstructed' or modular viral vectors, which involves the removal of viral genes that are not strictly necessary to the production of the recombinant protein. Usually, CP gene is the viral gene to be replaced with the gene of interest (GOI), as CP is usually expressed from a subgenomic RNA promoter and it is the most abundant viral protein. This system can express longer genes, usually up to 2.3kb inserts, equivalent to up to 80 kDa proteins; it exploits the cell-to-cell movement facilitating extension of the transient expression and reducing the requirement of *Agrobacterium* inoculum compared with non-viral systems. Also, large amounts of recombinant protein can be produced when compared with the virus-independent systems (Gleba et al., 2007, Scholthof et al., 1993, Gleba et al., 2005) (Figure 1c).

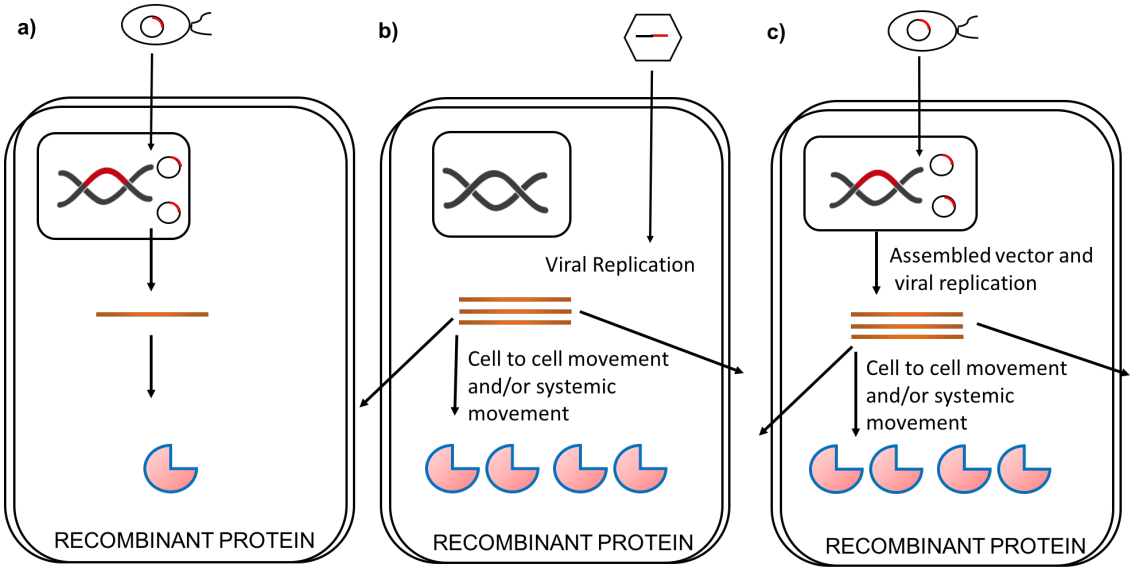

Figure 1. Comparison of expression vectors for transient expression of recombinant proteins in plants. a) Infiltration of Agrobacterium that contains the GOI into *transfer segment of DNA* (T-DNA). The T-DNA is transferred to the plant nucleus. The T-DNA is transcribed and the mRNA is exported to cytosol where it is translated and it encodes the protein of interest. **b)** Independent virus strategy. RNA or viral particles are infiltrated. The GOI is fused to a viral gene and the RNA viral replicates (amplified) at cytosol. The RNA is then generating the protein of interest. **c)** Minimal virus strategy. This strategy combines both previous strategies.

The first deconstructed virus expression system was the Magnifection system, developed by Icon Genetics. The Magnifection system is based on a replication-competent tobacco mosaic virus (TMV) vector. The vector has been engineered to divide the TMV genome into two major cDNA modules: a 5' module, which contains the viral RNA dependent RNA polymerase (RdRp) and the movement protein (MP), and a 3' module that carries the GOI and the 3' untranslated region (UTR). The viral functions are not complemented *in trans* but the two modules actually assemble together *in vivo* by a site specific recombinase delivered by a third *Agrobacterium* cell line. Once delivered to plant cells, the TMV genome is transcribed and spliced to generate a functionally infective RNA replicon. Levels of expression are impressive and can reach up to 5 mg of recombinant protein per gram of fresh weight (Gleba et al., 2005). Magnifection system was extensively tested with different genes, coding antibody-derivatives, growth hormones, bacterial and viral antigens, etc. (Lico et al., 2008, Gleba et al., 2007, Lomonossoff, 2001).

Another interesting deconstructed system is based on Geminivirus and it was developed by Chen and colleagues in 2011 (Chen et al., 2011). The geminivirus expression system is based in a circular DNA replicon derived from the bean yellow dwarf virus (BeYDV). The BeYDV based system involves two modules, one containing the GOI inserted between the BeYDV long and short intragenic regions, and the other being responsible for the expression of the Rep protein, which catalyzes various aspects of the circle replication. Delivery of the geminiviral vector to leaf cells via the Agrobacterium-mediated import procedure, produces very high levels of recombinant DNA in the nucleus that can acts a transcription template, yielding high levels of mRNA for the protein of interest (Chen et al., 2011). A humanized monoclonal antibody (mAb) against Ebola (6D8) was produced at 0,5 mg/g in *Nicotiana benthamiana* leaf mass using the BeYDV expression system, which is the same as that achieved in the Magnifection system.

A limitation of the viral expression systems is that they are basically suited for the production of monomeric proteins. The efficient expression of hetero-oligomeric proteins, such as antibodies is seriously hampered, as the efficiency of viral replication decreases sharply when the viral vector contains two ORFs encoding e.g. two antibody chains. Introducing both ORFs within a same viral genome exceeds the size limits for efficient replication (> 2.4 kb) and requires the duplication of subgenomic promoters, or the expression in the form of a polyprotein (Toth et al., 2001). Moreover, if the two polypeptide chains are cloned in two different vectors built around the same virus backbone, the efficiency of hetero-oligomeric protein formation is very low due to a phenomenon known as viral interference **or superinfection exclusion (SE)**.

The SE is a property inherent of plant virus infections and is defined as the ability of an established virus infection to prevent a secondary infection by the same or a related virus within the same cell (Folimonova, 2012). On the contrary, it has long been known in plant virology that viruses belonging to different groups can coexist in the same cell, probably because both viruses do not compete for the same cell resources. Therefore, the use of non-related (non-competing) viral vectors can be used as a strategy to circumvent SE and facilitate the expression of hetero-oligomeric proteins.

The non-competing vector technology was developed by Giritch and colleagues in 2006, and, similar to the seminal Magnicon technology, it was based on deconstructed vectors. The process relies on co-infection of two no related viral vectors, each one expressing a different polypeptide chain. By using vectors built from the backbones of TMV and potatovirus X (PVX), one expressing the heavy chain and the other expressing the light chain of an antibody, high yields of up to 0,5 mg/g FW of an assembled human a tumor-specific mAb were obtained in *N. benthamiana* leaves (Giritch et al., 2006).

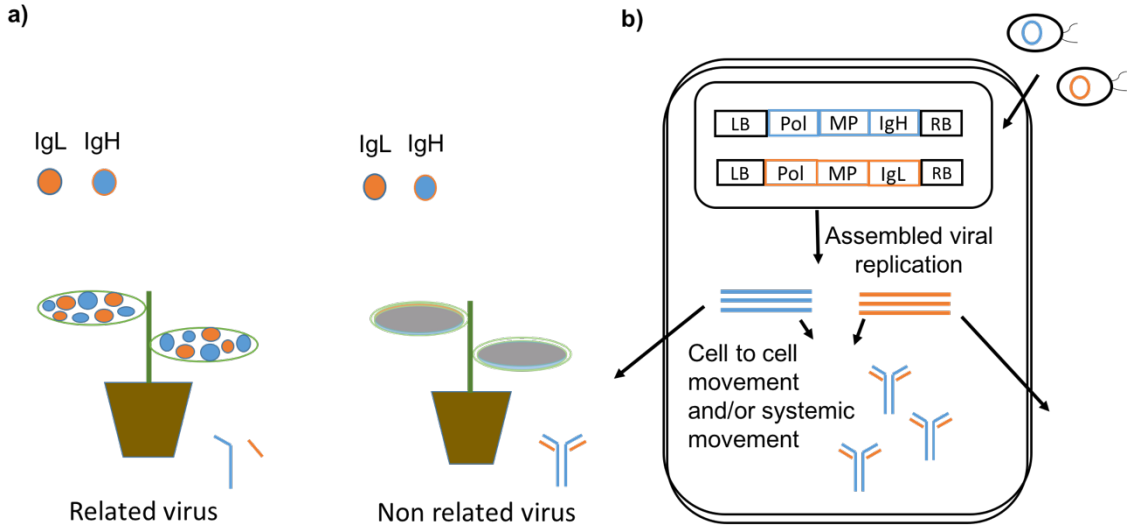

Figure 2. Scheme of the superinfection exclusion phenomenon. a) Co-expression of two related virus creates an expression mosaic, with each tile in the mosaic occupied by a single replicon with a minimal interaction, along the leaf surface due to the viral exclusion phenomenon. In contrast, the co-expression of two non related virus permits the co-expression of both viruses along the leaf surface. **b)** Non-competing strategy for antibody expression using Magnifection system. The variable regions of the heavy (IgH, blue) and light (IgL, orange) chains were subcloned in both tobacco mosaic virus (TMV, blue) and potato virus X (PVX, orange) vectors. The viral provectors are assembled in the nucleus of the plant cells and then spliced and exported to the cytoplasm. The viral vectors replicate (amplify) and they can move systematically or cell to cell.

4. Considerations for antibody productions in plants

The use of plants as biofactory requires multiple considerations involving the yield, quality and cost of the final product (Sarrión-Perdigones et al., 2011). Aspects as glycosylation, subcellular targeting, protein degradation and downstream processing are to be considered for the optimization of recombinant antibody production.

4.1. Glycosilation

Antibodies are glycoproteins with specific glycoforms that are involved in their folding, stability and activity. Plants have the ability to carry out post-translational modifications in a similar way as mammalian cells, such as N-glycosylation. However, N-glycosylation patterns processed in plant cells differ from those of mammals and humans. In the plant Golgi, N-linked glycans contain β(1,2)-xlyose and α(1,3)-fucose instead of α(1,6)-fucose as in mammals (Figure 3). In plants, the fucose and the β-1,3 galactose form a Lewis structure by a link in the terminal N-acetylglucosamine, while in mammals a β-1,4 galactose is frequently linked with sialic acid (Saint-Jore-Dupas et al., 2007, Catilho et al., 2012) (Figure 3).

Plant-specific glycans are considered potentially antigenic and/or allergenic epitopes (Bakker et al., 2001) and their removal is advisable for certain applications. Besides, many therapeutic proteins are glycosylated, and the presence or absence of the different sugar residues can produce a significant change in the protein function. For this, it is important to reproduce the mammalian glycosylation patterns in the recombinant therapeutic proteins produced in plants (Forthal et al., 2010, Shinkawa et al., 2003).

Different strategies to eliminate plant specific N-glycans have been performed. One approach is to retain the antibodies in the endoplasmatic reticulum (ER). The glycosylation in the ER is conserved in the plants and the animal kingdoms. When the ER-retention signal KDEL/HDEL is added to the C-terminal of a protein, this tetrapeptide retains proteins in the ER, yielding only oligomannose-type glycoforms (Bakker et al., 2012, Sriraman et al., 2004, Nutall et al., 2002). Nevertheless, the KDEL signal fused to the heavy chain does not completely retain antibodies in the ER from escaping to the Golgi, forming heterologous forms of glycan structures. A second more elaborated

glycoengineering approach is based on the genetic knock-out of plant-specific xylosyl- and fucosyl-transferases. RNAi procedures to downregulate the transcript of the unwanted glycosyl-transferases, or complete knock-outs by T-DNA insertion, have proven to be successful strategies (Koprivova et al., 2004, Strasser et al., 2004, Strasser et al., 2008, Sourrouille et al., 2008, Shin et al., 2011). Glycoengineered *N. benthamiana* plants lacking xylosyl and fucosyl transferases were used to express ZMapp antibodies with a humanized glycosylation (Loos et al., 2014). New gene editing technologies as the sequence-specific transcription activator-like effector nucleases (TALENs) were used to create targeted knock out mutations in two fucosyltransferases and two xylosyltransferases in *N. benthamiana,* demonstrating the ability and the efficacy of the method to create bi-allelic knock outs in four genes in a single generation (Li et al., 2016).

Recent studies demonstrate how even entire human glycosylation biosynthetic pathways can be introduced (Loos et al., 2014). Plants do not have the enzymes to synthesize the sugar precursor of the sialic acid necessary for the sialytation of the terminal galactose glycans. However, the simultaneous over-expression of the whole genes set involving the full mammalian N-acetylneuraminic acid (Neu5Ac) biosynthetic pathway permitted the sialylation of the recombinant monoclonal antibody 2G12 in *N. benthamiana* (Castilho et al., 2011).

Besides N-glycosylation another type of glycosylation, the O-glycosylation requires also engineering efforts. The O-glycosylation occurs in the Golgi and consists in the addition of N-acetyl-galactosamine (GalNAc) to serine or threonine residues. The role of the O-glycosylation is less understood than the N-glycosylation, but changes in the O-glycosylation pattern are related with several diseases (Tarp et al., 2008, Ju et al., 2011). The first step to add the machinery to transfer GalNAc residues for O-glycosylation in plants was reported by Dascalova and colleagues in 2010 (Dakaslova et al., 2010).

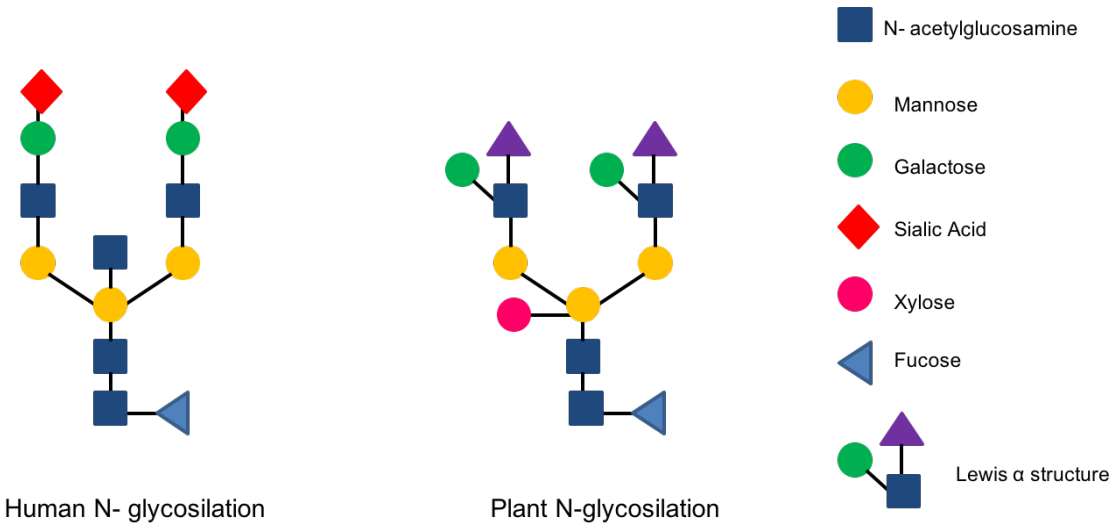

Figure 3. Schematic representation of plant and human N-glycosylation patterns. a) human N-glycosylation pattern; b) Plant N-glycosylation pattern.

4.2. Subcellular location

Extracellular secretion is the natural route for antibodies in mammals; targeting antibodies chains to specific compartments in the plant cell can result in advantages in terms of stability, yield or downstream processing (De Muynck et al., 2010). For instance, it might happen that a recombinant protein directed to the cytoplasm accumulates at moderate levels whereas the same protein targeted to the secretory pathway accumulates at high levels (Conrad et al., 1998, Schillberg et al., 1999). Among the different plant cell compartments (chloroplast, vacuole, apoplast, plasma membrane), the secretory pathway seems to be the most convenient route for a correct antibody folding, due to the oxidizing environment required for the formation of the formation of the disulphide bond, the low abundance of proteases and the presence of molecular chaperones found in the ER (Stoger, 2002, Ma, 2003). The heavy and light chain of recombinant antibodies can be targeted to the secretory pathway by adding a N-terminal signal peptide (a native signal or a plant signal) (Sainsbury et al., 2008, During et al., 1990, Hiatt et al., 1989).

4.3. Plantibody degradation

An important problem associated with the production of antibodies is the presence of degraded antibody fragments. The antibody heavy chain, more specifically the hinge region, is the most sensitive region to proteolytic activity (Sharp et al., 2001, De Muynck et al., 2009). Proteases are abundant in various subcellular compartments, including the vacuole and the apoplast, the final destination for plantibodies targeted to the secretory pathway (De Wilde et al., 1998).

To reduce the antibody degradation, several approaches have been developed. The first approach is the addition of protease inhibitors to extraction buffers, but these are expensive and not viable for extraction at larger scale (Hehle et al., 2011). However, by default plantibodies are accumulated in the apoplast and the vacuole, where the proteases are plentiful, so it would be better to create a strategy that would reduce the level of proteases *in planta.* The approaches *in planta* are complicated because the proteases play important roles in the plant cell. Proteases are involved in classical biological processes such as plant development, defense against pathogens, and nutrient remobilization for reproductive processes (Schaller, 2004). Up until now, several strategies have been proposed, as for example, the co-expression of a recombinant protease inhibitor with specificity for cysteine, serine or aspartic proteases (Benchabane et al., 2008, Goulet et al., 2010). The constitutive expression of the tomato cystatin protease inhibitor resulted in an increased accumulation of the murine C5-1 IgG antibody in *N. benthamiana* leaves. Another strategy to reduce the proteolytic degradation is to knockdown the expression of protease-enconding genes (van der Hoorn, 2008). For instance the RNAi-mediated silencing of cysteine protease-encoding gene, CysP6, improved the accumulation of recombinant full-length IgG1κ antibody (Mandal et al., 2014).

4.4. Downstream processing

Downstream processes are defined by three stages, namely extraction, protein recovery and purification. These stages can represent over 80% of the overall processing costs (Buyel, 2015, Cramer, 1999). The initial phase of extraction is the most important stage of the downstream processing because it determines the level of recombinant

antibodies available for purification, the level of plant protein and other contaminants that require removal and the subsequent downstream processing stages. At the laboratory scale, the most common disruption technique for the extraction of recombinant antibodies in tobacco leaf tissue is the manual grinding in buffer with a pestle and mortar because high levels can be released quickly in this way (Menkhaus 2004, 2008). However, the need of a grinding step is economically unfavorable, except if the recombinant antibody accumulation is extremely abundant to compensate the extra costs during the grind. (Vézina et al., 2009, Sainsbury et al., 2008).

The most suitable method for the extraction of a recombinant antibody also varies with the target location of the antibody in the plant cell (Fabian et al., 2011, Georgiev et al., 2009). For products that have been secreted, for example to hydroponic medium, cell culture supernatant or apoplast, no specialized extraction operations are required because the target is present in an accessible aqueous solution (Drake et al., 2003). In the case of the apoplast targeting, the apoplastic fluid can be isolated from the harvested agroinfiltrated leaves, by vaccum-infiltrating ice-cold extraction buffer and subsequent centrifugation of these infiltrated leaves (Lozano-Torres et al., 2014). In contrast, antibodies intracellular-targeted require a grinding or mechanical shearing (Hassan et al., 2008).

Once the mAb is extracted, centrifugation coupled with depth filtration has been employed for the recovery of mAb. Disk-stack continuous centrifuges are capable of removing cells and large cell debris in the cell culture fluid. Traditionally, depth filters are employed after the centrifugation process because there is a practical lower limit to the particle size that can be remove by centrifugation. Moreover, the use of depth filters in the clarification of cell culture broths protect the chromatography columns used during the last downstream process, the purification of mAb (Liu et al., 2010).

In the biopharmaceutical industry, the majority of purification processes for mAb are based in an affinity chromatography, which results in a high degree of purity and recovery in a single step. The purity of the final product obtained by this method is around 95-98% (Liu et al., 2010).

5. New strategies for recombinant antibody production based on different antibody formats

Advances in Modular Cloning technology and in the understanding of the modular architecture of antibodies has allowed the design of different forms of recombinant antibodies with different pharmacokinetic and pharmacodynamic properties (Figure 4). Each antibody format brings different properties as the binding valency for each antigen, pharmacokinetic half-life and effector functions (Spiess et al., 2015). To optimize the properties of an antibody for a particular application, it would be preferable to improve, or even delete, some particular characteristics. For example, it would be advantageous to have a small antibody with a short half-life in order to achieve tumor penetration or for a better visualization of the tumors (Chames et al., 2009). In other instances, it would be preferable to have an antibody with constant heavy chains that would avoid a rapid clearance immediately after its application. The fragment crystallizable region (Fc) of immunoglobulin binds to the salvage receptor (FcRn), thereby providing the potential for a long half-life *in vivo* (Roopenian et al., 2007).

To date, the great majority of antibodies produced in plants are the human IgG. The simplicity of the downstream processing method and its use for immunotherapy are the reasons for this choice. IgA and secretory IgA (sIgA) have also been produced in plants due to its relevance for passive immunotherapy (i.e. the transfer of active antibodies from one organism to another that can prevent or treat infectious diseases) (Juarez et al., 2013). In addition to full size antibodies, a number of engineered antibody fragments have been successfully produced in plants such as single-chain variable fragments (scFv), variable domain or nanobody (V_HH), fragment antigen binding (Fab), small immunoprotein (Li et al., 1997) and their fusions with the Fc (Figure 4).

Despite the large number of antibody-formats produced in plants, very little is known on how different formats of the same antibody affect the stability *in planta* and therefore the productivity of a given antibody. Up until today, there has not been a comparative studies of a same antibody in different formats. For that reason, the objective of one chapter of this thesis (Chapter I) is to compare the stability, productivity and the activity of an antibody in different formats.

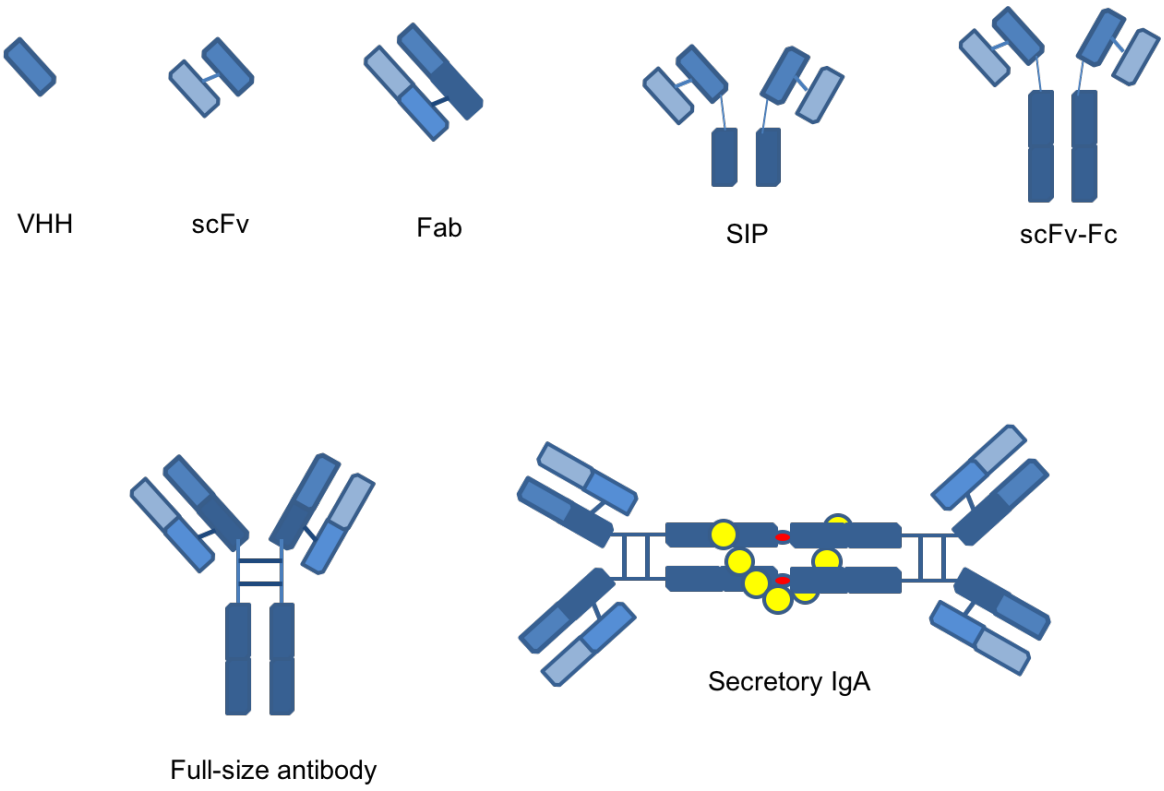

Figure 4. Alternative antibodies formats produced in plants. The modular domain of immunoglobulin allows a large range of alternative antibody formats that span a molecular weight range of at least 12 to 150 kDa. The secretory IgA has a molecular weight of 450 kDa.

6. New strategies based on antibody cocktails

The treatment or prevention of certain diseases such as some infectious diseases caused by highly variable agents, some oncology diseases or the envenomation with complex toxins, cannot be ensured with mAb therapy (Bregenholt et al., 2006, Nowakowski et al., 2002), and instead, the best therapy to provide protection is a combination of multiple monoclonal antibodies (mAb cocktail) (Jost et al., 2014). A mAb cocktail has the advantage of offering multiple specificities, binding several epitopes and acting in synergy (Marasco et al., 2007, Flego et al., 2013). MAbs cocktails can also provide superior blockade of receptor signaling and may provide a mechanism to prevent drug resistance in oncology (Koefoed et al., 2011). For example, three conventional mAbs are used for targeting HER/neu in breast cancer (Nejatollahi et al., 2014). Moreover, the use of a mAb cocktails provides great flexibility in the ratio and in

the amount of antibodies, allowing the dosing time to be varied (Spiess et al., 2014). The first mAb cocktail was used in clinical trials was in 1982 (Logtenberg, 2007). Since then, the use of mAb cocktails has been increasing. The Table 2 shows the mAb cocktails that are currently in clinical trials.

Table 2. Targets for which mAb cocktails have shown increased efficacy *in vitro* and *in vivo* preclinical testing. Adapted from Logtenberg, 2007.

Target	***Number of mAb included***	***References***
Viruses		
Rabies virus	*2 or 3*	*Bakker et al., 2008*
HIV	*3*	*Ferrantelli et al., 2007*
SARS-CoV	*2 or 3*	*Meulen et al., 2006*
Influenza virus	*2*	*Prabakaran et al., 2009*
Ebola virus	*3*	*Davidson et al., 2015*
Soluble molecules		
IL-6	*3*	*Montero-Julian et al., 1995*
IFN-2	*3*	*Kontsek et al., 1991*
Tetanus toxin	*2*	*Lang et al., 1993*
Cell-bound molecules		
HER/neu	*2-3*	*Nahta et al., 2004*
CD4xTNF-	*2*	*Williams et al., 2000*

There are different approaches for manufacturing mAb cocktails. The first strategy is the individual production of each mAb followed by their combination in a single drug

product. Today, this is the general strategy followed for the expression of mAb mixtures (Spiess et al., 2014, Pedersen et al., 2010). An alternative approach would consist in the simultaneous production of all mAb in the cocktail using a single cell line or by single-cell batch (Fradsen et al., 2011). For clinical studies, mAb cocktails can be created by co-formulating individually purified antibodies (the first strategy explained above) (Nayak et al., 2014), or streamlining the purification process by co-culturing the individual antibody expressing cells (the second strategy exposed before) (Pedersen et al., 2010).

One example of individually manufacturing three different antibodies in plants is the case of ZMapp, a cocktail that provided protection against Ebola virus infection, used in non-human primates and allegedly, also in humans under compassionate treatment (Qiu et al., 2014, Davidson et al., 2015, William et al., 2000). Plants-derived-mAb cocktails are produced, released and characterized separately. Then, the different mAbs are mixed in a final drug product. This approach is expensive for low number of antibodies in the cocktail, but prohibitive for large cocktails as the addition of each new mAb there increases dramatically the cost per gram and it is impractical for scaling up (Qiu et al., 2014, Wang et al., 2013).

Therefore, an ideal platform for mAb cocktail production should have the capacity to produce multiple mAbs at the same time, while providing low manufacturing costs, high yield, and, most importantly, good batch-to-batch reproducibility. One possibility to reduce production costs, and improve reproducibility batch to batch would be the co-expression in plants of the various antibodies that composed the mAb cocktail via agroinfiltration. However, the main drawback of this strategy would be the shuffling between heavy and light chains of different antibodies. The chains shuffling relies on the inability to ensure that vectors that are established in a cell encode both the light and the heavy chains pair of the original selected antibody. In other words, the agroinfiltration solution contains as many different bacteria as mAbs are to be expressed. However, the T-DNAs are randomly transferred to the plant cells often resulting in the expression of a combination of heavy and light chains different from any of the intended ones. Consequently, non functional antibodies can be obtained because of the chain **shuffling phenomenon** (Figure 5).

It is not clear to what extent the shuffling of antibody chains can affect the ability to produce effective mAb cocktails. If that is the case, it would be possible that viral exclusion, which keep clones segregated, could be exploited as an alternative strategy to circumvent this limitation and to efficiently produce cocktails of recombinant antibodies efficiently (see later in this introduction, section 7). In this thesis I explored the limitations of cocktail expression using non-replicative systems (chapter II) and I explored the possibilities offered by exclusion systems to produce recombinant antibody cocktails using so-called Pluribodies Technology (chapter III).

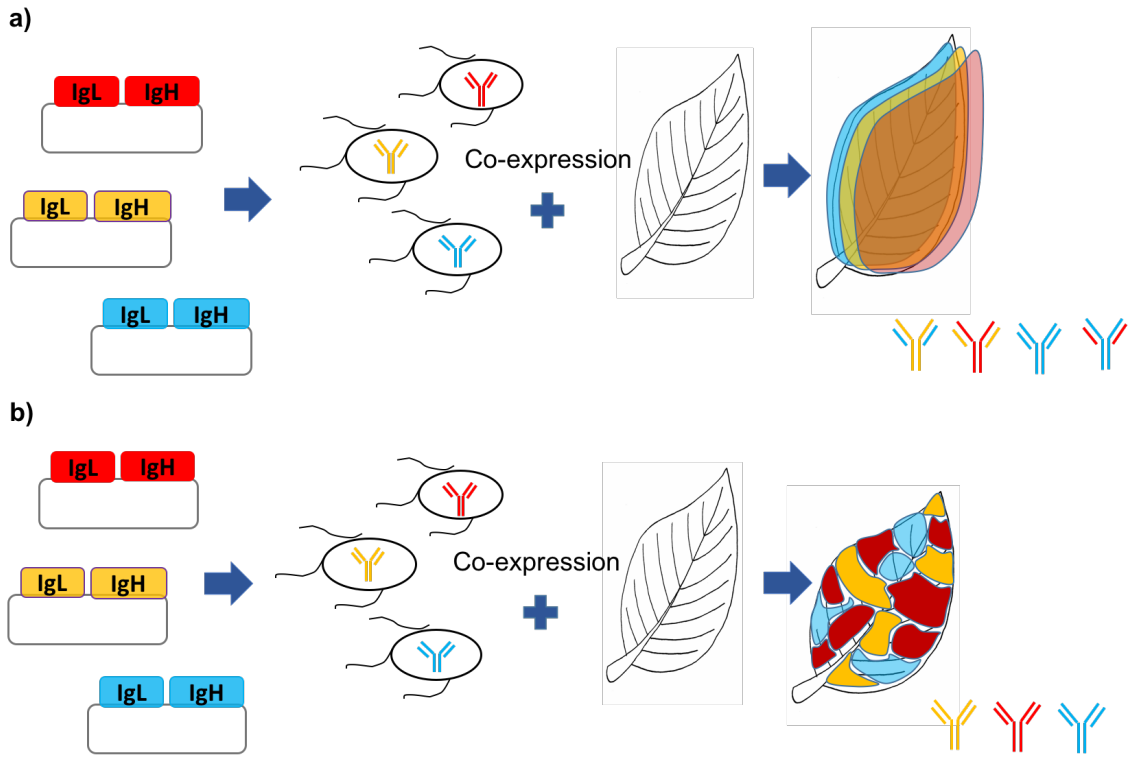

Figure 5. Schematic representation of co-expression of a mAb cocktail in the same leaf. a) The co-expression of several antibodies in a single transformation event using non replicative system could affect the specificity and functionality of the antibody. The promiscuity with the heavy and light chain could produce various combination possibilities, some of them with low specificity by the shuffling chain phenomenon. **b)** The co-expression of several antibodies in a single transformation event using replicative system could produce functional recombinant antibodies efficiently due to the induction of somatic expression mosaics in plant leaves.

7. Pluribody technology

In an attempt to overcome limitations in the production of antibody cocktails, our group has recently developed a new strategy for affordable production of recombinant polyclonal antibodies (pAbs) in plants. This strategy takes advantage of an intrinsic property of many plant viruses known as Superinfection Exclusion (SE) or Homologous Interference, which prevents the infection of a challenge virus into a previously infected cell (Folimonova, 2012). An important consequence of SE is that population variants in plant virus infections are not uniformly distributed along the plant, but structured in a mosaic-like pattern due to the limitation to superinfection imposed by resident viral clones (Elena et al., 2011). As a result, plant co-infection with two or more viral clones induces the formation of infection mosaics, with different sectors dominated by mutually exclusive viral clones. Our group proposes the use of virus-based expression systems inducing the formation of somatic expression mosaics in plant leaves for the production of recombinant polyclonal antibody cocktails. In a work carried out in parallel to this Thesis, a strategy was developed which ensures that high levels of recombinant polyclonal antibodies are produced with outstanding batch-to-batch reproducibility regardless of the complexity of the polyclonal composition.

Initially, a first study of how mosaic-like patterns of recombinant protein expression could be induced experimentally in somatic plant tissues was carried out by co-delivering three viral infective clones encoding GFP, BFP and DsRed fluorescent proteins in *Nicotiana benthamiana* leaves following a deconstructed virus strategy mediated by agroinfiltration (Julve et al., 2013). Upon experimental agrodelivery, successfully established infectious foci appeared approximately 48 hpi, and immediately began cell-to-cell expansion bringing along its corresponding fluorescent color. Clone expansion continues during at least seven days unless halted by a competing neighboring clone. As expected, the abundance of initial infection foci, and consequently the final average size of the tiles in the mosaic, was dependent of the *Agrobacterium* concentration in the infiltration culture (measured as the optical density, OD) (Figure 6). Based on this procedure, we hypothesized that above a certain infiltration OD, the segregated (mosaic) distribution of viral clones imposed by SE will offer a competition-limited

microenvironment that will ensure the survival of low-fitness clones, which would be otherwise displaced by high fitness competitors in an environment without SE.

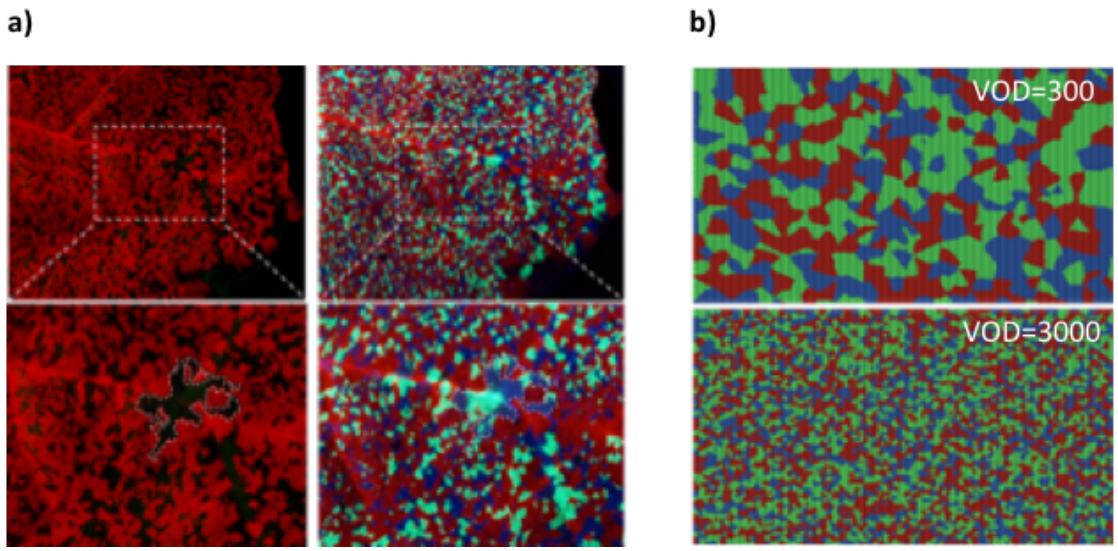

Figure 6. Principles of superinfection exclusion. Adapted from Julve and colleagues (submitted). **a)** *N. benthamiana* leaves showing somatic expression mosaics produced by three Magnicon viral clones encoding DsRed, GFP and BFP proteins agroinfected simultaneously at OD600=0,01. Mosaics are observed either with red/green filters for DsRed (left) or with UV light (right). **b)** Netlogo computer simulation of a triple viral co-infection (Red, Green, Bkue) subjected to SE in a virtual area comprising 62.500 virtual cells. The virtual fitness for each clone was adjusted to 1.0:0:1.0:0.7 (R:G:B) and virtual OD600 was adjusted to 300 (upper image) and 3000 (lower image) arbitrary units to match the natural infection.

Next, we explored if the robustness of low-competition environments in virus-induced expression mosaics could be applied to more complex mixtures, e.g. for the production of recombinant polyclonal antibodies in a reproducible manner and without compromising the diversity of the final product. To test this, we first assayed the *in planta* expression of an antibody library obtained from camel Peripheral Mononuclear Blood Cells (PMBC), thus comprising a fraction of the antibody repertoire present in circulating B cells of a camel individual. Camel antibodies were preferred because camelids display large immune repertoires in single chain antibody format (V_HHs), a feature that greatly simplifies library construction and plant expression using viral vectors. V_HH variable antibody regions were PCR-amplified from PMBC DNA, and cloned in batch into a binary MagnIcon vector containing an in-frame histidine tag for

detection. The library was then transferred to *Agrobacterium tumefaciens* cells and transiently expressed in *N. benthamiana* leaves using vacuum agroinfiltration. After seven days incubation, V_HHs from leaf apoplast fluid were recovered without disrupting the leaf tissue, purified and resolved in a 2D electrophoresis showing a complex pattern of >200 distinguishable spots, indicative of the polyclonal nature of the sample (Figure 7). The resulting plant-made recombinant polyclonal antibody is referred to as a "pluribody" sample.

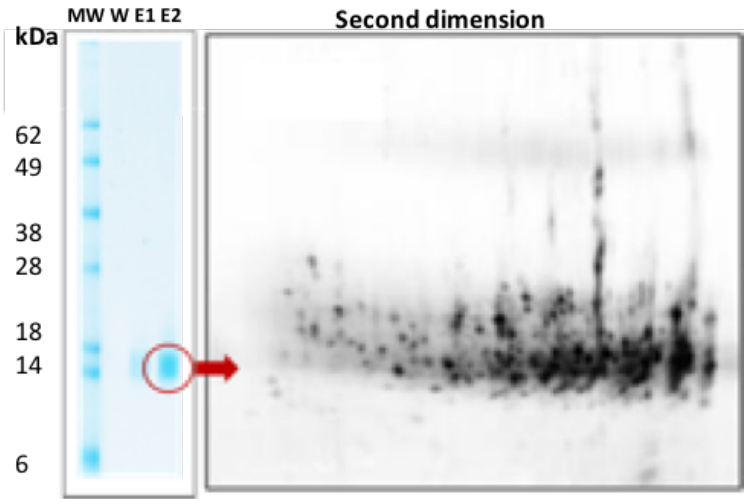

Figure 7. First and second dimension electrophoresis separation of V_HH -His clones purified from *N. benthamiana* leaves using Ni-NTA affinity columns.

Pluribody technology has been subsequently used in our group to produce in plants a mammalian immunized polyclonal antibody repertoire. To do so, a similar approach to that described above was conducted to obtain a new pluribody sample, but in this case camels were previously immunized against a snake venom, aiming to produce a plant-made recombinant polyclonal antibody that could serve as an antidote against snake envenomation. Snake envenomation, due to the complexity of the toxin composition, can only be treated with antidotes made of polyclonal antibodies, usually obtained from immunized animals. Currently, there is no technology available to produce snake anti-venoms in a recombinant manner. Pluribody technology could result in a recombinant alternative to animal polyclonal anti-sera-based anti-venoms if sufficient yield and batch-to-batch reproducibility can be ensured for this technology. To test this, PMBCs from immunized camels were isolated, and their antibody variable regions were cloned in a phage display vector for enrichment in toxin-binding antibodies. Next, anti-toxin-selected antibody variable regions were cloned "in batch"

in Magnicon vectors fused to the constant region of a human IgG1. As a consequence, a number of "cocktails" of antibodies ready to be transformed in plants was generated, which need to be produced and assayed for yield, reproducibility and their ability to neutralize snake toxins *in vivo* and *in vitro*. In chapter III of this thesis I studied the ability of "pluribody" technology to produce recombinant polyclonal antibodies formulations able to neutralize snake venoms.

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Objectives

Plants are an alternative expression system for the manufacturing of recombinant antibodies (rmAbs). Next to the classical monoclonal immunoglobulin G format, which predominated in recent years, rmAbs are now required in different presentations for an increasing array of applications, e.g. incorporating new antibody isotypes, exhibiting a variety of structural formats from single chain to full-size, or displaying expanded antigen-binding capacities, from monoclonal to bi-specific, oligoclonal or polyclonal. The incorporation of Transient Gene Expression and Modular Cloning techniques in Plant Biotechnology facilitates the design, production and functional comparison of new antibody formats in a reasonable period of time.

The overall objective of this thesis is to evaluate the production of innovative antibody formats in plants taking advantage of Modular Cloning and Transient Gene Expression, comparing production yields, manufacturing requirements and assessing their functionality.

To fulfil this general objective, the following specific objectives are proposed:

1. To produce in *N. benthamiana* five different formats of a commercial mAb (Infliximab) against the human Tumor Necrosis Factor α (h-TNF-α), by combining variable and constant regions in different arrangements by means of Modular Cloning, and to compare the different formats in terms of yield and TNF neutralizing capacity.

2. To employ Modular Cloning to produce a small cocktail of three human rmAbs against Ebola virus in full IgG format (ZMapp), and to use the resulting constructs to compare two manufacturing alternatives for antibody cocktails in *N. benthamiana*: simultaneous co-expression versus the establishment of parallel production lines.

3. To produce a complex cocktail of 36 rmAbs in *N. benthamiana* raised against *Bothrops asper* snake venom toxins, taking advantage of the Superinfection Exclusion imposed by a Magnicon viral vector, and to assay the functionality of the resulting product to neutralize snake venom toxicity *in vivo* and *in vitro*.

CHAPTER 1 Production of Infliximab in plant: Comparative analysis of different antibody formats

1. INTRODUCTION

Recombinant monoclonal antibodies (rmAbs) have several applications including research, diagnostic tests or biological therapy. The first recombinant therapeutic antibody, Orthoclone OKT3 used for prevention of kidney transplant rejection, was approved by the FDA in the mid-1980s (Hiatt et al., 1989). Since then, 30 rmAbs were approved by the FDA for treating human diseases such as cancer, infectious diseases, cardiovascular diseases and inflammatory bowel diseases (IBD) (Liu, 2014, Hansel et al., 2010). Nowadays, the use of rmAbs represents over 30% of the biopharmaceutical treatments with a market value of $99 billion per year worldwide.

An example of rmAb approved by the FDA for the treatment of IBD is Infliximab. Infliximab is a recombinant IgG1 antibody against the human Tumour Necrosis Factor (TNF-α), a cytokine with multiple biologic actions including mediation of inflammatory responses and modulation of the immune system (Niazi, 2016). Infliximab is produced in mammalian cells, to be more specific in mouse myeloma cells, and commercialized by Johnson & Johnson/Merck & Co/Mitsubisho Tanabe, which patent has expired in Europe in August 2014 and in the US in September 2016 (Peter Lakatos, 2016). The use of Infliximab revolutionized the treatment of IBD, such as Chron's disease, psoriasis arthritis, rheumatoid arthritis, and ankylosing spondylitis, during the last two decades because it reduced the immunogenicity of TNF-α and led to sustained remission in many patients (Shirota et al., 2008, Raychaudhuri et al., 2009, Choy, 2011).

Generally, Infliximab is administered intravenously to control the signs and symptoms of inflammation. An infliximab injectable dose of 3-5 mg/kg is recommended as initial treatment, but some patients who require maintenance need to increase the Infliximab dose to 5-10mg/kg at 0, 2 and 6 weeks (Sandborn et al., 2002). Topical administration of Infliximab is also possible either as a gel formulation or as a solution. Streit and colleagues reported that topical administration of Infliximab produced complete recovery of patients with chronic ulcers. In this case, they showed that Infliximab is safe and effective when it is topically applied (Streit et al., 2006). In topical

administration, Infliximab was applied to ulcers either as a 0.1-4.5mg/gel formulation or as a 10mg/mL solution (Streit et al., 2006).

Despite the currently widespread use, Infliximab is an expensive medication, costing about $958.2 for a 100 mg dose. Thus, the treatment using Infliximab carry a significant economic burden, with an annual cost of $24,273 per treated patient in USA (Schabert et al., 2013).

Plants can overcome some of the limitations of other rmAb production platforms, such as mammalian cells, yeasts and bacteria, offering a cost-effective platform for the production of rmAb. The advantages of plants are low production cost, easy scaling up production, absence of animal and human pathogens, high production and the ability to engineer posttranslational modifications *à la carte* (Ma et al., 2003, Orzáez et al., 2009). Large scale production of rmAb could also serve to explore alternative administration routes like mucosal or topical applications, known to require higher doses than parenteral, intramuscular or intravenous (i.v.). Moreover, advances in recombinant DNA technology and in bioengineering antibodies technology enable the design, construction and expression of different antibody formats (Orzáez et al., 2009, Ko et al., 2005, Moussavou et al., 2015). The development of alternative antibody formats facilitates different therapeutic applications. For instance, for local administration, smaller formats are preferred rather than full-length antibodies (150kDa) because its ability to penetrate epithelial tissue barriers more rapidly and efficiently than full-length and they have a very short-life (< 5 hours). Thus, small Infliximab format would be desirable for the topical treatment of diseases that are manifested at a particular location and do not require a systemic treatment, as for example in the local arthritis or chronic wound healing (Streit et al., 2006). In last years, Vandenbroucke and collaborators demonstrate that orally administration of small anti-TNF-α antibody reduced signs of colitis in patients with ulcerative colitis (Vandenbroucke et al., 2009). Moreover, small antibody formats can be fused to the fragment crystallizable (Fc) of immunoglobulin granting the same properties as a full-length antibody, although they have a minor size. This is advantageous, due to the fact that minor sizes allow penetration in epithelial tissues, and the addition of Fc prolongs the biological half-life, thus the intermediate antibody

format becoming ideal for some systemic diseases like Chron's disease (Li et al., 1997, Yokota et al., 1992, Hutt et al., 2012, Suzuki et al., 2010, Cortes, 2013).

Although antibodies in plants have been produced in several formats, little is known on how yield and stability are affected by the format of choice. In most studies, a single antibody format is studied and few comparative studies have been carried out. It is well known that idiotype affects dramatically expression levels and therefore to understand the influence of the antibody structure in the accumulation *in planta* it is necessary to produce the same antibody in different formats. Here, we compare the production of five recombinant versions of Infliximab in *N. benthamiana* leaves: scFv, scFv fused to the Fc of human heavy alpha 1chain (scFv-Fcα1), scFv fused to a human Fcγ1 (scFv-Fcγ1), and full length human immunoglobulin γ1 with the two isotypes forms from the light chain, designated kappa (k) and lambda (λ). Moreover, we also assayed the production of Infliximab scFv-Fcα1 format in tomato fruit. Fcα1 is the most abundant constant regions in the mucosal surfaces and therefore is specially indicated for oral administration (Raiola et al., 2014). We evaluate accumulation, expression, degradation, cellular location and purification of all the different antibody formats. With this study, we compared the biological active *in vitro* activity of the different plant-derived anti-human TNF- α antibodies.

2. RESULTS

2.1. Construction of several Infliximab formats by GoldenBraid

The building of the different Infliximab antibodies was performed using GoldenBraid 2.0 technology (GB). GoldenBraid is a modular DNA assembly system for plant multigene engineering based on type IIS restriction enzymes (Sarrión-Perdigones et al., 2013). A number of standard basic DNA elements, named GB parts, were used as starting point in the building of alternative Infliximab antibody format. The process of adapting a raw DNA sequence to the Goldenbraid syntax, converting it to a GBpart is known as domestication, and consists in (i) the removal of internal BsaI and BsmsBI restriction sites by site-directed mutagenesis PCR; (ii) PCR-mediated addition of flanking GB standard barcodes that specify the relative position of each GBpart in the assembly; (iii)

cloning of the resulting domesticated PCR products into an entry (pUPD1) vector. Basic GBparts as the CaMV 35S constitutive promoter (35s), the pectate lyase signal peptide (SP) and the nopaline synthase terminator (Tnos) were already available in the GBparts collection. Therefore, it was only required to domesticate the constant regions of the human γ1 heavy chain (HCγ), the Fc region of human α heavy chain (HCα) and the constant regions of human light chain (LC) lambda (LCλ) and kappa (LCκ). The heavy and light variable regions and the scFv of the anti-human TNF-α were also domesticated as GBparts. All these GBparts were made available in the GBcollection (Figure 1a). A number of steps were followed to construct each alternative Infliximab antibody. Firstly, multipartite assembly reactions were performed to assemble together the different GBparts into a binary destination vector (α-GB vectors) to produce individual transcriptional units (TUs) as Light chain, Heavy chain, small and intermediate antibody format (Figure 1b). To construct full monoclonal antibodies, a second GB-step was carried out: two TUs (LC+HC) were assembled together into a binary destination vector (Ω-GB vector) through a so-called binary assembly reaction (Figure 1c).

2.2. Anti-human TNF-α antibodies production in N. benthamiana leaves and in tomato fruit

In order to evaluate the production of the different generated Infliximab antibody formats in *N. benthamiana* leaves*,* all binary plasmids containing the Infliximab formats were transferred to *Agrobacterium tumefaciens* and transiently transformed in *N. benthamiana* by means of agroinfiltration. Five days post infiltration, leaves were collected and an initial screening was performed with an antigen-ELISA test to detect the presence of recombinant antibodies in the clarified extract using an anti-HC antibody for detection. Antibody production levels were quantified assaying the clarified extract with an ELISA test. Several differences in production were observed between alternative Infliximab formats (Table 1). Of all the packed formats, the intermediate format (scFv-Fcγ1) accumulated the most, with an accumulation level reaching the 2.05% of the total soluble protein (TSP), followed by both full length antibodies, HCγ1-LCκ and HCγ1-LCλ, with accumulation levels higher than the 1.2% of TSP. Surprisingly,

a low accumulation level was observed for HCγ1-LCκ. Finally, the accumulation of the small Infliximab format, scFv, was undetected (ND).

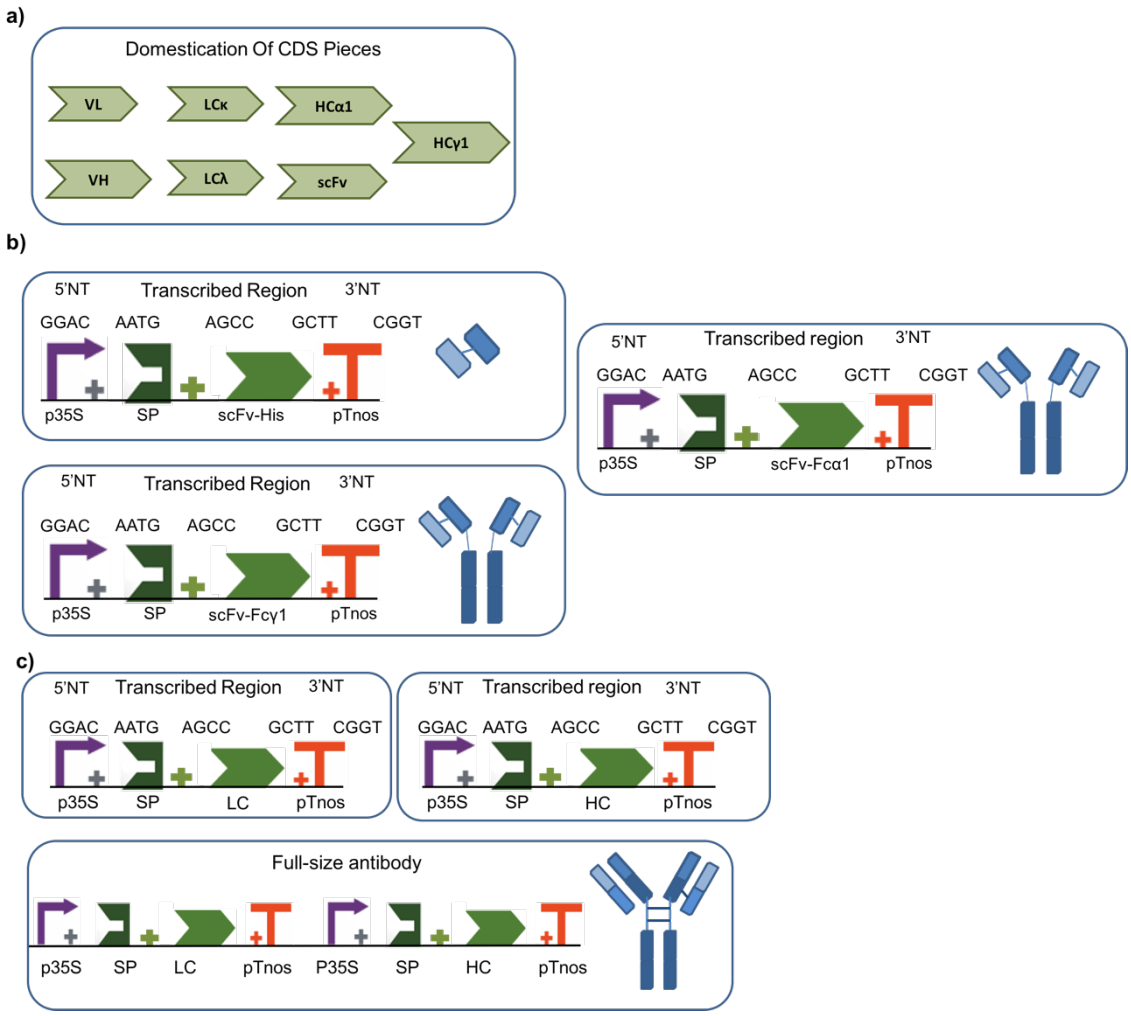

Figure1. Assembly process of five Infliximab formats with Golden Braid. **(a)** Collection of GBparts necessary to build the different antibodies. **(b)** Multipartite reactions of the GBparts required to construct the different Transcriptional Units (TUs). The multipartite reactions consist in assembling the GBparts in a GBα-vector. **(c)** To construct the full length antibody requires a binary assembly of two TUs, the heavy chain TU and the light TU, in a GB Ω-vector.

All these Infliximab formats were targeted to the apoplast compartment with a signal peptide. In order to know if they were differentially accumulated in the apoplast, an extraction of the apoplastic fluid was carried out for each version of the Infliximab by the infiltration-centrifugation method (Lohau et al., 2001). The results showed that only the intermediate Infliximab format, scFv-Fcγ1, was extracted from the apoplastic fluid

while both full-length antibodies, HCγ1-LCκ and HCγ1-LCλ, were retained in the leaf tissue.

One of the TNFα antibody format under analysis, scFv-Fcα1, included a KDEL signal for endoplasmic reticulum (ER) retention in the C-terminus of the protein. In some cases, ER retention is known to allow higher accumulation levels than those obtained when the antibodies are secreted to the apoplast (Juárez et al., 2013). This format was assayed both in *N. benthamiana* and in tomato. We found that the accumulation level of scFv-Fcα1 was different depending on the platform. The scFv-Fcα1 expressed in stable lines of tomato represents 0.06% of TSP fruit extract. However, the same antibody expressed in *N. benthamina* leaves in transient has a higher level, representing the 1.03% of TSP.

Table 1. Comparative evaluation of anti-TNFα antibodies.

Construct	Platform	% Antibody (% TSP)	Yield (μg/g FW)	Location	Expression
HCγ1-LCκ	*N.benthamiana*	1.22 ± 0.2	74 ± 0.17	Intracellular	Transient
HCγ1-LCλ	*N.benthamiana*	1.55 ± 0.15	86 ± 0.12	Intracellular	Transient
scFv-Fcγ1	*N.benthamiana*	2.05 ± 0.1	122 ± 0.06	Apoplast	Transient
scFv-His	*N.benthamiana*	ND	ND	ND	Transient
scFv-Fcα1	*N.benthamiana*	1.03 ± 0.2	63 ± 0.2	Intracellular	Transient
scFv-Fcα1	*S.lycopersicum*	0.06 ± 0.1	9 ± 0.2	Intracellular	Stable

The levels of antibody are reported in percentage of total soluble protein (%TSP) and the yield in micrograms per gram of fresh weight (μg/g FW).

The supernatants of the different plant protein extracts were analysed on Western blot under reducing conditions to examine the expression of the different Infliximab

versions. The Western blot was developed using anti-heavy chain antibody. The intensity of the bands is in accordance with the accumulation levels observed above. The scFv-Fcγ1 format had the most intense band at 50 kDa corresponding to the heavy chain region. In the case of the both full length Infliximab, HCγ1-LCλ antibody showed a higher-intensity band than HCγ1-LCκ at 50 kDa (Figure 2a). In case of the scFv-Fcα1 format, a lower intensity band was observed for tomato fruit stable lines than for clarified extract of agroinfiltrated leaves in *N. benthamiana* (Figure 2b). Degradation products were also observed on the Western blot analysis under reducing conditions. Antibody fragmentation was present in all *N. benthamiana* leaves extracts and the antibody fragments were visible at around 28 kDa (Figure 2). These fragments belong to heavy chain domains as the Western blot was developed using anti-heavy chain. In the clarified extracts from *N. benthamiana* leaves, minor degradation bands were observed for the full-length antibody HCγ1-LCκ. In contrast, high intensity bands of a smaller fragment were observed in scFv-Fcγ1 and HCγ1-LCλ formats. These degradation observations indicate that a significant proportion of the total antibody was present in the form of Fab' fragments, probably as a result of the cleavage on the hinge region of the heavy chain.

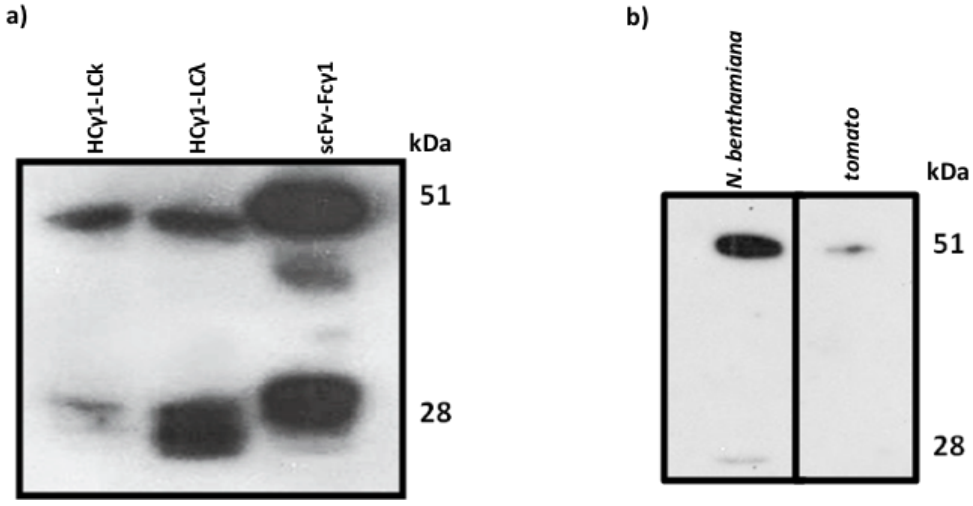

Figure 2. Western blot analysis of the recombinant antibodies in crude plant extracts. Protein extracts of supernatants. Samples were resolved under reducing conditions and developed using **(a)** anti-HCγ and **(b)** anti-HCα.

2.3. Functional comparison of the different anti- TNF-α formats

Due to low accumulation of the Infliximab derivative produced in tomato, only the functionality of the Infliximab derivatives produced transiently in *N. benthamiana* leaves was further analysed. All of them were purified using affinity chromatography. The three antibody formats that contain the isotype γ1 from the HC (scFv-Fcγ1, HCγ1-LCλ and HCγ1-LCk), were purified using the protein A affinity chromatography, which binds the Fcγ portion of the HC (Yang et al., 2003). In contrast, the scFv-Fcα1 antibody, which contains the isotype α1 from the HC, was purified using the SSL7-affinity chromatography, which binds the Cα2/Cα3 domain junction of the Fc portion of the HC (Langley et al., 2005). Upon SDS-PAGE separation of the elution fractions, a 23.5 kDa band corresponding to the full size LC, a 50 kDa band corresponding to the full size HC and a 55kDa band corresponding to the scFv-Fcγ1 and scFv-Fcα1 were observed in Coomassie stained gel (Figure 3).

The Coomassie-stained gel showed also degradation products of the purified antibodies. Notably, a part of the total antibody purified from plants expressing Infliximab derivatives containing the isotype γ, was present in form of degraded fragments. Smaller fragments were present around 28 kDa. This observation suggests proteolytic cleavage during extraction and purification. However, degradation fragments were not observed in Infliximab scFv-Fcα1 format. This could be due to the fact that smaller fragments do not bind SSL7 since they lack the Fc domain, specifically the hydrophobic Cα2/Cα3 domain junction.

The functionality of the crude and the purified antibodies was initially determined by a direct ELISA test. The plate was coated with human TNF-α, followed by detection with an anti-human HCγ antibody for the HCγ1 antibodies or anti-human HCα antibody for the Fcα1 antibody (Figure 4). A dilution series of purified antibodies was probed with identical molar amounts. No difference in binding was observed between the three different Infliximab derivatives HCγ1-LCk, scFv-Fcγ1 and scFv-Fcα1. Contrary, ELISA analysis showed that the HCγ1-LCλ antibody format was more sensitive binding the antigen than the other three Infliximab derivatives.

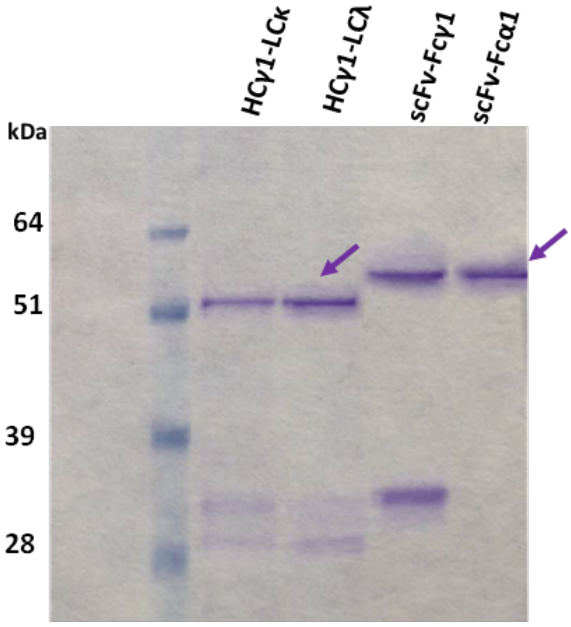

Figure 3. Coomassie stained SDS-PAGE of HCγ1-LCκ, HCγ1-LCλ, scFv-Fcγ1 and scFv-Fcα after affinity purification. Expected band sizes are 50kDa for the HC, 28kDa for the degradation fragments, 25kDa for the LC and 55kDa for the scFv-Fc. 500 ng of purified antibodies were loaded per lane.

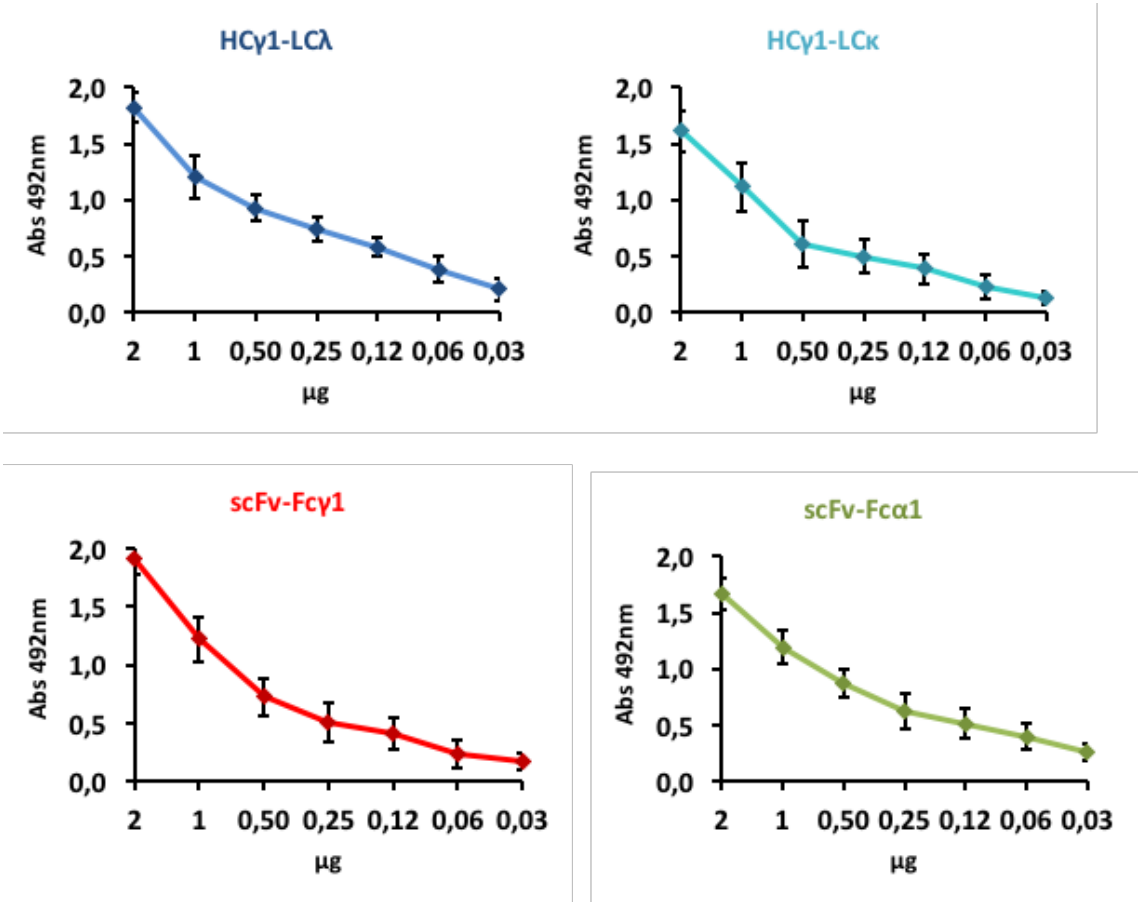

Figure 4. ELISA test of the purified plant-derived-antibody versions. Comparison between the several Infliximab formats produced in *N. benthamiana* leaves. The ELISA was coated with 1µg of TNF-α/well. Samples were developed with anti-HCγ and anti-HCα.

2.4. Comparison of the TNF-α neutralization activity of the different anti-TNF-α antibody formats

Members of the tumour necrosis factor ligand family (TNFs) induce the apoptosis pathway via the caspase cascade and via mitochondrial proteins. Currently, TNF pathway is one of the best-characterized apoptotic signalling pathways (Ślebioda et al., 2014). Briefly, the binding of soluble TNF-α to this cognate receptor, tumor necrosis factor receptor 1 (TNFR1), results in the binding of the TNRF1-associated death domain protein (TRADD) with recruitment of Fas-associated death domain (FADD) and receptor-interacting protein (RIP). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signalling complex (DISC) is formed, resulting in autocatalytic activation of procaspase-8. Once caspase-8 is activated, the apoptosis pathway is triggered (Elmore, 2007). The addition of anti-TNF-α prevents the binding between TNF and its cognate receptor reducing the induction of the apoptosis pathway (Sedger et al., 2014).

The activity of our recombinant anti-human TNFα antibodies to block the biological activity of hTNF-α was tested in a cell viability assay with Trypan Blue. U973 cells were treated with a constant concentration of hTNF-α and increasing amounts of anti-TNF-α, HCγ1-LCλ, HCγ1-LCκ, scFv-Fcγ1, scFv-Fcα1, for 24h. We used a chemotherapeutic agent as doxorubicin as a death-control because it has been known to cause cell death by inducing apoptosis in U973 cells (Demidenko et al., 2006). An anti-hTNFα commercial antibody that is used in biological therapy to block TNF-α activity was used as a positive control. After the treatment, cells were stained with Trypan Blue which only stains death cells. The cell viability was manually counted in a microscope Neubauer chamber. Figure 5 shows the inhibition of the cell death in a dose-dependent manner. Significant differences were observed between the different Infliximab derivatives produced in plants. HCγ1-LCλ and scFv-Fcγ1 antibody formats showed higher percentage of cell viability (around 75%) demonstrating a higher antigen binding capacity in an *in vitro* assay. In contrast, the HCγ1-LCκ format showed less capacity to block TNF-α activity showing minor survival rate (nearly 60%) than the other recombinant antibodies that contain HCγ. Curiously, scFv-Fcα1 antibody format showed a low capacity to bind TNF-α resulting in a survival rate of approximately the 50% (Figure 5) .

Significant differences were observed between the commercial mAb and the plant-produced variants. The commercial mAb showed higher capacity to bind TNF-α than the antibodies produced in plant (Figure 5).

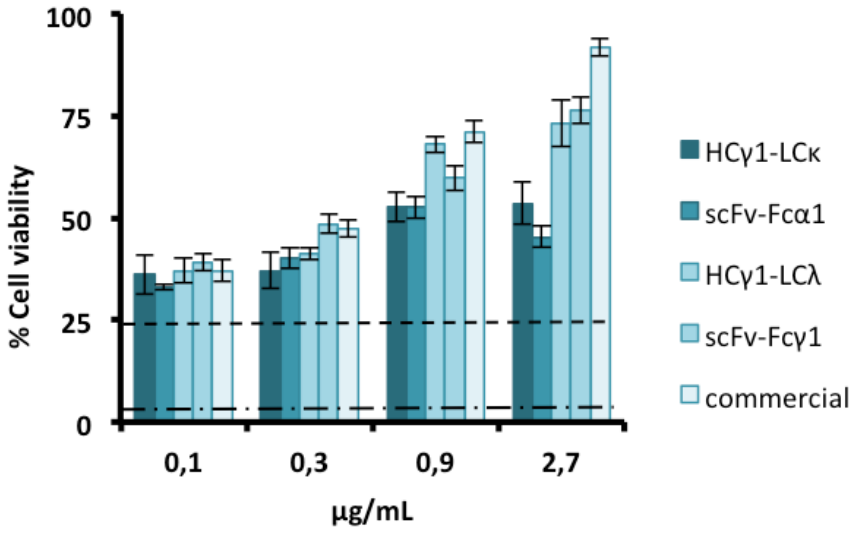

Figure 5. *In vitro* TNF-α neutralization. Human U937 cells (10,000cells/well) were treated with a 10 ng/mL of hTNF-α and increasing amounts of the plant-derived-anti-TNF-α and the equivalent commercial mAb. Plant-derived-mAb blocks the hTNF-α, the inhibition of the apoptosis mediated by hTNF-α was quantified by the Trypan Blue method. The horizontal broken line represents the survival rates related to a TNF-α control and the dotted line corresponds to a doxorubicin (cell death positive control).

3. DISCUSSION

The incidence and prevalence of IBDs have been increasing over the last few years and in different regions around the world (Na, 2012, Qin, 2012). Over 1 million people in the USA and 2.5 million in Europe have an IBD, according to estimation of Kaplan and colleagues (Kaplan, 2015). Moreover, in the past few years, IBD has emerged in new industrialized countries in Asia, Middle East and South America, and it has grown into a global malady, becoming a global disease (Kaplan, 2015).

Despite the IBD's cause is unknown, there are evidences suggesting that TNF-α plays an important role in most of the autoimmune and inflammatory diseases (Niazi, 2016).

Inhibitors of human TNF-α, such as the anti-TNF-α antibody Infliximab, offer a targeted strategy that differ from the nonspecific immunosuppressive agents traditionally used to treat most of the IBD (Raychaudhuri et al., 2009). Currently, the treatment with Infliximab is quite expensive (Schabert et al., 2013). Plants could be a cheaper platform for manufacturing the Infliximab antibody because they are considered to be a cost-effective and an efficient expression system for pharmaceutical proteins (Ma et al., 2003).

In this study, we compared the expression and activity of five different versions of Infliximab produced in plants. To determine which is the most appropriate version of Infliximab, we focused our comparison on the accumulated protein levels and on the ability of the plant derived antibodies to neutralize human TNF-α.

We observed that the expression of most of the recombinant antibody formats was successful. The Infliximab versions with HCγ1 transiently produced in *N. benthamiana* leaves, reached about 0.1mg/gFW (or about more than 1.2% TSP), which is the order of the accumulation levels previously described for other antibodies (De Meyer et al., 2015, Richard et al., 2013). However, we could observe strong differences in the accumulation levels of the different Infliximab's versions that contain the HCγ1. The highest expression was observed in the scFv-Fcγ1 version, with around the 0.12 mg/gFW representing the 2% of TSP. In contrast, the HCγ1-LCκ showed the lowest accumulation levels with around the 0.074mg/gFW equivalent to the 1.22% of TSP. The scFv-Fcα1kdel format transiently expressed in *N. benthamiana* leaves, reached about 0.065mg/gFW; an accumulation level higher than that earlier described for a recombinant IgA (Paul et al., 2014). However, infliximab scFv-Fcα1 produced in a stable tomato fruit, reached only 0.009mg/gFW; less than was described in Juarez and collaborators for a rotavirus IgA produced in fruit. In this case, the full length IgA against the VP8 peptide of rotavirus reached 1.2mg/gFW. The differences could be the result of the different sequence of the variable regions (Juárez et al., 2012). We could not detect the production of the smaller version of Infliximab, scFv-His. This observation was surprising, as we could not find any element wrong in the genetic design. Probably, our scFv was an unstable configuration and it was degraded, Schouten and colleagues speculate that the addition of a KDEL sequence to the C-terminal enhances retention, stabilization and

accumulation of scFv while reducing the level of proteolytic degradation (Schouten et al., 1997).

In conclusion, the different accumulation levels of the several recombinant anti-TNF-α antibodies expressed in *N. benthamiana* leaves were strongly dependent on the immunoglobulin constant chain sequence, with the IgG isotype expressing slightly better than the IgA. In the case of scFv-Fcα1, we observed different accumulation levels depending on the plant platform used. This heterogeneity in antibody quantity is in line with previous reports (De Muynck et al., 2010, Westerhof et al., 2014, De Meyer et al., 2015).

Degradation has been reported for full-length human IgG1 antibodies expressed in tobacco, being the majority of the cleavage sites clustered near the antibody interdomain regions of the heavy chain (Hehle et al., 2015). In this work, we observed small degradation fragments in all three recombinant Infliximab versions that contain the HCγ1. These fragments have the same size (28 kDa) as the antibody degradation fragments reported in previous studies (Hehle et al., 2015). For the scFv-Fcα1 format, fewer degradation products were observed in the Infliximab produced in *N. benthamiana* and any was observed in tomato fruit. In contrast to our observations, previous studies had reported the presence of several degradation fragments when α chains antibodies were expressed in plants (Juarez et al., 2012). The small fragments reported in the literature are likely to be the result of the cleavage of the full-length IgA, especially in the hinge region. However, the antibody expressed in this Chapter contains only the Fcα1 and this could be the reason why we observed less degradation products. The fact that we could not observe any degradation fragments in tomato fruits is probably due to the low accumulation of the antibody.

In terms of protein immunoreactivity, all the antibodies showed binding activity in ELISA tests, but we observed differences between the different versions. The best version according to the ELISA assay results was the HCγ1-LCλ. The other three antibody formats had a similar activity, with reached endpoint titers of 60ng. Curiously, the HCγ1-LCκ format showed a lower activity level than HCγ1-LCλ, Juárez and colleagues obtained similar results. In that report, several IgA with different isotype in HC and LC were transiently expressed in *N. benthamiana*. The IgA that contained LCκ presented lower

activity than the IgA with LCλ (Juárez et al., 2013). However, the LCκ had been successfully expressed before in the production of other plant-derived antibodies (Ramessar et al., 2008, McLean et al., 2007). The LCκ used in this assay was optimized for *N. benthamiana* expression, so an inefficient codon-usage can be discarded as a possible cause. Probably, the variable light region (VL) is affecting the stability of LCκ and the lower activity observed is caused by an idiotype feature.

To determine the antigen-binding capacity of the plant-produced antibodies, a cell-based assay was performed. The *in vitro* assay demonstrated that all Infliximab derivatives produced in plants have capacity to bind TNF-α. However, differences were observed between the commercial antibody, produced in murine myeloma cell (SP2/0), and plant-derived antibodies. The mAb commercial antibody presented a higher binding capacity than the Infliximab-plant-derived. The best plant-derived anti-TNF-α antibodies were the scFv-Fcγ1 and HCγ1-LCλ formats, showing the two of them the same capacity to neutralize TNF-α, about a 15% less capacity than the commercial antibody. These two antibodies are followed by the HCγ1-LCκ format that presented around a 20% lower capacity than scFv-Fcγ1 and HCγ1-LCλ. A low antigen-binding capacity was observed in the scFv-Fcα1 plant-derived-antibody format. The lower binding capacity observed between the plant-derived-antibodies when compared to the mAb commercial one may be due to (i) a higher content of degradation fragments in plant-made Infliximab since smaller fragments are also purified with the employed method; (ii) different antibody glycosylation patterns. These drawbacks could be resolved by improving the affinity purification method and by using glycoengineered plants that resemble human glycosylation.

In conclusion, we have demonstrated that plants can be used for the production of different antibody formats. However, the production of immunoglobulins in terms of yield and functionality is determined by the isotype and the antibody format chosed.

4. MATERIALS AND METHODS

4.1. Cloning and assembly of DNA parts

GBparts were obtained by PCR amplification using suitable templates. The Phusion High-Fidelity DNA Polymerase (ThermoScientific, Waltman, MA, USA) was used for amplification following the manufacturer's protocol. Primers were purchased from Sigma-Aldrich. The variable sequence of the anti-human TNF-α was obtained from the IMGT database and it was synthesized as a gBlock by IDTDNA (Coralville, IO, USA). Amplified bands were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were quantified in the Nano Drop Spectrophotometer 2000. Assembly reactions were performed as described by (Sarrión-Perdigones et al., 2013) using BsaI and BsmBI restriction enzymes (New England Biolabs, Ipswich, MA, USA) and T4 ligase (Promega, Madison, WI, USA) in 50-cycle digestion/ligation reactions. The reaction was then transformed into DH5α electrocompetent cells. Positive clones were selected on kanamycin, for multipartite constructions, or streptomycin, for binary construction, XGal, IPTG-containing plates. Plasmid DNA extractions were performed using the E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA). Correct assemblies were confirmed by restriction analysis and sequencing.

4.2. Strains and growth conditions

Escherichia coli DH5α was used for cloning. *Agrobacterium tumefaciens* strain GV3101 was used for transient expression and transformation experiments. Both strains were grown in Luria-Bertani medium under agitation (200 rpm) at 37°C and 28°C, respectively. Kanamycin (50 μg mL^{-1}), and spectinomycin (100 μg mL^{-1}) were used for *E. coli* selection. Rifampicin, tetracycline, and gentamicin were also used for *A. tumefaciens* selection at 50, 50, and 30 μg mL^{-1}.

4.3. Nicotiana benthamiana transient expression

For transient expression experiments, plasmids were transferred to *A. tumefaciens* strain GV3101 by electroporation. Agroinfiltration was carried out as previously described in Orzaez and colleagues (Orzaez at al., 2099). Overnight-grown bacterial cultures were pelleted and resuspended in agroinfiltration medium (10

mM MES, pH 5.6, 10 mM $MgCl_2$, and 200 µM acetosyringone) to an optical density at 600 nm of 0.2. Infiltrations were carried out using a needle-free syringe in leaves of 4 to 5-week-old *N. benthamiana* plants (growing conditions: 24°C day/20°C night in a 16-h-light/8-h-dark cycle). Leaves were harvested 6 to 7 days post infiltration and examined for transgene expression.

4.4. Tomato stable transformation

The scFv-Fcαkdel construct was transferred to *Agrobacterium tumefaciens* LBA4404 strain for stable tomato transformation. Tomato (var. Money Maker) transformation was carried out as described by Ellul et al. (2003) with minor modifications. Briefly, cotyledons of 10 days tomato plants were cut and explants were submerged in the *Agrobacterium* culture for half an hour. After that they were transferred to coculture medium and were kept in the dark 48 hours. Then explants were transferred to the organogenic medium with kanamycin. Individual shoots were excised and transferred to elongation medium prior to being transferred to the rooting medium for root regeneration.

4.5. Sample preparation

Plant tissues (*N. benthamiana* leaves and tomato fruit) were ground with a mortar and pestle to a fine powder under liquid nitrogen and stored at -80°C until used. Proteins were extracted with three volumes (w/v) of PBS pH 7.5. After mixing, the suspension was centrifuged twice at 4°C at maximum speed and the supernatant was immediately used for further analysis.

Apoplastic fluid was extracted by vacuum infiltration-centrifugation. Leaves were excised from the plant, rinsed in distilled water, and blot dry on tissue paper. Afterwards, the leaves were submerged in cold extraction buffer; vacuum was applied to a pressure of -0.9 bar (Vacuum Degassing Chamber DP118, Applied Vacuum Engineering, Thornbury, UK), held for 1 min, and then slowly released to allow the buffer to infiltrate the leaf blades. Na_2HPO_4 40mM, 50mM ascorbic acid, EDTA 10mM, pH7 was used as the extraction buffer. Vacuum infiltrated leaves were then blot dry on tissue paper and centrifuged in swing-rotor centrifuge (10 min, 1200xg) at 4°C to extract the

apoplast wash fluid (AWF). The AWF were clarified at 10,000xg for 15 min at 4°C. The supernatant was collected and immediately used for further analysis.

4.6. ELISA for the detection of human TNF-α binding activity and recombinant immunoglobulin determination

Plates (Corning, New York, NY, USA) were coated overnight at 4 °C with coating buffer (50 mM carbonate buffer pH 9.8) with 1 μg/mL of recombinant human TNF-α (Prepotech Inc, London) for the detection of human TNF-α binding activity. Plates were then washed three times in PBS (0.1M phosphate and 0.1M NaCl pH 7.4) and blocked with a 2% (w/v) solution of ECL AdvanceTM Blocking agent (GE Healthcare, Buckinghamshire, UK) in PBS-T [0.1% (v/v) Tween 20 in PBS]. Samples were diluted in PBS as required for each assay and incubated in the coated plates for 1 h at room temperature. After incubation, plates were washed three times with PBS, and the antibodies for detection were added in PBS-T-2% blocking buffer (GE Healthcare, Buckinghamshire, UK). Different antibodies were used for detection: anti-human HCγ 1:2000 non-conjugated (Sigma Aldrich, St Louis, MO, USA), anti-LCλ 1:5000 non conjugated (Sigma Aldrich, St Louis, MO, USA), anti-LCκ 1:5000 non conjugated (Pierce Thermo Scientific) and 1: 5000 anti-human HCα (Sigma Aldrich, St Louis, MO, USA). Anti-rabbit-HRP (GE Healthcare, Buckinghamshire, UK) secondary antibody (1:5000) was used after the non-conjugated detecting antibodies. The detection of scFv was incubated with 1:2000 mouse Penta-His antibody (Qiagen, Hilden, Germany) as a primary antibody, followed by a secondary antibody anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK). After three PBS washes, the substrate O-phenylenediamine (Sigma Aldrich, St Louis, MO, USA) was added, and the reactions were stopped with 3 M HCl. Absorbance was determined at 492 nm.

A standard curve from 0.8 to 0.1 μg/mL of commercial immunoglobulin was obtained to calculate the concentration of immunoglobulin in the different samples. The concentration in each sample was obtained by interpolation with the immunoglobulin standard curve.

4.7. SDS-PAGE and Western blot analysis

Proteins were separated by SDS/PAGE in 10% denaturing gels (Invitrogen, Carlsbad, NM, USA). Gel staining was carried out with coomassie following standard procedures.

For Western blot analysis, blots were incubated with 1:20,000 anti-human HCγ (Sigma Aldrich, St Louis, MO, USA), 1:10,000 anti-LC (Sigma Aldrich, St Louis, MO, USA). After, 1:20000 anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) was used for the detection. Blots were developed with an ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

4.8. Affinity chromatography purification

Protein extracts, prepared as explained previously, were further clarified using a 0.22 µm filters Stericup (Millipore, Billerica, MA) on ice. Purification of HCγ1-LCλ, HCγ1-LCκ and scFv-Fcγ1 were performed with protein A (GE Healthcare, Buckinghamshire, UK) that specifically binds the Fc of the HCγ1 following the manufacturer's instructions. Purification of scFv-Fcα1kdel was performed with SSL7 protein (InvivoGen, San Diego, CA) that specifically binds the hydrophobic interface between the Cα2 and Cα3 domains of the HCα1 following the manufacturer´s procedure.

4.9. Human U937 cell viability

U937 cells were seeded onto a 96-well plate at a density of 10.000 cells/well and culture for 24h. Thereafter, the cells were incubated for additional 24h either without or with 20ng/mL human TNF-α and either with or without known concentrations of the different recombinant plant anti-TNF-α antibodies. After the treatment, cells were stained with Trypan Blue (Sigma Aldrich, St Louis, MO, USA) and manually counted in a Neubauer chamber under the microscope.

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6. SUPPLEMENTARY MATERIAL

6.1. Supplementary Table 1. Primer sequences for DNA constructs

Primer	*Sequence*
PJ11DIC07	GCGCCGTCTCACTCGCGGCCCCCGACTGTCACTGCACCG
E12OCT03	GGGGTCTCAAAGCGAGTTCGTCCTTGTAGCAGGTG
E14NOV01	GCGCCGTCTCAGGTCAACCAAAGGCCGCCCCCTCTGTC
E14NOV02	GCGCCGTCTCT CTCGAAGCTGAACATTCTGTAGGGGCCAC
E15FEB09	GCGCCGTCTCACTCGAGCCGAAGTTAAACTTGAAGAATCTGGAGG
E15FEB10	GCGCCGTCTCTCTCGACGCAACAGTAAGAGTAGTTCCTTGTCCC
E15FEB11	GCGCCGTCTCACTCGAGCCGATATTCTTCTTACTC
E15FEB12	GCGCCGTCTCTCTCGTCCTTTTAACTTCAAGATTAGTTCCAGATCC
E15FEB13	CGCCGTCTCACTCGAGGACTGTTGCTGCTCCATC
E15FEB14	GCGCCGTCTCTCTCGAAGCACATTCTCCTCTATTAAAAGATTTAG
E15MAR01	GCGCCGTCTCACTCGGCGTCCACCAAGGGC
E15SEPT01	GCGCCGTCTCTCTCGAAGCAGTGATGGTGATGGTGGTGAGAAAC
E15SEPT02	GCGCCGTCTCTCTCGAAGCTCATTTACCCGGAGACAGGGAG

CHAPTER 2: Design and production of a ZMapp antibody cocktail in N. benthamiana using modular cloning

1. INTRODUCTION

In August 2014, an experimental plant-derived-mAb cocktail against the Ebola Zaire Virus (EBOV), called ZMapp, was used to treat Ebola patients showing promising results. EBOV is one of the most virulent and deadliest pathogens in the planet, with mortality rates approaching the 90% in the last Ebola outbreak (Davidson et al., 2015, Pettit et al., 2013, Qiu et al., 2014). Until now, neither an approved vaccine or treatment is available to prevent or treat the EBOV infection (Mühlberger, 2007). ZMApp was the first drug that showed therapeutic efficacy against EBOV in non human primate models and in humans (Leroy et al., 2011, Rasmussen et al., 2012, de Kruif et al., 2010). ZMapp is a combination of three humanized monoclonal antibodies (mAbs) and it is manufactured in glycoengineered *Nicotiana benthamiana* plants by transient expression. On these glycoengineered plants, expression of specific glycosyltransferases (α1,3 fucosyltransferase and β1,2 xylosyltransferase) is supressed by RNAi (Strasser et al., 2008). The three mAb that comprise ZMapp (called c13C6, c2G4 and c4G7) were obtained by immunizing mice with a recombinant vesicular stomatitis virus (VSV), in which the VSV glycoprotein had been replaced by the homologous from EBOV. Antibodies that were able to bind the viral glycoprotein (GP) and protected mice from infection were identified, and the variable chains of three of them were used to assemble humanized antibodies that were then produced in *N. benthamiana* plants (Qiu et al., 2014). It is known that c2G4 and c4G7 bind to the membrane-proximal part of the GP neutralizing viral infection. In contrast, mAb c13C6 binds to the tip of the GP and it does not neutralize viral infection suggesting that c13C6 may work in concert with the complement system to block the virus infection (Lee et al., 2008). The three antibodies that comprise ZMapp were also produced in CHO cells. In the mouse-adapted Ebola model, the plant-derived antibodies provided protection superior to CHO-derived mAbs, likely due to the increased antibody-dependent cellular cytotoxicity (ADCC) activity conferred by the N-glycans lacking core fucose present on the fragment crystallisable (Fc) region (Zeitlin et al., 2011).

ZMapp was produced in plants by expressing each of the plant-derive-anti-EBOV mAbs using the Magnifection system. Each viral vector encoded a polypeptide antibody chain, either the heavy chain or the light chain, subcloned into the 3' TMV and the 3'

PVX provector constructs, respectively (Gleba et al., 2005). In this case, the method for generating ZMapp consists on the expression and functional characterization of individual therapeutic mAbs, which were then combined in a single drug product (Leroy et al., 2011, Davidson et al., 2015). Unfortunately, this approach is expensive because of the high cost associated with the development and the manufacturing of the composite mixture.

The case of Zmapp serves as a hallmark of how plant platforms can play an important role in the defence against emerging diseases. A second predominant example is the plant-based production of recombinant vaccines in *N. benthamiana* against pandemic flu using Virus-like Particles (Marsian et al., 2016, D'Aoust et al., 2008). In both cases, an important advantage of plants systems, particularly those based in transient expression, is the capacity to react rapidly to the manufacturing needs imposed by emerging diseases. In the case of recombinant antibodies, rapid manufacturing responses benefit from the establishment of modular cloning systems that allow the exchange of variable antibody regions in plant expression constructs. Modular DNA assembly methods are even more important when antibody cocktails are to be produced, as the easy combination of constant and variable regions reduces the requirements for *de novo* gene synthesis.

Whereas Zmapp antibodies were originally produced using the Magnicon system, an alternative option is the use of a non-replicative T-DNA transient expression strategy. Co-expression of two or more antibodies in a single batch using the Magnifection system is not possible due to the phenomenon of superinfection exclusion (SE), the mechanism in which a viral infection prevents the subsequent infection of the same cell with the same or a closely related virus preventing the co-infection with competitor virus (Beperet et al., 2014). Magnicon uses non-competing PVX and TMV viruses to encode heavy and light antibody chains respectively, therefore circumventing SE in the co-expression of a full antibody. However, the introduction of a second antibody in the system would activate SE among PVX (and TMV) clones themselves, this preventing the formation of correct pairings between cognate chains. In contrast, the absence of a viral system that activates SE would allow the co-expression of two or more antibodies in the same cell, as it has been widely described that the agroinfiltration method in *N.*

benthamiana produces high levels of co-transformation (several T-DNAs entering simultaneously in the same cell) (Kapila et al., 1997). However, special considerations need to be taken into account in the case of non-viral systems regarding the possible shuffling of antibody chains during the manufacturing process. This approach brings along as a drawback the possibility of illegitimate chain paring: each heavy chain does not necessarily bind its cognate light chain; instead it can associate with a light chain from another antibody in the cocktail, resulting in an antibody with reduced affinity. We reasoned that the level of illegitimate antibody chain association due to chain shuffling is dependent on the co-transformation rates, which in turn depends on the optical density of the *Agrobacterium* cultures used during the agroinfiltraton method.

In this chapter, we first tested the flexibility of GoldenBraid (GB) modular cloning system (Sarrión Perdigones et al., 2013) for the rapid and inexpensive production of the three antibodies in the Zmapp cocktail in *N. benthamiana*. The GB collection of standard DNA parts (phytobricks) contains a number of codon-adapted human antibody regions, next to well-characterized regulatory regions as promoters, terminators, etc. which can be easily assembled together to create new transcriptional units for recombinant production. First, we analyse the individual production of each antibody in the cocktail and showed how the versatility of the cloning system allows easy exchange of variable regions, enabling the production of a Zmapp cocktail in less than three weeks. Furthermore, we evaluated the strategy of non-replicative co-transformation to produce antibody cocktails in *N. benthamiana*. To this end, we constructed three T-DNAs each one encoding one of the three antibodies comprising the ZMApp cocktail and compared individual mAb production with the mAb mixture in co-transformation strategies using different concentrations of *Agrobacterium* cultures. We analysed the effect of the optical density of the agroinfiltrated products on the levels of chain shuffling and consequently on the antigen-binding activity of the final cocktail product and propose strategies to minimize shuffling effect using non-replicative systems.

2. RESULTS

2.1. Construction of the three individual antibodies comprising ZMapp with GoldenBraid

The assembly of the plasmids for the expression of the three recombinants antibodies that composed ZMapp, was performed with GoldenBraid (GB) (Sarrión-Perdigones et al., 2013). Basic GBparts as the CaMV 35S constitutive promoter (35s), the pectate lyase signal peptide (SP) and the nopaline synthase terminator (Tnos), the constant human γ heavy chain (HC) and the constant human light chain lambda (LCλ) were already available in the GBpart collection (https://gbcloning.upv.es). The heavy and light variable regions (VH and VL) were obtained from the patent application US2004/0053865 A1 and US20120283414 and they were also domesticated as a GBpart (Figure 1a). Once all the basic GBparts were created, six different multipartites reactions were performed, three of them to assemble the transcriptional units (TUs) for the expression of heavy chains, and three for the expression of the light chains. Each multipartite reaction combined a promoter, a variable antibody region, a constant antibody region (heavy or light), and a terminator next to destination vector. The multipartite assembly reactions resulted in TUs cloned in a binary destination vector (α-GB vectors) (Figure 1b). Next, binary reactions were performed to combine each LCs with its cognate HC into a Ω-GB destination vector, producing the 2X TUs genetic modules required for the expression of a full-length antibody (Figure 1c). All six TUs and all three 2X TU gene modules were successfully assembled in onte-tube-one reaction GB assemblies following procedures obtained in (www.gbcloning.org), producing the expected constructs as shown in the Figure 1d.

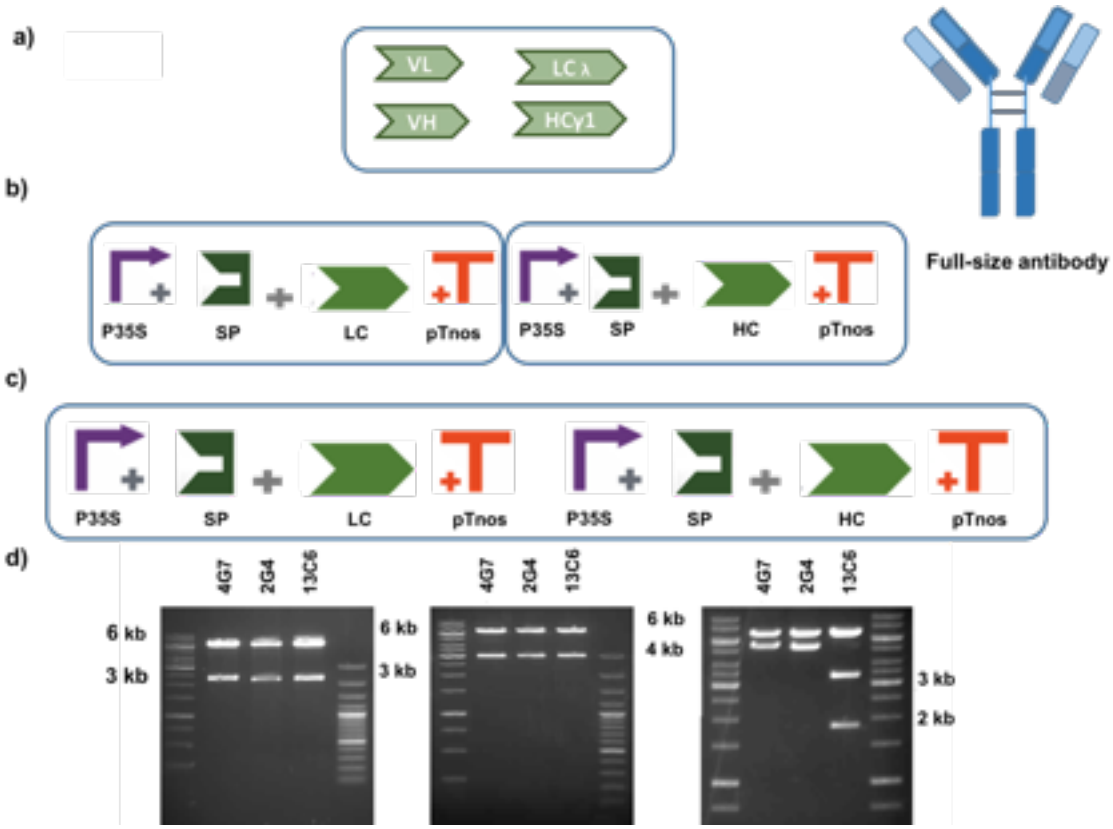

Figure 1. **Assembly processes of the three individual antibodies which composed ZMapp cocktail. (a)** Collection of basic GBparts generated for building the different antibodies. **(b)** Basic GBparts conforming the heavy (HC) and light chain (LC) transcriptional units. **(c)** Result of the binary GB assembly in a GB Ω-vector of the HC and LC transcriptional unit. **(d)** Left, restriction analysis of one colony of each LC transcriptional unit with EcoRI (expected bands of 2250 + 6322); middle, Hind III restriction analysis of the HC transcriptional unit; middle, (expected bands of 2975 + 6322); right, BamHI restriction analysis of the full-length antibody (expected bands of 5213 + 6652 for 4G7 and 2G4 and 3370 + 1840 + 6652 for 13C6).

2.2. Transient expression in Nicotiana benthamiana of three individual antibodies and a three-antibodies cocktail against EVOB

The three binary plasmids encoding three different antibodies against the glycoprotein of EBOV were transferred to *Agrobacterium tumefaciens* and they were individually agroinfiltrated into leaves of *N. benthamiana*. The individual anti-EBOV-mAb were co-expressed with the GB1203 module of GoldenBraid collection (containing the constitutive expression of the bushy stunt virus-TBS-19, suppressor of gene silencing). Leaves were harvested at 5 days post infiltration (dpi).

For each of the three plant-derived-mAb five days post-infiltrated leaves were harvested and their total protein extracts were tested in an antigen ELISA assay against recombinant glycoprotein of EBOV. Antibody binding titres proved to be consistent, as three different antibodies reached endpoint titres at 1/128 (Figure 2). Besides analysing the binding activity of each antibody in the clarified extracts, we also quantified the absolute production levels of each antibody in a sandwich ELISA using a standard human IgG curve for calculations. These levels correspond for 13C6 and 2G4 were up to 50μg of IgG per gram of fresh leaf weight (FW). In the case of the antibody named 4G7, the observed yield is quite low compared to the other two antibodies, concretely 38μg/g FW (Table 1).

Table 1. Comparative evaluation of anti-EBOV antibodies.

Construction	% Antibody (% TSP)	Yield (μg/g FW)
13C6	1.5 ± 0.1	55.7 ± 2
2G4	1.4 ± 0.05	51.2 ± 1.1
4G7	1.1 ± 0.15	37.8 ± 1.6

The levels of antibody are reported in percentage of total soluble protein (%TSP) and the yield in micrograms per gram of fresh weight (μg/g FW).

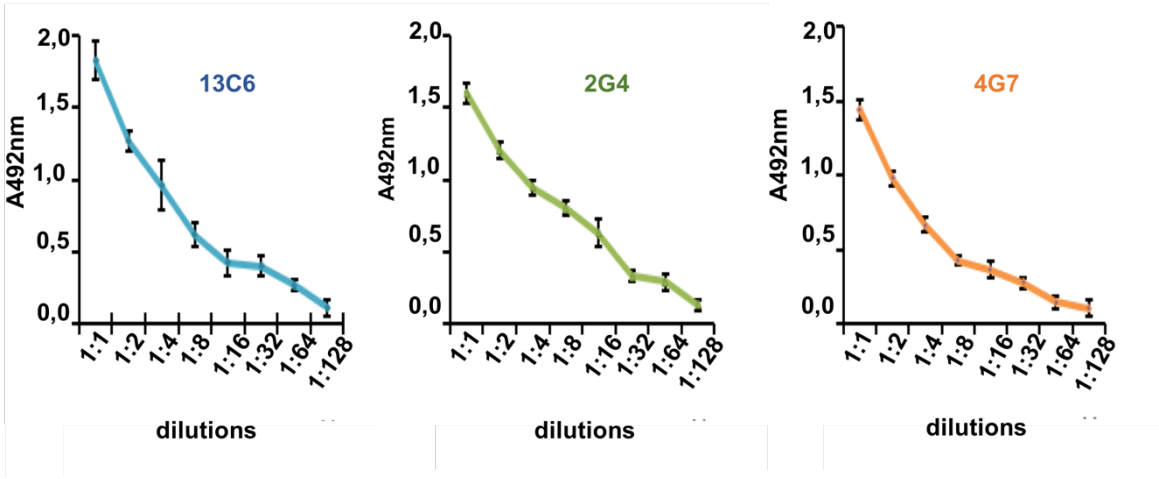

Figure 2. End-point antigen-ELISA titering of the clarified total extract protein of *N. benthamiana* leaves expressing three antibodies against the EBOV glycoprotein. Activity comparison of the three anti-EBOV mAbs produced in *N. benthamiana* leaves. The ELISA was coated with 1µg of glycoprotein EBOV/well. And samples were developed with anti-HCγ.

Once it has been confirmed that the three humanized mAbs when they are produced individually into *N. benthamiana* leaves are functional, we decided to co-express them to determine the suitability of the co-expression as an alternative expression system to manufacture mAb cocktails. To do this, the individual antibodies and the mAb cocktail were transiently transformed in *N. benthamiana* leaves using a final Agrobacterium Optical Density (OD) of 0.1, and samples were harvested at 5 dpi. The expression of the individual antibodies and of the mixture was corroborated by the Western blot (Figure 3). The Western blot assay shows a similar degradation level and the same degradation fragments for all the expressed antibodies.

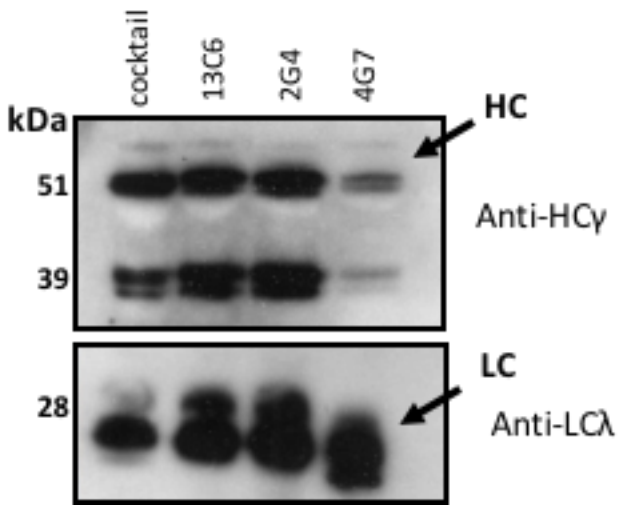

Figure 3. Western Blot analysis under reducing conditions of clarified crude extract samples of the individual antibodies of ZMApp and the ZMApp cocktail. The samples used in the Western Blot were agroinfiltrated at an 0.1 OD. Western Blot assay was developed with anti-HCγ and anti-LCλ.

Next, we assessed the activity of the three individual mAb and the mAb cocktail at five different OD_{600} (0.006; 0.0125; 0.025; 0.05 and 0.1). Agroinfiltrated leaves were harvested at 5 dpi and the activity was assayed in an antigen ELISA test using plates coated with the glycoprotein of the EBOV. Figure 4 shows the comparison of the binding activities measured for each individual mAb and for mAb mixture. The highest activity was observed for the individual mAb agroinfiltrated at the highest OD (0.1). For the individual mAbs, higher ODs showed higher activity. However, the functionality of the mAb cocktail increases only until 0.05 OD. From that OD, the activity of mAb mixture remains stationary with a value of about 0.5 absorbance at 492nm, independent of the OD agroinfiltrated (0.05 or 0.1). Interesting, at low OD, which correspond to low co-transformation levels, we found minimal differences between the overall binding activities of the individual antibodies and that of the mixture. However, significant differences were observed between the activity of the single mAbs and the mAb mixture with increased cell suspension densities. In the highest OD assayed, namely 0.1, individual mAbs showed three times the functionality activity of the mAb cocktail.

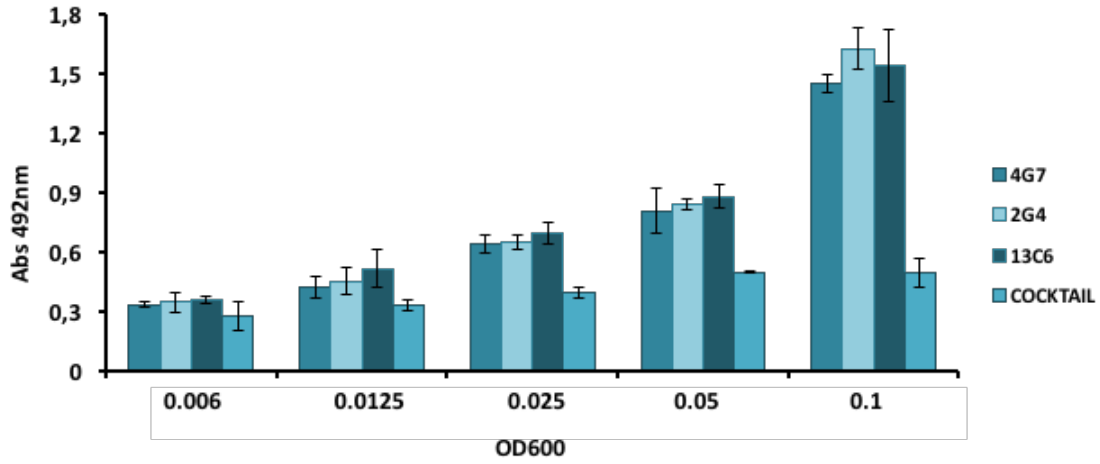

Figure 4. **Functional activity of the three individual antibodies comprising ZMapp (4G7, 2G4, 13C6) and the ZMapp cocktail.** The individual antibodies and its mixture were agroinfiltrated at different ODs, 0.006 to 0.1. The functional activities of the infiltrated antibodies at different ODs were tested in an antigen ELISA assay. The ELISA was coated with the EBOV glycoprotein and developed with anti-HCγ and all the samples were performed by triplicates.

3. DISCUSSION

Recombinant monoclonal antibodies (rmAb) are the biomolecules of choice for biological therapy. There are being used on the treatment for cancer, autoimmune disorders and infectious diseases (Moussavou et al., 2015, Raychaudhuri et al., 2009, Shirota et al., 2008, Choy, 2011). High target specificity makes mAbs-based drugs the most used in conventional therapy since they have an increased patient tolerance and higher efficacy than non-rmAb drugs (Thompson et al., 2014).

Although specificity is the strength of mAb, a bio-threat agent that may undergo rapid mutation to result in antigenic variation, such as a glycosylation, genetic polymorphism and denaturation, poses significant hurdle for individual mAb as therapeutics (Diamant et al., 2015). For example, the monospecifity of the mAbs may also limit their suitability for the treatment of complex diseases because of the high mutation rate of certain viruses enables them to escape neutralization (Reverbi et al., 2007). These problems may be overcome by using two or more individual antibodies (mAb cocktail) that target various areas of bio-threat agents. In fact, several studies demonstrated that the use of mAb cocktail are more potent than individual mAb. For instance, mAb mixtures can lead

to an increased effector response because they can have a synergistic effect by recognizing several epitopes limiting the chances of escape for variants that could propagate the disease (Thompson et al., 2014, Diamant et al., 2015, Robak et al., 2016). In addition, mAb cocktails are more stable than the individual mAbs over a range of salt concentration and pH (Reverbi et al., 2007).

For these reasons, currently, composite mAb cocktails are designed to combat diseases, as infectious diseases or oncology, present a new, rapidly, emerging technology in the field of biopharmaceuticals (Saylor et al., 2009, Dienstmann et al., 2015, Nejatollahi et al., 2014, Nandi et al., 2010, Logtenberg, 2007, Frenzel et al., 2013). For example, in oncology a mAb cocktail composed by two conventional mAb is used for targeting HER2/neu in breast cancer (Nejatollahi et al., 2014). A second example is the use of a cocktail composed also by two mAbs in people who have been exposed to rabies virus infection (Nandi et al., 2010).

We show here the production of a recombinant ZMapp mAb cocktail using GoldenBraid (GB) modular cloning tools and *N. benthamiana* agroinfiltration. Modular cloning in plant biotechnology greatly reduces hurdles associated with construct design, lowering the barriers for implementation. The whole process of creating all the constructs required for ZMapp expression in plants comprises six individual one-tube multipartite reactions (performed in parallel), followed by three parallel binary reaction (which can also be performed in parallel). Altogether, the whole cloning procedure starting from basic GB parts up to creating 2XTU constructs in agrobacterium ready for agroinfiltration, takes as little as 12 consecutive days, with five additional days required for the production of mAbs *in planta*. Consequently, with a continuous production flow chart in place, an oligoclonal cocktail of mAbs can be produced at the mg level in less than three weeks, including the time required for custom gene synthesis of variable antibody chains. Also interesting is the calculation of costs associated with ZMapp cloning and recombinant production using GB cloning. The variable regions employed in these work were purchased as gBLOCKs at IDT (https://eu.idtdna.com/site). Synthesis prices were 205 € (c2G4 and c4G7) and 265 € (clone 13G6) respectively, including both VH and VL chains. The GB cloning kit, including all the necessary parts for creating 2XTUs can be obtained from our lab or from the Addgene repository (www.addgene.org) (with

a bench fee of approximately 150 €). We calculated that each GB cloning reaction has an average cost of 25€, including the restriction analysis of resulting colonies. In sum, the DNA assembly of the 3 X mAb cocktail takes little more than 1000 €. Therefore, with antibody expression levels showed here, the first 50-100 mg of ZMapp, or an equivalent cocktail, can be produced in 3 weeks with little more than 1500 € plus purification costs, employing 100-200g of leaf tissue (equivalent to 5-10 seven-weeks old *N. benthamiana* plants) in a minimally equipped laboratory. The mg level obtained here is in the low range given the requirements of ZMapp as a drug aimed at passive protection. Experiments conducted with ZMapp in experimental animals make use of effective doses of 50 mg/Kg. Therefore for applications in passive therapy, Magnifection-based production and purification of individual clones followed by *in vitro* formulation of the combined drug are still necessary. However, for many other applications requiring antibody cocktails in the milligram range, as animal testing or diagnostics, the procedures showed produce sufficient antibody quantities in very short time.

Our results clearly show that co-expression via agroinfiltration of antibody cocktails using non-replicative systems is severely affected by chain shuffling. In a previous work performed using fluorescent proteins, we established that co-transformation levels in *N. benthamiana* are strongly dependent on the OD of the infiltrated Agrobacterium cultures, this dependence reaching a plateau close to OD= 0.1 (Vazquez-Vilar et al., 2017). In the same experiments it was shown that a multiplicity of transformation of one (MOT=1, the situation where the average number of transformed T-DNAs per cell equals one), is reached with agroinfiltration ODs values as low as 0.002. The results obtained here are consistent with those observations. At low ODs, where co-transformation levels are also low, each cell is transformed on average with a single antibody clone (comprising cognate heavy and light chains), and therefore the activity of the co-transformed cocktail does not significantly differs from that of the individual clones transformed separately. In these conditions, the cocktail co-transformation can be considered as an advantageous strategy. However, these conditions are suboptimal in terms of yield, as we clearly observed that higher gene dosage obtained by co-transformation of each cell with several T-DNA rises expression levels up to 10 times. It was earlier calculated that in an agroinfiltration experiment with an OD= 0.1, each cell

receives on average eight transcriptionally-active T-DNAs (Vazquez-Vilar et al., 2017). However, we show here that when this level of co-transformation is reached using a cocktail of three antibodies, the detrimental effect of chain shuffling counteracts any increases in yield, impeding any further raises in the overall binding activity of the cocktail product. This effect is likely to be intensified when larger cocktails (>3) are produced. We conclude that infiltration ODs producing a MOT of 1, (usually around OD=0.005) are most convenient for cocktail production in non-replicative systems, and therefore further increases in yield should be obtained by other means. There are several strategies described elsewhere for rising up production yields in a non-replicative, SE-free environment as those making use the pEAQ expression system (Sainsbury et al., 2009). Alternatively, the use of a replicative system where HC and LC chains are linked in the same viral vector (e,g; expressed from separate sub-genomic promoters or as a polyprotein) (Roy et al., 2011) could be conceived as an strategy to take advantage of SE for optimizing mAb cocktail production.

4. MATERIALS AND METHODS

4.1. Cloning and assembly of parts

GBparts were obtained by PCR amplification using suitable templates. The Phusion High-Fidelity DNA Polymerase (ThermoScientific, Waltman, MA, USA) was used for amplification following the manufacturer's protocols. Primers smaller than 30 nucleotides were purchased from Sigma-Aldrich. The sequences of the variable antibody regions against the glycoprotein of EBOV were obtained from the US2004/0053865 A1 and US20120283414 patents and they were synthetized as a gBlock by IDTDNA (Coralville, IO, USA). Amplified PCR bands from gBlocks were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were quantified in the Nano Drop Spectrophotometer 2000. Assembly reactions were performed as described by Sarrión-Perdigones et al., (2013) using BsaI and BsmBI restriction enzymes (New England Biolabs, Ipswich, MA, USA) and T4 ligase (Promega, Madison, WI, USA). Assembly reactions were transformed into home-made DH5α electrocompetent cells. Positive clones were selected on ampicillin-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

acid, and isopropylthio-β-galactoside-containing plates. Plasmid DNA was extracted using the E.Z.N.A Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA). Correct assemblies were confirmed by restriction analyses and sequencing.

4.2. Strains and growth conditions

Escherichia coli DH5α was used for cloning. *Agrobacterium tumefaciens* strain GV3101 was used for transient expression. Both strains were grown in Luria-Bertani medium under agitation (200 rpm) at 37°C and 28°C, respectively. Ampicillin (100 µg mL^{-1}), kanamycin (50 µg mL^{-1}), and spectinomycin (100 µg mL^{-1}) were used for *E. coli* selection. Rifampicin, tetracycline, and gentamicin were also used for *A. tumefaciens* selection at 50, 50, and 30 µg mL^{-1}.

4.3. Nicotiana benthamiana transient expression

For the transient expression experiments, plasmids were transferred to *A. tumefaciens* strain GV3101 by electroporation. Agroinfiltration was performed as described in Orzáez and colleagues (Orzáez et al., 2009). Overnight-grown bacterial cultures were pelleted and re-suspended in agroinfiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 200 µM acetosyringone) to an optical density at 600 nm of 0.2. Infiltrations were carried out using a needle-free syringe in leaves of 4 to 5-week-old *N. benthamiana* plants (growing conditions: 24°C day/20°C night in a 16-h-light/8-h-dark cycle). Leaves were harvested 5 days post infiltration and examined for transgene expression.

4.4. Sample preparation

Plant tissues (*N. benthamiana* leaves) were ground with a mortar and pestle to a fine powder under liquid nitrogen and stored at -80°C until used. Proteins were extracted with three volumes (w/v) of PBS pH 7.5. After mixing, the suspension was centrifuged twice at 4°C at maximum speed and the supernatant was immediately used for further analysis.

4.5. ELISA for the detection of anti-glycoprotein of ebov activity of individual and mab mixture

Plates (Corning, New York, NY, USA) were coated overnight at 4 °C in coating buffer (50 mM carbonate buffer pH 9.8) with 1 µg/mL of recombinant glycoprotein of EBOV (IBT Bioservices, Gaithersburg). Plates were then washed three times in PBS (0.1M phosphate and 0.1M NaCl pH 7.4) and blocked with a 2% (w/v) solution of ECL AdvanceTM Blocking agent (GE Healthcare, Buckinghamshire, UK) in PBS-T Tween 20 [0.1% (v/v). Samples were diluted in PBS as required for each assay and incubated for 1 h at room temperature. After incubation, plates were washed three times in PBS, and the antibodies for detection were added in PBS-T-2% blocking buffer (GE Healthcare, Buckinghamshire, UK). The different antibodies were used for detection: anti-human HCγ 1:2000 non-conjugated (Sigma Aldrich, St Louis, MO, USA). Anti-rabbit-HRP (GE Healthcare, Buckinghamshire, UK) secondary antibody (1:5000) was used after the non-conjugated detecting antibodies. After three PBS washes, the substrate O-phenylenediamine (Sigma Aldrich, St Louis, MO, USA) was added, and the reactions were stopped with 3 M HCl. Absorbance was determined at 492 nm.

A standard curve from 0.8 to 0.1 µg/mL of control immunoglobulin was obtained to calculate the concentration of immunoglobulin. The concentration in each sample was obtained by interpolation with the immunoglobulin standard curve.

4.6. SDS-PAGE and Western blot analysis

Proteins were separated by SDS/PAGE in 10% denaturing gels (Invitrogen, Carlsbad, NM, USA). For Western blot analysis, blots were incubated with 1:20,000 anti-human HCγ (Sigma Aldrich, St Louis, MO, USA), 1:10,000 anti-LC (Sigma Aldrich, St Louis, MO, USA). After, 1:20000 anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) was used for the detection. Blots were developed with an ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

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6. SUPPLEMENTARY MATERIAL

6.1. Supplementary Table 1. Primer sequences for DNA constructs

Primer	*Sequence*
E14SEPT16	*GCGCGCCGTCTCTAAGCTGAACATTCTGTAGGGGCCAC*
E14SEPT17	*GCGCGCCGTCTCTAAGCTCATTTACCCGGAGACAGGGAG*
E14OCT01	*GCGCCGTCTCACTCGAGCCGCAGCTGTTGCAGTCTGCAG*
E14OCT02	*GCGCGCCGTCTCTCTCGAAGCTCATTTACCCGGAGACAGGG*

CHAPTER 3 Functional evaluation of plant-derived-mAb cocktail against a snake venom

1. INTRODUCTION

During the last two decades, the use of recombinant monoclonal antibodies (rmAb) in clinical trials has emerged as an important therapeutic modality. However, in the last years, the use of mAb cocktails represents a new trend which consists of mixing mAbs with different binding specificities. Antibody mixtures offer as an advantage that several antigens can be target simultaneously, mimicking natural polyclonal immune responses (Thompson et al., 2014, Diamant et al., 2015, Robak et al., 2016, Saylor et al., 2009, Nandi et al., 2010, Nejatollahi et al., 2014, Robak, 2013). Recombinant mAbs cocktails are currently used to treat infectious diseases, to neutralize natural toxins and in oncology treatment (Moussavou et al., 2015, Qiu et al., 2014, Nowakowski et al., 2002, Bregenholt et al., 2006).

The strategy commonly used for manufacturing recombinant mAb cocktails is the establishment of separate production and purification lines, one per each antibody, and subsequently formulate the cocktail (Chartrain et al., 2008, Frenzel et al., 2013). However, mixing independently mAbs, especially when the number of components of the cocktail is high, might not be commercially viable because of the high development and manufacturing costs (Rasmussen et al., 2012, Spiess et al., 2015). An alternative option is the co-expression of several recombinant antibodies in the same transgenic plant. However, chain shuffling affects the functionality of the mAb cocktail, as already described in Chapter 2.

A way to eventually overcome chain shuffling effect and to ensure high reproducibility in the expression of antibody cocktails is to take advantage of an intrinsic property of virus known as superinfection exclusion (SE) or viral interference (Folimonova, 2012). In a work developed in parallel to this Thesis in our laboratory, a recombinant expression system for antibody cocktails based on SE called Pluribody technology was developed. The so-called Pluribody technology takes advantage of the SE to facilitate the co-expression of several antibodies in leaves in a reproducible manner. SE prevents the superinfection of cells with a second virus when they are already infected with a closely related virus arrived first (Folimonova, 2012). An important consequence of this is that population variants in plant virus infections are not uniformly distributed along the plant. Instead, they are structured in a mosaic-like

pattern due to the limitation to superinfection imposed by resident viral clones. As a result, the co-infection of a plant with two or more viral clones induces the formation of an infection mosaic, with different sectors in the plant soma dominated by mutually exclusive clones (Julve et al., 2013, Tscherne et al., 2007). Most importantly, mosaic-like distribution ensures high population diversity as compared to the fitness-driven quasi-species-like distributions typical of unstructured population dynamics (Elena et al., 2011).

Our group has investigated the use of virus-based expression system for the induction of somatic expression mosaics in plant leaves, aiming that each tile in the mosaic will function as an independent micro-production line, leading globally to the production of recombinant polyclonal antibody cocktails. This strategy ensures that high yields of recombinant polyclonal antibody cocktails comprising hundreds of idiotypes are produced simultaneously. The lack of physical interaction between the different clones excludes mutual competence, and as a consequence the methodology ensures high batch-to-batch reproducibility regardless of the complexity of the polyclonal composition. In a parallel work, our group demonstrated batch-to-batch reproducibility of Pluribodies independently of the mAb mixture.

This chapter describe the characterization of a 'Pluribody' designed as a potential snake antivenom. Currently, treatments for envenoming by poisonous snake is based in hyper-immunized animal sera, normally obtained from horse or sheep (Chippaux, 2013, Gutiérrez et al., 2011). Snake venom is not composed of a single compound, instead, it is a complex mixture of different proteins or peptides that exert hemotoxic, hemorrhagic, cytotoxic and neurotoxic pathologies in envenomed prey and humans. Nowadays, snakebites cause 125,000 deaths and 400,000 amputations and permanent disabilities per year worldwide (Gutiérrez, 2016, Krifi et al., 1993, Krifi et al., 1992). Unluckily, the limitations of polyclonal antibodies prepared from pools of sera are manifold; they include batch-to-batch variation, hypersensitivity reactions, potential risk of infectious disease transmission and an increasingly regulatory hurdle (Chippaux, 2013, León et al., 2013, Gutiérrez et al., 2011). Antivenins made of recombinant human or humanized antibody cocktails would be a highly desirable alternative, as they would reduce secondary effects while facilitating product standardization and reproducibility.

Unfortunately, manufacturing costs for complex antibody cocktails are very high because they require maintenance of parallel production lines for each component in the cocktail to ensure consistency. Conversely, the simplification of the cocktail composition is technologically challenging due to the high diversity of toxins comprising venom's composition. Furthermore, efforts in cocktail simplification would enter in conflict with a major trend in antivenim formulation, which is the expansion of the spectrum of snakebites covered by a single shot (Lavona et al., 2012, Stock et al., 2007). The development of a recombinant platform (eg. pluribodies) that permits the reproducible expression of mAb cocktails could be the solution of problems associated with animal-derived-antivenoms (Diamant et al., 2015, Aubrey et al., 2006, Figueiredo et al., 2014, Logtenberg, 2007).

Pluribodies can be produced either as stand-alone VHH moieties, or fused to the constant region of a human immunoglobulin γ1 chain (VHH-Fcγ1 format). The fusion of a human immunoglobulin γ constant chain allows the 'humanization' of the recombinant antibodies, reducing possible adverse effects associated with the presence of non human (e.g horse) constant antibody regions. Fcγ1 regions also contribute to an increased stability and facilitates purification using standard affinity chromatography procedures.

In this Chapter, we analyze the ability of a pluribody cocktail formulation in VHH-Fcγ1 format to neutralize *in vitro* and *in vivo* the toxin activities *of Bothrops asper* venom. The pluribody cocktail was obtained through a selection procedure carried out previously in our lab and described in Julve and colleagues (submitted). Briefly, a phage display antibody library in VHH format was prepared from camels immunized with *B. asper* venom. Subsequently, a group of 36 selected VHH sequences showing strong binding activity against several *B. asper* venom toxins were transferred from phage display plasmids into a binary Magnifection-derived plasmid fused in-frame to the human Fcγ1 antibody chain. The resulting antibody clones were co-infiltrated in *N. benthamiana* leaves for simultaneous expression in the form of a somatic mosaic taking advantage of SE phenomenon. The resulting plant-made recombinant anti-venom (Plantivenom) was shown capable to neutralize most of the relevant toxic effects induced by the venom of *B. asper* snake, namely phospholipase A2 activity, haemorrhagic activity, proteolytic

activity and coagulant activity. Furthermore, the Plantivenom was shown able to neutralize the lethality activity of the full venom of *B. asper* in mice.

2. RESULTS AND DISCUSSION

2.1. Production and purification of polyclonal plant-derived recombinant antibody in N. benthamiana leaves

A polyclonal mixture comprising 36 different antibodies selected by phage display against *B. asper* venom was co-infiltrated transiently in *N. benthamiana* leaves. Plant-made VHH-Fcγ1 samples were recovered from the leaf apoplast without disrupting the leaf tissue and purified by an affinity chromatography, resulting in a >95% purified single band product of the expected size, 42 kDa in reducing conditions (Figure 1). One kilogram of agroinfiltrated *N. benthamiana* leaves yielded 0.2g of recombinant polyclonal antibody collected from the corresponding leaf apoplastic fluid.

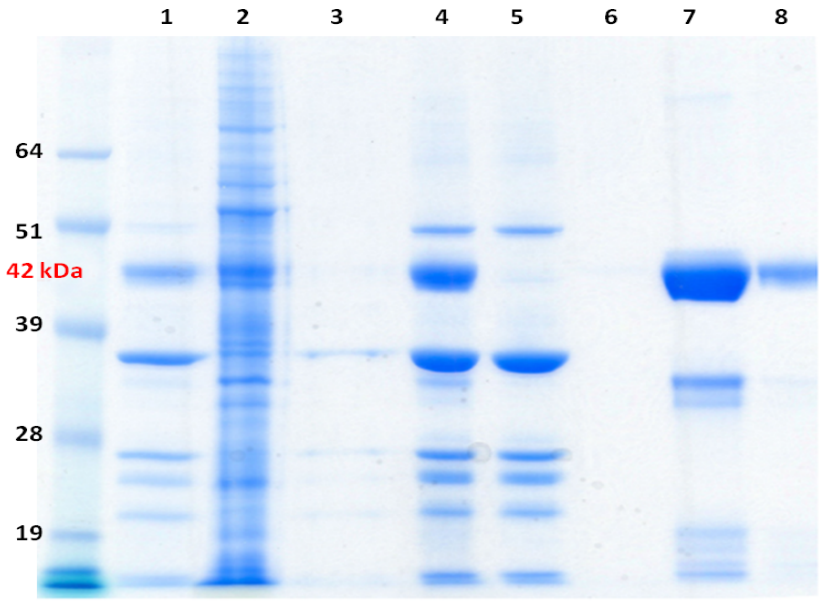

Figure 1. Coomassie of Plantivenom purification. The figure shows the different purification steps in a SDS-PAGE gel in reducing conditions. (1) Crude extract from apoplast liquid. (2) 20% ammonium sulfate precipitation. (3) Pellet of the 60% ammonium sulfate precipitation. (4) Supernatant of the 60% ammonium sulfate precipitation. (5) Flow fraction obtained during the affinity purification step. (6-8) Elution fractions, fraction 18 to fraction 20 respectively.

The purified antivenom was tested in an ELISA assay to verify the functionality activity of the Plantivenom against the *B. asper* venom after the downstream process (Figure 2). The starting concentration of the antivenom-plant-derived was 5 µg. Several dilutions were performed obtaining an end-point at 0.5 ng of Plantivenom.

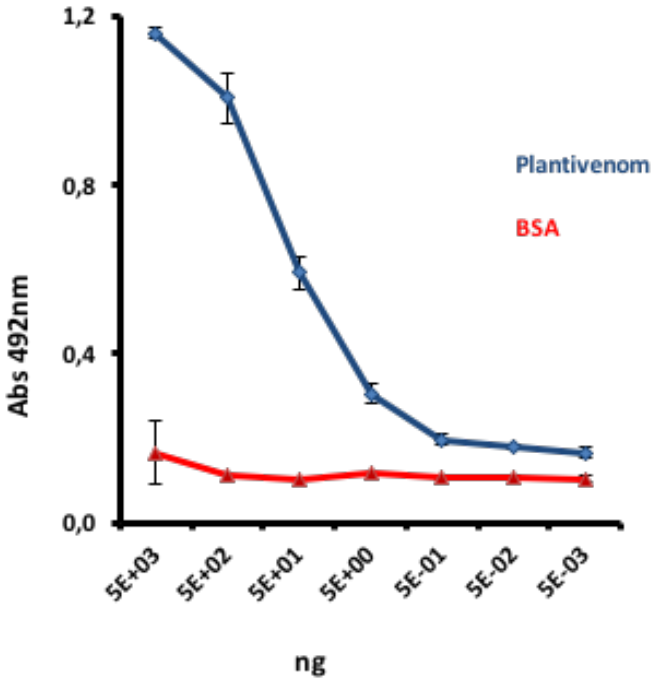

Figure 2. ELISA test. The figure shows the functional activity of the purified Plantivenom. The ELISA was coated with 2µg of full venom of *B. asper* snake per well. Samples were developed by anti-HCγ and anti-rabbit antibody. The background was determined of the bovine serum albumin (BSA) coated wells.

2.2. Immunoreactivity of antivenoms

Following affinity purification, an antivenom solution in PBS was prepared at a concentration of 46.5 mg/mL, similar to the typical concentration of equivalent commercial antivenom (55 mg/mL), and used for further characterization. Immunoaffinity chromatography was employed to assess the affinity of Plantivenom toward *B. asper* venom components. Briefly, a chromatography column is bounded with the Plantivenom and subsequently used to affinity-capture venom toxins. *B. asper* venom comprises different type of toxins, namely phospholipases, serine proteases,

metalloproteinases, L-amino acid oxidase and disintegrins. These toxins are well characterized in detail in Calvete and colleagues and Gené and collaborators (Calvete et al., 2009, Gené et al., 1989). The profile of captured toxins is indicative of the effective anti-toxin composition of the Plantivenom. Phospholipase (PLA2), and to lesser extent serine proteinases (SP), PI- and PIII snake venom metalloproteinases (SVMP) and L-amino acid oxidase (LAO) molecules, were efficiently retained by the immobilized plantivenom. However, the affinity matrix was poor in capturing disintegrins (DISI). Such pattern of immunorecognition mirrors the immunological most P-III SVMP, serine proteinases, L-amino acid oxidase, but only to a lesser extent medium-size disintegrins, PLA2 molecules, some serine proteinases and P-I SVMP BaP1 (Figure 3).

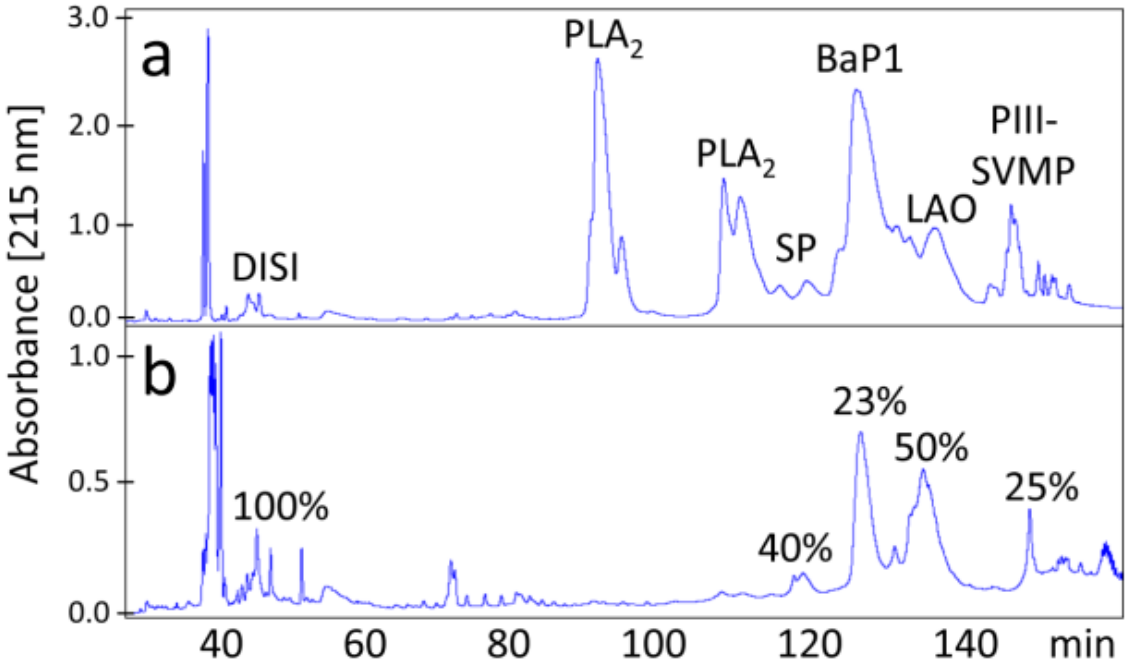

Figure 3. Antivenomic profile of Plantivenom. Upper (a) and lower (b) panels display, respectively, chromatographic profiles of whole Costa Rican *B. asper* (Pacific population) venom, and the venom fraction non-immunoretained in the immobilized affinity column.

2.3. Toxic and enzymatic activities and their neutralization by antivenoms

2.3.1. Inhibition of protease activity

Most of the toxins of snake venoms are proteolytic enzymes. Full protease activity was assayed using the azocasein assay, a colorimetric method based on the digestion of a solution of azocasein. By this method, the total proteolytic activity of *B. asper* was calculated as 4.1 µg/0.2UA/min. Next, the Plantivenom formulation was co-incubated with the full venom in order to calculate the median effective dose (ED_{50}) required to neutralize *B. asper* proteolytic activity. Plantivenom was shown effective in the partial neutralization of proteolityc activity of venom, showing and ED_{50} 120.5 ± 4.2 mg/mg (Table 1).

Table 1. Neutralization of the proteolytic activity of *B. asper* by the Plantivenom. The table shows the percentage of neutralization of the Proteolytic activity. We used as challenge dose 5µg of full venom

Dose Plantivenom/venom mg/mg	% neutralization of proteolytic activity
25	12
50	29.6
100	34.6
150	46.2
Snake venom control	0

2.3.2. Inhibition of metalloprotease activity.

SVMPs participate in the hemorrhagic process by proteolytic degradation of extracellular matrix components (Herrera et al., 2015, Guitérrez et al., 2005, Chambers et al., 2003). To asses the ability of Plantivenom to neutralize SVMP activity, we first calculated the Minimal Hemorrhagic Dose (MHD) of *B. asper* venom using an ex-vivo mice assay based on intradermal injections. The MHD was calculated 3.1 ± 0.1 µL/mg. The Plantivenom was shown to neutralize the hemorrhagic activity produced by B. asper venom with ED_{50} 22.2 ± 0.05 mg/mg (Figure 4).

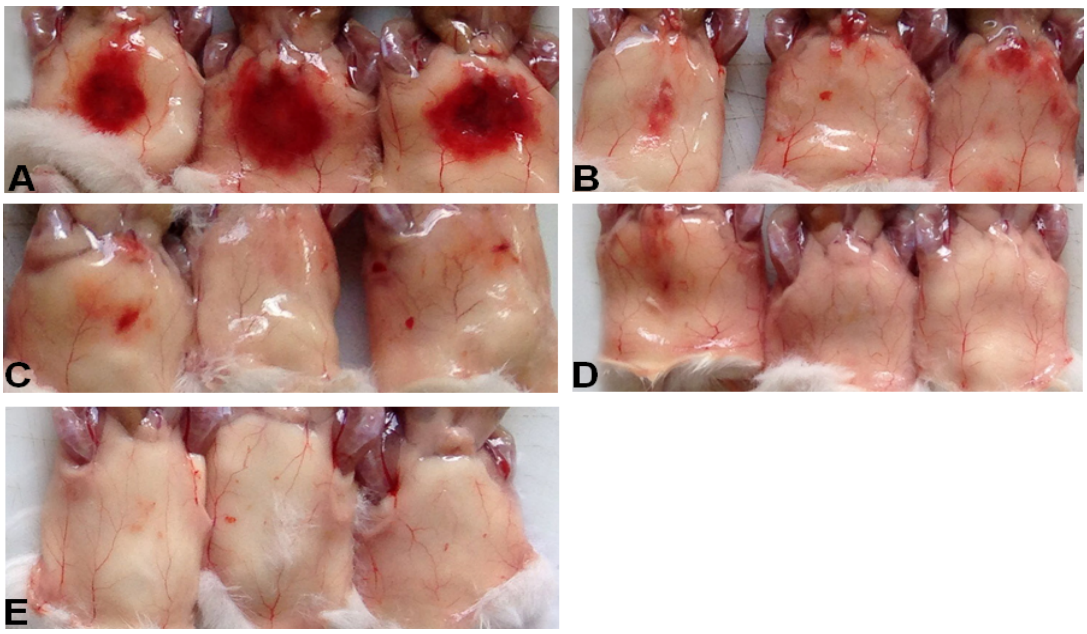

Figure 4. Neutralization of hemorrhagic activity of *B. asper* venom by the Plantivenom The figure shows the abdominal surface of mouse skin after 2h injection with a constant dose of full venom and different amounts of Plantivenom. Mice were injected intradermally with 15µg of *B. asper* venom (5MHD). **a)** positive control (5 MHD); **b)** hemorrhagic spot appear in 12.5mg Plantivenom /mg venom; **c)** hemorrhagic spot appear in 25mg Plantivenom /mg venom; **d)** hemorrhagic spot appear in 37.5mg Plantivenom /mg venom; **e)** negative control (Plantivenom diluted in PBS).

2.3.3. Inhibition of coagulant activity.

SPs act at the level of the coagulation cascade, such as thrombin-like activity on fibrinogen, induction of platelet aggregation, coagulation and fibrinolysis (Teixeira et al., 2009, Serrano et al., 2005). The coagulant activity of *B. asper* venom was tested on human citrated plasma and on bovine fibrinogen solution. Clotting times were recorded, and the Minimum Coagulant Concentration (MCC) was determined in both cases. In the case of human citrated plasma the MCC was 0.8µg ± 0.25 and the MCC for bovine fibrinogen solution was 89.6µg ± 0.1.

In order to test the ability of Plantivenom to neutralize SPs, different amount of Plantivenom were incubated with one MCC. Unfortunately, Plantivenom failed in this venom activity. Plantivenom was not capable to neutralize the coagulation of human citrated plasma and the coagulation of bovine fibrinogen solution. The inability of the Plantivenom to neutralize fibrinogen activity coagulant effect of the venom correlates with the antivenomic observation, as most of the non-retained peaks correspond to SPs.

2.3.4. Inhibition of phospholipase activity

PLA_2 from snake venoms are among the most aggressive toxic proteins, playing a role in prey immobilization and killing. Most presynaptic neurotoxins and myotoxins from snake venoms are PLA_2 or contain PLA_2-like subunits in their molecules (Ghazaryan et al., 2015, Gutiérrez et al., 1987, Alagón et al., 1980). We analyzed the PLA2 activity by the titrimetric method, an acidimetric assay based on egg yolk phospholipids as substrate, which resulted in a PLA_2 activity of 20 µg of *B. asper* venom in a value of 10.54µg/min/mg. We analyzed the neutralization of the PLA_2 activity using the Plantivenom. Plantivenom was effective in the neutralization of PLA_2 activity, showing an ED_{50} 224.1 ± 13.2 mg/mg (Table 2). The neutralization of PLA_2 is in agreement with the antivenomic observation showing as most of the chromatographic retained peaks corresponded to PLA_2.

Table 2. Neutralization of the phospholipase A2 activity of *B. asper* by the Plantivenom. The table shows the percentage of neutralization of the PLA_2 activity. We used as challenge dose 20µg of full venom.

Dose plantivenom/venom mg/mg	% Inhibition of phospholipase activity
56.25	20
112.5	38.9
225	50.5
250	56.5
Snake venom control	0

2.3.5. Inhibition of lethal activity

Finally, we tested the capacity of Plantivenom to neutralize lethality induced by *B. asper* venom in mice, using $2LD_{50}$ of venom as 'challenge dose' and employing different doses of Plantivenom (Table 3). Plantivenom prevented lethality in all injected mice at ratio af 61.24 mg Plantivenom/mg venom. The estimated Median Effective Dose (ED_{50}) for the Plantivenom was 43mg plantivenom/mg venom (95% confidence limits: 33-56mg/mg) (Table 3). All neutralization activities were assayed in parallel with a commercial horse-derived antivenom, and the corresponding neutralizing activities are also summarized in Table 4. Horse-derived antivenom was effective in the neutralization of all enzymatic and toxic effects assayed.

Table 3. Estimation of the intraperitoneal ED_{50} of Plantivenom. The table shows the data obtained in this experiment. We use as challenge dose $2LD_{50}$ of venom with different amounts of Plantivenom and $2LD_{50}$ as venom control.

Dose plantivenom/venom mg/mg	Animals injected	Dead animals after 48h
26.28	4	4
34.78	4	3
46.5	4	2
61.84	4	0
Snake venom control	4	4

3. DISCUSSION

Envenomations by snakes of the family Viperidae, as *B. asper* the most poisonous snake in Central America, induce severe pathological alterations at the site of venom injection, such as edema, necrosis, hemorrhage, coagulopathy and blistering, which may lead to permanent tissue damage and disability (Teixera et al., 2009, Gutiérrez, 2016, Alam et al., 1996). Snakebite envenomation constitute a public health in many regions of the world (Gutiérrez, 2014, Chippaux, 2013). Parenteral administration of equine or ovine-derived antivenoms is the validated treatment to confront this envenomation. However, animal-derived antivenom is not reproducible batch-to-batch, cause hypersensitivity reactions, it has a high risk of infectious disease transmission and can be an ethic problem (Chippaux, 2013, León et al., 2013). The development of genetic recombinant engineered antivenom production can be a solution.

In a previous study developed in our group, it was created a polyclonal mixture against *B. asper* snake venom. In this study, we produced data which strongly suggest that Plantivenom is effective in neutralizing some of the toxic activities of the venom. Plantivenom neutralize proteolytic activity, phospholipase activity, hemorrhagic activity and i.p. lethality. We compared neutralization activities of *B.asper* by Plantivenom and the commercial antivenom (Equine-derived-antivenom) (Table 4). Plantivenom is capable to neutralize the SMVP hemorrhagic-PIII preventing the local hemorrhage in the skin of the mice and therefore contributing to the prevention of i.p. lethalithy. These neutralization results are congruent with the antivenomic profile, where the Plantivenom specifically recognizes the PLA_2 peaks and some of the SVMPs proteins. These successful outcomes demonstrate the good selection by phage-display done against the PLA_2 and the SVMP in the previous work. This good selection may be explained because SVMP and PLA_2 represent 89% of the total venom proteins in *B. asper*, which 44% are SVMP and 45.1% are PLA_2 (Calvete et al., 2009).

Camelid antibodies are promising therapeutic agents with an excellent safety profile in clinical trials. Most importantly, heavy-chain antibodies show 80% sequence homology to human VH fragments and therefore exhibit immunogenicity. V_HHs were shown earlier to inhibit the BthTX-II phospholipase A2 activity, and to neutralize the myotoxic effects induced by *B. jararacussu* venom in mice (Prado et al., 2016). Other anti-toxin V_HHs include scorpion and botulinum toxins (Rossotti et al., 2015, Lausten et al., 2016). Fusion constant IgA regions to camelid antibodies made in plants enhanced passive immunotherapy for the prevention of diarrhea in pigs. In our approach, V_HHs fusions with the IgG1 constant heavy chain was designed as a strategy to reduce adverse reactions and improve the pharmacokinetic profiles of recombinant antivenoms. Other combinations including free V_HHs could also be considered for the neutralization of low molecular wight toxins in deep tissues.

The result obtained in the coagulation activity, in particular in the fibrinogen bovine solution, shows how the Plantivenom failed in the serinprotease neutralization activity. It is described than thrombine-like serine proteinase display fibrinogen-clotting activity *in vitro* and it plays an important role in the coagulopathy characteristic of envenomation inflicted by *B. asper* bites (Rucavado et al., 2004, Núñez et al., 2004).

Probably, during the selection by phage-display this thrombin-like serine proteinase was not selected, provided that the SP only represent 4% of the full venom.

Taken together the results of this work demonstrated that the Pluribodies Technology is a powerful recombinant tool for manufacturing functional mAb cocktail *à la carte* in a single transformation event. The cocktail described here showed a reasonable spectrum of neutralizing activities with relatively little effort in antibody selection. With consistency and reproducibility ensured, the opportunities for formulation improvements through the implementation of successive design-build-test cycles are manifold. The low efficiency of our Pluribody formulation to neutralize *in vitro* coagulant activity, can be solved with the introduction of new serine protease neutralizing clones from improved antibody selection procedures, synthetic repertoires or alternative single-cell selection methods (Beerli et al., 2010). A streamlined formulation should also involve the elimination of neutralizing activities against other snake venoms from the same region. Our recombinant antivenom is capable to neutralize *in vivo* and *in vitro* most toxics activities of *B. asper* venom. Therefore, the results show a clear way for improving the formulation of the plant-derived-mAb mixture to obtain efficient recombinant antivenom.

Table 4. Neutralization of toxic activities of *B. asper* venom by Plantivenom and Horse-derived-antivenom. The ED_{50} was expressed as mg of antivenom per mg of venom. (a) Neutralization of hemorrhagic activity is expressed as Median Effective Dose (ED_{50}). Challenge doses correspond to 5MHD for the *B. asper* venom. Determinations were performed in triplicates. (b) Neutralization of phospholipase activity is expressed as Median Effective Dose (ED_{50}). Challenge doses correspond to constant dose (20µg) of the *B. asper* venom. Determinations were performed in triplicates. (c). Neutralization of protease activity was expressed as Effective Dose (ED) Challenge dose of venom corresponds to 5µg of *B. asper*. (d). Neutralization of coagulant activity was expressed as Effective Dose (ED). Challenge dose of venom corresponds to 2 MCDs. Determinations were performed in triplicates. (e). Neutralization of coagulant activity was expressed as Effective Dose (ED) Challenge dose of venom corresponds to 1 MCDs. Determinations were performed in triplicates. (f). Neutralization of lethal activity is expressed as Median Effective Dose (ED_{50}). Challenge doses correspond to 2 LD_{50}s for the *B. asper* venom. Determinations were performed in triplicates.

Toxic activities of *B. asper*	ED_{50} Plantivenom	ED_{50} Horse-derived
Hemorrhagic [a]	22.2 ± 0.65	6.9 ± 0.01
Phospholipase A_2 [b]	224.1 ± 13.2	37.4 ± 0.4
Proteolytic [c]	120.5 ± 4.2	43 ± 2
Coagulant citrated human plasma [d]	>> 372	11.3 ± 0.13
Coagulant bovine fibrinogen [e]	>>11.6	0.165 ± 0.01
Peritoneal lethality [f]	43.25 (33.4-56)	3.1 (33.4-56)

4. MATERIALS AND METHODS

4.1. Infiltration of N. benthamiana leaves

For the transient expression experiments, all clones were transferred to *A. tumefaciens* strain GV3101 by electroporation. Agroinfiltration was performed as described in Gutiérrez, 2014. Overnight-grown bacterial cultures were pelleted and re-suspended in agroinfiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 200 µM acetosyringone) to an optical density at 600 nm of 0.1. in a viral system, to be more specific in MagnICON expression system. Infiltrations were carried out using the vacuum infiltration technique in leaves of 5-6 week-old *N. benthamiana* plants (growing conditions: 24°C day/20°C night in a 16-h-light/8-h-dark cycle). Leaves were harvested 7 to 10 days post infiltration and examined for transgene expression.

4.2. Extraction and purification mAb mixture from plant leaf tissue

Recombinant mAb mixture from agroinfiltrated plants were extracted from the leaf apoplast by vacuum infiltration-centrifugation. Leaves were excised from the plant, rinsed in distilled water, and blot dry on tissue paper. Afterwards, the leaves were submerged in cold extraction buffer; vacuum was applied to a pressure of -0.9 bar (Vacuum Degassing Chamber DP118, Applied Vacuum Engineering, Thornbury, UK), held for 1 min, and then slowly released to allow the buffer to infiltrate the leaf blades. Na_2HPO_4 40mM, 50mM ascorbic acid, EDTA 10mM, pH7 was used as the extraction buffer. Vacuum infiltrated leaves were then blot dry on tissue paper and centrifuged in swing-rotor centrifuge (10 min, 1200xg) at 4°C to extract the apoplast wash fluid (AWF). The AWF were clarified at 10,000xg for 15 min at 4°C. The supernatant was collected and ammonium sulfate was added to 20% of saturation at 4°C during 1 h mixing gently. After the incubation, the solution was centrifuged at 10,000xg for 30min at 4°C, the supernatant was collected. Ammonium sulfate to 60% was added to the supernatant at 4°C during 2 h. After the incubation, the solution was centrifuged at 10,000xg for 30min at 4°C. The pellet was resuspended in buffer PBS (20mM NA2HPO4 and 10mM ascorbic acid) in 1/5 the original volume of extraction buffer and dialyzed against binding buffer (20 mM phosphate, 10mM ascorbic acid, pH:7). The solution was collected and it was filtrated using a 0.22 µm membrane filter on ice. Affinity purification was carried by FPLC using an ÄKTA purifier (GE Healthcare, Buckinghamshire, UK) equipped with a HiTrap Protein A HP affinity column (GE Healthcare, Buckinghamshire, UK) following manufacturer recommendations. Samples were eluted with 0.1 M citric acid pH 3.0 and neutralized with 1.0 M Tris-HCl pH 9.0.

4.3. SDS-PAGE

Proteins were separated by SDS/PAGE in 10% denaturing gels (Invitrogen, Carlsbad, NM, USA). Gel staining was carried out with coomassie following standard procedures.

4.4. ELISA

Costar 96 Well EIA/RIA plates (Corning, NY, USA) were coated (o/n, 4°C) with 2 µg/mL of whole *B. asper* venom in coating buffer (50 mM carbonate buffer pH 9.8). Blocking was performed for 2 h at RT in a 2% (w/v) solution of ECL Advance™ Blocking Reagent (GE Healthcare, Buckinghamshire, UK) in PBS-T (20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 100

mM NaCl, pH7.4, supplemented with 0.1% (v/v) Tween 20). Purified plantivenom samples diluted in PBS (20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 100 mM NaCl, pH7.4) were then added to the wells and incubated for 45 min at RT. After 4 washings, 1:2000 rabbit anti-human IgG (SIGMA, St. Louis, USA) in PBS-T was added and incubated for 1 h at room temperature. Plates were washed 4 times and incubated with ECL™ peroxidase labeled anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK) at a 1:5000 dilution for 1 h at RT. After washing the plates, o-phenylenediamine dihydrochloride substrate (SIGMAFAST™ OPD tablet, SIGMA, St. Louis, USA) was added for detection; reactions were stopped with 3 M HCl. Absorbance was determined at 492 nm. As a control for binding specificity, plates were coated with 2 µg/mL of BSA. All washing steps were performed with PBS.

4.5. Determination of the immunoreactivity profile

Affinity chromatography columns were prepared by immobilizing 16 mg of Plantivenom on 10 mL of cyanogen bromide-activated Sepharose 6MB (Sigma; catalog Number C9267), following the manufacturer's instructions. Columns were equilibrated with PBS, and absorbance was recorded at 280 nm. Then, each column was loaded with 3mg of *B. asper* venom and washed with PBS until the non-bound fraction was eluted. The bound fraction was eluted by changing the mobile phase to glycine, pH 3.0, and the pH of the collected fractions was adjusted by the addition of 0.5 M NaOH to reach pH 7.0–7.5. Subsequently, the bound fractions and the non retained fractions were analyzed by HPLC, using an Agilent Technologies 1100 series system (Santa Clara, CA, USA) equipped with a chromatographic data management system (ChemStation Data Analysis and Reporting, Agilent Technologies) and using a Lichrosphere RP 100 C_{18}column (250 × 4.6 mm, 5 µm particle size). Fractions were eluted at 1 mL/min with a linear gradient of buffer A (95% water, 0.1% TFA) and buffer B (95% acetonitrile, 0.1% TFA) (5% B for 10 min, followed by 5–15% B over 20 min, 15–45% B over 120 min, and 45–70% B over 20 min). Detection was set at 215 nm. Chromatographic runs of whole *B.asper* dissolved in PBS were used as controls.

4.6. Neutralization of hemorrhagic activity

Hemorrhagic activity was assessed according to the method of Gutiérrez et al., 1985. The minimum hemorrhagic dose (MHD) of *B. aper* snake venom (3.1 µg) was determined by intradermal (ID) injection in the ventral abdominal region, to groups of three CD1- mice (18-20g). Mice of both sexes of CD1 strain were used for the assessment of hemorrhagic activity. A "challenge dose" of venom was selected and incubated with various amounts of plantivenoms proteins for 30min at 37C. Controls included venom incubated with PBS instead of plantivenom. The inhibition of hemorrhagic activity was assayed by ID injection of five MHD with various amounts of Plantivenom. After two hours, mice were sacrificed with CO_2, the skin was removed and the diameter of the hemorrhagic lesion was measured. Crude venom (five MHD) was used as control.

4.7. Neutralization of intraperitoneal lethality activity

Lethality was assessed by intraperitoneal route. Groups of four CD1- mice (18-20g) were injected with various doses of *B. asper*, dissolved in 0.5ml of phosphate-buffered saline solution, pH 7.2 (PBS). Mice of both sexes of CD1 strain were used for the assessment of lethal activity. A "challenge doses" of venom was selected and incubated with various amounts of plantivenoms proteins for 30min at 37ºC. Controls included venom incubated with PBS instead of plantivenom. The median lethal dose (LD_{50}) of *B. asper* is 73µg (33µg-125µg) and it was determined by Spearmen-Karber method. The neutralization of lethality was assessed by the same method, but substituting the venom solutions by venom/Plantivenom mixtures containing two LD_{50}. The neutralizing activity was expressed as Median Effective Dose (ED_{50}), the Plantivenom/venom ratio in which the venom-induced lethality was inhibited by 50%. Full venom (two LD_{50}) was used as control. Deaths were recorded during the following 48h.

4.8. Neutralization of phospholipase A_2 (PLA_2) activity

PLA2 activity of *B. asper* venom was measured according to the method of Dole (1956) as modified by Gutiérrez et al., 1986. The activity venom was assessed by 100µL of different amounts of *B. asper* with one mL of chicken egg yolk diluted 1:5 in 1% Triton X-100, 0.1M Tris, 10mM $CaCl_2$ buffer, pH 8.5. The neutralization was assessed by 100µL of a venom/Plantivenom mixture (containing a constant dose of 20 µg of *B. asper*) with

one mL of chicken egg yolk diluted 1:5 in 1% Triton X-100, 0.1M Tris, 10mM $CaCl_2$ buffer, pH 8.5, and incubating the mixture during 30min at 37ºC. The neutralization was expressed as the median effective dose (ED), Plantivenom/venom ratio in which the venom induced phospholipase A_2 activity was inhibited by 50%. Determinations were performed in triplicates.

4.9. Neutralization of coagulant activity

The coagulant activity was assessed on citrated human plasma obtained from healthy volunteers and on a 4mg/mL solution of bovine fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS. The citrated human plasma or the solution of bovine fibrinogen was mixed with 100 µL of different amounts of *B. asper*. Coagulant activity was expressed as the Minimum Coagulant Dose (MCD), the amount of venom that induces clotting of plasma in 60 s (Gené et al., 1989). The neutralization of coagulant activity was determined by the same method, but substituting the venom solutions by venom/Plantivenom mixtures containing two MCDs of venom as challenge dose and incubating the mixture during 30min at 37ºC. Neutralization was expressed as Effective Dose (ED), the Plantivenom/venom ratio at which coagulation time was increased three-fold when compared to coagulation time of plasma incubated with the venom alone (Gené et al., 1989). Determinations were performed in triplicates.

4.10. Neutralization of protease activity

Proteolytic activity was determined using azocasein as substrate (Wang et al., 2004), as modified by Gutiérrez et al., 2008. One unit of proteolytic activity corresponds to the amount of venom that induces a change in absorbance at 450nm of 0.2. Neutralization of venom was based on the incubation of a fixe dose of *B. asper* venom (5µg) with different amounts of Plantivenom for 30min at 37ºC. Determinations were performed in triplicates.

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General Discussion

In the last fifteen years, recombinant antibodies have grown to become the dominant product class within the biopharmaceutical market. Today, they are used in several applications such as research, diagnostics and treatment of several major diseases due to their ability to bind to a wide variety of molecules with high specificity and affinity (Moussavou et al., 2015, Hansel et al., 2010, Chames et al., 2009, Janeway, 2001). BioProcess Technology Consultants reported that in 2014 forty-seven recombinant antibody products had been approved in the USA or Europe for the treatment of several diseases (de Marco, 2015). Currently, global sales for all recombinant products are nearly $99 billion per year and the therapeutic antibody market represents more than 50% of this sales. With the current approval rate of new products per year, approximately seventy recombinant antibodies will be on the market in 2020, meaning a world-wide sale of nearly $125billion (Ecker, 2013; de Marco, 2015).

The increasing demand for therapeutic antibodies has resulted in a significant development in both the recombinant production systems and the antibody manufacturing process. Among the variety of systems that are being used for recombinant antibody production, plants are the ones that experienced a bigger development on the last few years. However, the use of plants to produce recombinant antibodies is not new. In 1989, Hiatt and colleagues reported for the first time the expression of a full-size IgG in plants (Hiatt et al., 1989). Nowadays, there are nine plant-derived pharmaceutical products used in clinical trials and there are thirteen companies using plants to produce recombinant pharmaceutical products (Jian et al., 2015). In this thesis we explored the expression of alternative mAb formats in plants and their expression as mixtures for specific therapeutic application. From the standpoint of the experimental data produced here, we can affirm that plants represent a promising system for the production of alternative mAb formats as therapeutic drugs.

1. Recombinant plant-derived-mAb expression levels

The first part of this thesis shows the comparison of the expression of different recombinant mAb formats in plants. The different mAb versions were obtained by combining different antibody constant regions with the same variable region. The use

of the GoldenBraid assembly system (Sarrión-Perdigones et al., 2013) facilitated this task. Thanks to the combinatorial possibilities that offers this system, three binary plasmids ready for plant transformation were created in three weeks days. Results of the expression of the different mAb formats in *N.benthamiana* indicate that the production levels of the mAbs are affected by the antibody format and the antibody isotype. The scFv-Fcγ1 antibody format accumulated around 120 µg of antibody per gram of fresh weight in *N. benthamiana*. The full-length γ1 antibodies formats, HCγ1-LCλ and HCγ1-LCκ, accumulated on average at around 75 µg of antibody per gram of fresh weight. The scFv-Fcα1 showed lower levels in transient expression than IgG-like antibodies. In general, when produced transiently, the expression levels of the different mAb formats represent around 1-2% of the TSP. These expression levels support the use of plants as an alternative expression system since previous reports estimated that yields of 1% TSP are generally sufficient to make plants cost effective (Fernandez-del-Carmen et al., 2013). The recommended dose of Infliximab for IBD treatment is 3-5mg/kg as initial treatment and increases up to 10 mg/kg at the end of the treatment. Considering these amounts, an adult (assuming an average weight of 70kg) wound need a dose of 210 to 700mg of Infliximab. With our current expression levels of Infliximab on its best format scFv-Fcγ1, a 1000m^2 greenhouse facility growing 15Kg biomass/m^2/year could produce 18000 vials per year containing 100mg. Of special interest in this context is the possible use of plant-made recombinant antibodies in topical applications. An example is the application of infliximab for the treatment of inflammatory skin conditions such as psoriasis or in the healing of chronic wound (Streit et al., 2006). Local applications of plant-made mAbs or even semi-purified plants extract could become an affordable and safe alternative to current systemic treatments. If Generally-Regarded-As-Safe (GRAS) production system are employed (as exemplified here with the use of tomato fruits, or alternatively using edible plant organs), partially purified formulations reaching high antibody concentrations can be produced without disturbing the GRAS status of the final composition. This option could be accomplished by industrial processes for protein concentration as partial precipitation, cross flow filtration or membrane chromatography yielding mAb-enriched formulations (Giese et al., 2013, Li et al., 2013, Baruah et al., 2005, Lee et al., 1992, Oelmeier et al., 2013).

Additional strategies could be considered in order to boost antibody expression with the enhancement of the *in planta* expression levels. Higher expression levels have been reported using viral vectors or viral-derived transcriptional enhancers in transiently infiltrated leaves (Dugdale et al., 2013, Gleba et al., 2007). An interesting possibility would be to transform the best antibody format, scFv-Fcγ1 using a viral vector as the MagnICON system (Gleba et al., 2006) or a non-replicative system such as the pEAQ system (Sainsbury et al., 2009). Expression levels achieved with the Magnifection technology were as high as 4.5mg per g of fresh-leaf biomass (Giritch et al., 2006), and similar levels are reported for non-replicative systems. If our estimation is confirmed, and given the low capital investment required for the establishment of the infrastructures needed to use plants as recombinant proteins expression platforms, it is possible to achieve the estimated quantity required for the clinical use at low price using plants.

However, the agroinfiltration method at a large scale is laborious and time consuming. Since several studies have suggested that levels of mAb generated via transient expression can be reached using stable expression, an alternative would be the hydroponic culture of transgenic plants. Hydroponic culture is suitable for large scale production since is simple, reproducible and has a controllable production. Recently, a new replicative system based in the in-planta activation of a DNA virus was reported (Dugdale et al., 2013). The tobacco yellow dwarf geminivirus (TYDV)-based production in-plant activation system (Dugdale et al., 2014) used plants stably transformed with a replicative system that, once triggered with ethanol, activates the amplification of the gene of interest, reaching production yields similar to those reported for transient replicative systems. The combination of stable transformation and switchable replicative systems could provide an interesting alternative for the manufacturing of mAb in plants, particularly for topical applications using semi-purified formulations.

2. Manufacturing plant-derived-mAb cocktail.

The feasibility of producing functional mAb in oligoclonal formats in plants was addressed in Chapter 2. As it was described in the introduction section of that Chapter, treatments used in autoimmune, oncology, poisonous bites and autoimmune and infectious diseases are based on mAb mixtures (Boes et al., 2015, Nejatollahi et al., 2014, Wang et al., 2013, Logtenberg, 2007). For that reason, the production of a mAb cocktail in plants was set as the following goal.

Unfortunately, current recombinant production systems are not well adapted to the manufacturing of mAb cocktail. The production and purification of each individual mAb separately is unaffordable when making for complex cocktails since the cost is proportional to the number of antibodies included in the cocktail. Alternatively, the simultaneous production of all the components of the mAb mixture could reduce cost compared to manufacturing each mAb individually. For example, it is estimated that the batch failure for the manufacturing process of a six mAb cocktail when processed individually will be higher (35%) than when a single batch manufacturing approach is used 7% (ES Langer, Bioprocess Inc, 2008). For this reason, it would be interesting to express a mAb cocktail in a single batch event.

During the development of Thesis, in 2014, an outbreak of Ebola virus began in Africa becoming a global health threat. At that time, ZMapp cocktail, an experimental plant-derived-mAb mixture against Ebola virus, was the only effective treatment against the Ebola virus in nonhuman primates and in human patients (Qiu et al., 2014). That is why, ZMapp cocktail was selected as state-of-the art for manufacturing a mAb cocktails in a single transformation event in plants. ZMapp cocktail comprises three mAb, each of them with different heavy chains and light chains.

The experimental work described in Chapter 2 of this Thesis showed differences in the functionality of a three mAb mixture when the three mAb are co-expressed in a single batch compared to individual production. The results obtained where in accordance to our expectations, the production of the mAb cocktail by co-expression of the two light and two heavy chains in a single transformation event can be challenging due to the phenomenon called chain shuffling, where light chains miss-pairs with non-

cognate heavy chains (Kang et al., 1991). Consequently, co-expression of different antibodies can result in the production of unwanted antibodies species. In this work, we indirectly confirmed chain shuffling phenomenon in the mAb mixture by verifying that co-transformation severely affects the binding activity of the ZMapp cocktail. Interestingly, we observed a residual activity level independent of the OD used in agroinfiltration mixture. Probably, this residual activity corresponds with the correct pairing between the antibodies' light and heavy chain. The existence of this residual activity opens the possibility to use the co-expression at low ODs as an alternative expression strategy for simple mAb cocktails, as in certain circumstances this approach could be more convenient and robust than the separate production of individual mAb followed by its formulation in a single product.

An option to overcome this miss-pairing of non-cognate antibody chains would be to use a common light chain or heavy chain for all mAb in the cocktail. An example is the Oligoclonics™ technology that produces a mixture with a combination of a single light chain and two or three heavy chains conferring different antibody specifities (Thompson et al., 2014). But, with this strategy, the activity of the cocktail would probably decrease because there are less epitope-binding-sites in the mixture. Another strategy was by Symphogen. This company has created a single batch manufacturing approach using the Sympress™ method, which reportedly allows the production of any number of specific antibodies in a single bioreactor as a one drug substance. To produce the mAb cocktail, seed material is prepared from a polyclonal cell bank by mixing the CHO individual stable cell lines, each of them expressing a different mAb. In the Sympress™ method, mAb mixtures can be produced under predictable, reproducible and stable conditions. However, the process of culture stabilization is long and expensive and does not include the additional advantages provided by plant production systems.

Another alternative strategy to overcome chain shuffling and the associated high costs could consist in exploiting the superinfection exclusion phenomenon, which prevents a second infection to happen when a cell is already infected by a closely-related virus (Folimonova, 2012). The results described in Chapter 3 demonstrate that the Pluribodies Technology, based on the use of plant viral vectors, permits the expression of a functional recombinant complex mAb mixture of single-chain mAbs produced in a

single transformation event, where each foci expresses an independent antibody. With this proof of concept, we proposed Pluribodies Technology as a cheap platform for expression of complex mAb cocktails, with considerable reductions in manufacturing, transformation and downstream process costs.

A similar approach, based on the use of plant viral vectors, could be envisioned to avoid chain shuffling during the production of cocktails of full size mAbs. In theory, if both heavy and light chains are encoded in the viral genome, viral superinfection exclusion would keep clones separately, ensuring that no chain shuffling takes place (see Fig 1 for an explanatory scheme). However, the principal limitation for this approach derives from the difficulty to encode both heavy and light chains simultaneously in a viral genome. Indeed, the expression of full-length antibodies using viral vectors is usually achieved by using two noncompeting viral vectors as a way to circumvent viral exclusion (e.g. TMV and PVX). In these cases, each plant viral vector expresses a separate antibody chain (HC or LC) (Giritch et al., 2006). Nowadays, thanks to the development of Synthetic Biology, the co-expression of two proteins in the same viral vector can be achieved through the introduction of viral 2A peptide, IRES sequence and related subgenomic promoter sequences (Roy et al., 2011, Daniels et al., 2014, Li et al., 2007). These three strategies would enable the expression of a full-length mAb mixture using viral vectors. The use of bicistronic viral or dual viral vectors would prevent the chain shuffling through the viral exclusion phenomenon (Figure 1). In fact, in previous reports the use of bicistronic viral vectors showed the co-expression of two fluorescent proteins in the same cell (Daniels et al., 2014, Roy et al., 2011). We therefore propose the use of bicistronic viral vectors as a future alternative for the production of antibody cocktails in full size format.

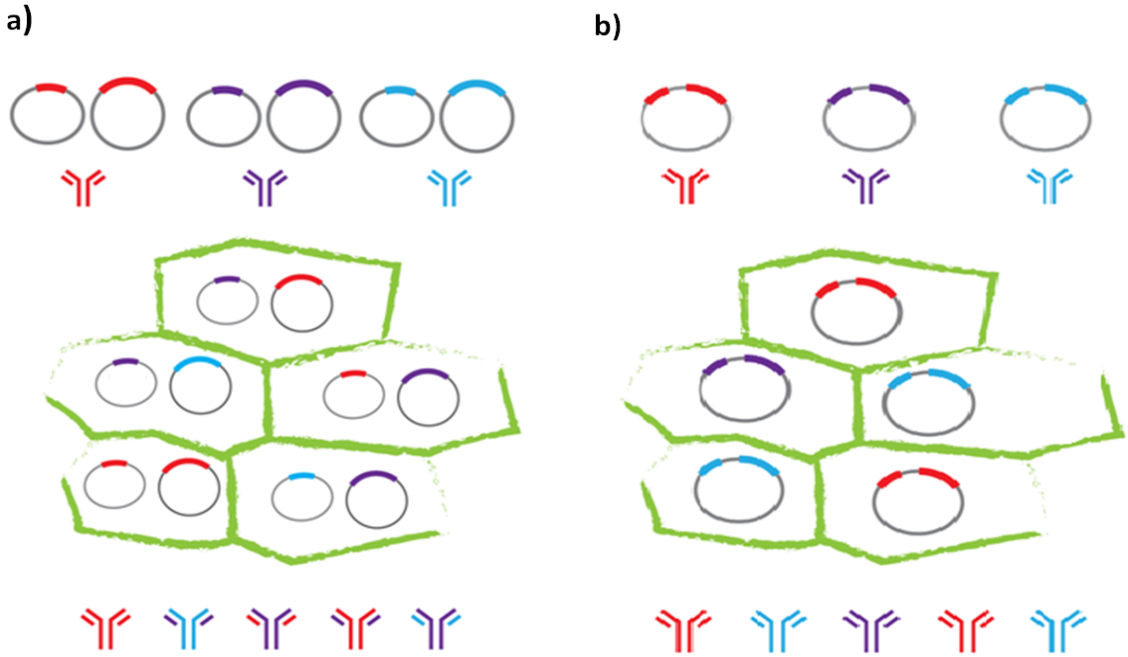

Figure 1. Schema of several strategies for manufacturing full-length antibodies mixture into viral vectors. (a) The use of non competing virus permits the expression of the two polypeptides that composed a full-length antibody within the same cell (Giritch et al., 2006). However, the co-expression of full-length mAb mixture using non related virus can affect the functionality due to miss-pair between the different antibody chains. **(b)** The co-expression of two polypeptides into a dual viral vector permits the co-expression of the two antibody chains in the same cell. Taking advantage of the viral exclusion phenomenon, the co-expression of mAb mixture using dual vectors can solve the chain shuffling.

However, for certain therapeutic applications, the production of full size antibodies is not necessary and it can be substituted with single chain antibodies decorated with different constant regions as for instance, the human Fc of gamma immunoglobulin 1. Based on this, our group developed the Plantivenom concept based on Pluribody Technology, which ensures high reproducibility in the production of single-chain polyclonal cocktails by exploiting the viral exclusion phenomenon (submitted). Since the XIX century, the only available treatment against venomous snake bites is based on polyclonal Abs (pAbs) obtained from hyperimmunized animals sera, usually horses or sheep (Gutiérrez, 2016, Chippaux, 2013, Krifi et al., 1992). Differences in the reproducibility, pharmacokinetics, the induction of adverse reactions in patients and

ethical problems promote the development of technological alternatives for manufacturing antivenoms, for example the development of a recombinant mAb cocktail technology against snakebites (Aubrey et al., 2006). In the third part of this work, we demonstrated that the production of a functional complex mAb cocktail composed by thirty-six different antibodies using Pluribodies Technology is possible. The cocktail can be purified by affinity chromatography in a single purification step reducing the downstream costs (Chon et al., 2011). The purified mAb cocktail is capable of neutralizing most of the toxic effects of the venom as haemorrhagic activity, phospholipase activity, proteolytic activity and intraperitoneal lethality (Alam et al., 1996). These results showed that Plantivenoms can neutralize a wide range of toxin activities providing protection against lethal venom doses in mice. In a wider sense, this opens the possibility to produce recombinant "polyclonal" neutralizing antibodies in a consistent and highly reproducible way, which is particularly important for therapeutic approaches requiring neutralization of complex toxins and/or highly diverse and variable serotypes.

3. Final remarks

Despite all the advantages that plants can offer as antibody expression systems, very few antibodies have been used for clinical trials (Yusibov et al., 2011, Obembe et al., 2011). The well established mammalian system for manufacturing recombinant antibodies has indirectly contributed to hold up the development of plant-derived antibodies technology. Currently, plant expression systems cannot replace mammalian system as those have taken an advanced position in cGMP production and drug regulation (Li et al., 2010). However, new opportunities for plant-made antibodies emerged in recent years with the need for effective treatments for infectious/poisonous/immune diseases requiring mAb cocktails (Qiu et al., 2014). Plants are known for long to be cost-effective production systems free of mammalian pathogens. Recently we have learnt that plant glycosylation patterns can be engineered *à la carte* using Synthetic Biology approaches. Now we also know that plants offer the possibility of manufacturing mAb cocktails, in several formats, in a consistent and reproducible way.

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Conclusions

1. Several human antibody formats carrying the variable regions of the infliximab mAb, can be produced transiently in *N. benthamiana* leaves, reaching accumulation levels up to 1.2% of the total soluble protein. All purified plant-made antibodies formats showed strong anti-h-TNF-α binding activity and inhibited *in vitro* the apoptotic cell death induced by TNF-α. Among the combinations assayed, HCγ1-LCλ and scFv-Fcγ1 formats, both carrying HCγ1 constant regions, accumulated at higher levels and showed stronger anti-h-TNF-α neutralizing activity.

2. A cocktail of three human mAbs in full length IgG1 format can be produced in *N. benthamiana* up to the milligram scale in only three weeks, using the DNA sequence of their variable regions and the Modular Cloning GoldenBraid collection as starting points. The co-expression of all three antibodies in a single co-agroinfiltration batch affected the functionality of the cocktail, apparently due to a shuffling effect resulting in mispairing of cognate antibody chains. Chain shuffling imposes a limit on the maximum activity of antibody cocktails when co-expressed transiently using agroinfiltration.

3. It is possible to produce a complex mAb cocktail in *Nicotiana benthamiana* in a single co-agroinfiltration batch by exploiting the viral interference phenomenon (also known as superinfection exclusion). A plant-made mAb cocktail comprising 36 camel-derived humanized mAb selected against *Bothrops asper* snake venom toxins was shown to neutralize most toxic activities and to neutralize venom lethality *in vivo*.