UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI DIPARTIMENTO DI AGROBIOLOGIA ED AGROCHIMICA

CORSO DI DOTTORATO DI RICERCA
BIOTECNOLOGIE VEGETALI – XXIV CICLO

PhD Thesis TITLE:

PLANT PRODUCTION OF A TUMOUR-TARGETING HUMAN IgG. CONSIDERATIONS ON EXPRESSION STRATEGIES, GLYCOSYLATION PROFILE AND IN PLANTA PROTEOLYSIS

(BIO/15)

Coordinator: Prof. Stefania Masci

Signature ……..

Tutor: Dott. Marcello Donini

Signature……

PhD Student: Raffaele Lombardi

Signature ……..

1. **ABSTRACT**

Compared to other expression platforms, plants present several advantages for the expression of recombinant molecules represented by the ability to assemble complex multimeric proteins such as antibodies, the possibility to achieve post-translational modifications similar to those found in mammalian cells, low management costs and ease of scalability. Despite these evident advantages, some drawbacks need to be addressed.

One of the factors limiting plant expression systems is the downstream purification in which many elements could influence the effectiveness of the purification, such as low antibody concentration, extraction methodologies and purification strategy. Another important issue is represented by the plant typical glycosylation patterns. In fact, a major drawback in the application of plant-derived immunoglobulins for human therapy is indeed represented by differences between sugar moieties harbored by proteins expressed in plants and mammals. The presence of typical plant sugars has been demonstrated capable of inducing immunogenic responses in humans, leading also to a remarkable reduction of pharmacokinetic properties and biological activity of plant derived proteins. An additional drawback of recombinant antibodies obtained from plants is represented by the final yield that can be, in some cases, reduced by unintended proteolytic processees taking place in the plant cell. Particularly, in the case of antibody overexpression degradation may account for up to ninety percent reduction of the final purified product.

In this work, we describe the expression of the human recombinant tumour targeting mAb H10 in *Nicotiana benthamiana* using a transient expression system based on vacuum agroinfiltration, focusing our research efforts on optimizing expression and purification strategies, glycosylation profile and *in planta* proteolysis follow-up. The optimization of the extraction/purification steps was carried out evaluating different strategies in terms of antibody yield, degradation and level of contaminants in the final purified product. We optimized a pilot-scale purification method, using a two-step purification protocol starting from 250 g of fresh agroinfiltrated leaves, achieving an average yield of 40 mg per Kg fresh weight of intact IgG at a purity of > 99.4%, with endotoxin levels < 1 EU/ml, clearly free of any phenolic, alkaloid compounds. In addition, the glycosylation pattern of the plant produced mAb H10 was modulated evaluating the effects of the different glycan moyeties on antibody quality and final yield. Results suggest that, among all factors contributing to protein stability, glycosylation might play an important role. Finally, we characterised the degradation profile of the tumour targeting H10 antibody, identifying specific proteolytic cleavage sites of the plant produced immunoglobulin localized in the three inter-domain regions of the H10 heavy chain. Moreover, based on a bioinformatics analysis, three proteolytic enzymes were identified, as belonging to the cystein proteases family. Overall data indicate that possible ‘fragile’ sites in the H10 antibody reside in the hinge region, particularly prone to cleavage by the plant protease machinery. The identification of these cleavage sequences is a first step towards the development of new strategies to reduce antibody degradation in plants and to increase the yield of intact IgG molecules.

2. INTRODUCTION

2.1 Heterologous expression systems for the production of biopharmaceuticals

During the past decade the need for high quantities of correctly folded, pure and biologically active proteins for both applied biological research and medicine has increased constantly. Biopharmaceuticals based on recombinant proteins (antibodies, vaccines, antigens, hormones) are used in research diagnostic and in current medicines and have reached about 10 % of the pharmaceutical market value in 2007 (Lowe and Jones, 2007). With fast-growing demand of recombinant biopharmaceuticals, the capacity of the current production systems (yeast, microbes, animals cells and transgenic animals) is often not sufficient to respond to the market requirements (Obembe et al., 2011). Therefore, it is crucial to increase the manufacturing capacity of heterologous proteins to satisfy the existing request.

There are several examples of molecules expressed using different hosts, these include monoclonal antibodies as anti-tumour agents, enzymes or hormones for replacement therapies, cytokines and growth factors for *in vivo* or *in vitro* systems, antigens for anticancer treatments, and vaccines against infectious diseases. The main heterologous expression systems used to produce recombinant proteins are based on bacteria, yeast, insect and mammalian cells (Demain et al., 2009). Not all systems are able to yield a final product with the required characteristics in terms of biological functionality. In fact, biochemical modifications occurring in the cell (formation of disulfide bonds, glycosylation, proteolytic cleavage, phosphorylation, acetylation, myristylation), generically referred to as post-translational modifications, are essential for the synthesis of correctly folded and bioactive proteins (Pogue et al., 2011). The most used organisms for heterologous protein production are bacteria such as *Escherichia coli* and the gram-positive *Bacilli* strains. These recombinant protein expression systems are widely used in laboratory practices because of their high growth rate on inexpensive substrates (Saida, 2007). Moreover, these organisms present a well characterized genome, easy to manipulate with a large number of cloning vectors and mutant strains available. However, bacterial systems have also several drawbacks. Prokaryotic cells are not able to perform most post-translational modifications necessary for the correct assembly and functionality of many complex mammalian proteins (Terpe, 2006). In particular, the production of proteins with disulfide bonds is difficult to achieve since bacteria lack most of the enzymatic machinery for this post-translational modification, often leading to the formation of inclusion bodies. Besides, bacterial systems produce proteins lacking glycosylation, which is often required for biological activity of some classes of molecules (Li, 2011).

For this reason, yeasts are often used to express heterologous proteins that require glycosylation or disulfide bond formation for their correct folding and function (De Pourcq et al., 2010). Yeast combines the advantages of bacterial systems, such as rapid growth, scalability, reduced media cost and relative ease of genetic manipulation, with its capacity to perform typical eucaryotic post-translational modifications (Gasser et al., 2006). *Saccharomyces cerevisiae* is the most important host used for the expression of recombinant proteins (Sleep et al., 1991), but it is gradually replaced by methylotrophic yeasts, in particular *Pichia pastoris*, for their capacity to grow in high cell density fermentations, without the risk of accumulation of toxic levels of ethanol (Jeong et al., 2011). However, also yeast has several disadvantages primarily due to undesirable post-translational modifications which are different from those performed by mammalian cells. One of the post-translational modification which could affect the quality of protein products produced in these microorganisms, is represented by glycosylation. In fact, it has been shown that yeasts, in many cases, could produce an over-glycosylation of N-linked sugar chains, leading sometimes to the production of recombinant proteins with impaired biological activity. Besides, the presence of these sugars may cause also immunogenicity problems if the protein is used for human therapy (Demain et al., 2009). Another efficient strategy for the expression of recombinant proteins is represented by insect cells. The most important expression system is based on baculovirus expression vectors. Baculoviruses are a rod-shaped double strand DNA viruses able to infect insect cells. These viruses are able to direct the production of proteins at high levels in insect cells and this natural ability has been exploited to create recombinant expression vectors. The most commonly used vectors are derived from the nuclear polyhedrosis virus *Autographa californica* that naturally contains a circular double-stranded DNA. This virus is a common pathogen of lepidopterans, and can infect and replicate in cell suspensions of the fall armyworm *Spodoptera frugiperda* (Hitchman et al., 2009). The baculovirus expression system is capable of post-translational modifications similar to those occurring in mammalian cells, including phosphorylation, N- and O-glycosylation, correct cleavage of the signal peptide, acylation, myristoylation, carboxymethylation (Luckow and Summers, 1988). The possibility to clone the gene of interest under the control of the strong viral polyhedrin promoter, can produce high levels of recombinant proteins with reported yields of up to 30 % of total soluble proteins (TSP) (Morrow, 2007). However, also insect cell systems show some limitations such as the need to verify case by case the specific patterns of post-translational processing, the improper folding of some recombinant proteins and some differences in the N-linked glycosylation patterns compared to their mammalian counterpart.

Production of proteins requiring specific mammalian post-translational modifications is usually performed in mammalian expression systems that ensure correct glycosylation, folding, fatty acid chain addition and phosphorylation (Griffin et al., 2007). The first biopharmaceuticals produced in mammalian cell cultures have been the tissue plasminogen activator (tPA) and erythropoietin (EPO) in the 1980s (Swartz, 1996). Among the cell types commonly used for protein expression, such as NS0 murine myeloma cells (Andersen and Krummen, 2002), baby hamster kidney cells (BHK), green monkey kidney cells (Wrotnowski, 1998) and human hembryonic kidney cells (HEK), the most employed are the chinese camster ovary cells (CHO). The expression yield of recombinant proteins in mammalian cells has dramatically increased in the last decades principally due to improvements in the *in vitro* cultural conditions (Aldridge, 2006). There are several examples of high yield production of recombinant proteins using this cell system and at industrial scale protein titers have even reached levels of 10 g/L (Ryll, 2008). A new expression platform is represented by the human cell line PER.C6 that was reported to produce up to 26 g/L of a monoclonal antibody (Jarvis, 2008). Despite high yields obtained in mammalian cells, this expression system requires high culture costs and time-consuming maintenance, with the need of complex processing techniques coupled by a high risk of contamination by human pathogens (Pogue et al., 2002).

An alternative production system for the expression of recombinant proteins, is based on the use of transgenic animals. Initially designed with the aim of improving the characteristics of the breed, genetically modified (GM) animals have proved suitable for large-scale production of recombinant proteins in milk, blood and eggs (Houdebine et al., 2009). The main disadvantage of this strategy is linked to high costs and time consuming procedure to obtain transgenic animals as well as the risks associated to contamination of the final product with human pathogens (Sands et al., 2003). A summary of the features of the previously described expression systems is reported in **Table 1**.

Table 1: Comparison between heterologous protein expression systems. The table reports the main features of each expression systems in terms of growth, costs, expression levels and post translational modifications (adapted from Yin et al., 2007).

Host system	***Cell growth***	***Cost of medium***	***Expres-sion levels***	***Post-translational modifications***					
				Protein folding	**N-linked glycosyla-tion**	**O-linked glycosyla-tion**	**Phospho-rylation**	**Acetyla-tion**	**Acylatio n**
E. coli	Rapid 30min	Low	High	Refolding usually required	None	No	No	No	No
Yeast	Rapid 90min	Low	Low-High	Refolding may be required	High mannose (mostly)	Yes	Yes	Yes	Yes
Insect Cells	Slow 18–24h	High	Low-High	Proper folding	Simple, no sialic acid	Yes	Yes	Yes	Yes
Mammalian Cells	Slow 24 h	High	Moderate	Proper folding	Yes	Yes	Yes	Yes	Yes

2.2 Production of biopharmaceutical proteins in plants

Genetic modification of plants has been extensively used in the last 30 years for the expression of heterologous proteins, transforming plants into “biofactories” of biopharmaceutical products. The first examples of expression of therapeutic proteins in plants were the Human Serum Albumin (HAS) produced in tobacco and potato (Sijmons et al., 1990) and a monoclonal antibody expressed in tobacco leaves (Hiatt et al., 1989). These pioneering studies were the premises to the development of a new biotech area known as “molecular farming”. This term generally refers to the production of valuable recombinant proteins in plants (Schillberg et al., 2003). The capacity of plant cells to synthesize, process and target large complex mammalian proteins in a similar manner to their natural hosts makes them an attractive alternative system for the expression of heterologous proteins. Moreover, plants offer the possibility to produce proteins at lower costs compared to mammalian cell based bioreactor systems and their well established technologies for processing and harvesting favor the production scale-up of heterologous proteins in many crops (Mett et al., 2008). Different plant species have been used for the production of biopharmaceutical proteins in leaves and fruits (such as tobacco, tomato, alfalfa and lettuce) (Twyman et al., 2003), tubers (potato) or in seeds, including cereals (rice, wheat and maize) and legumes (pea and soybean) (**Table 2**) (Stoeger et al., 2000). Several crucial factors need to be considered when choosing a production crop including biomass yield per hectare, yield of recombinant protein per unit of biomass, ease of transformation, and scalability. An alternative expression platform to the use of transgenic plants is represented by plant cell suspensions. Plant cell cultures are commonly used for the production of small molecule drugs, but they are proving also advantageous in ‘molecular farming’ for the high-level of containment and the possibility of producing proteins under current good manufacturing practice (cGMP) conditions. Interesting examples are represented by the BY-2 tobacco cells, carrot cell cultures and the moss *Physcomitrella patens*. The latter perfectly suits the requirements for the production of complex biopharmaceuticals. In fact, this eukaryotic system offers an outstanding genetic accessibility and proteins can be produced safely in scalable photobioreactors without the need animal-derived medium compounds (Büttner-Mainik et al., 2011).

Biosafety issues and public acceptance are essential concerns for the successful use of plants to produce biopharmaceuticals. During the last years, it has become clear that transgenic food and feed plants producing biopharmaceuticals present a certain risk of spreading transgenes to wild type edible species increasing the possibility to contaminate food chain with unwanted heterologous products. Therefore, in the last years much effort has been focused on the use of non-food plants in molecular farming. Among the species used for heterologous protein expression, *Nicotiana tabacum*

is certainly the most used and best characterized (Ma et al., 1995). There are several advantages in the use of tobacco such as genetic manipulation, prolific seed production, high biomass yield and the existence of a well estabilished large-scale processing infrastructure (Ma et al., 2003).

Table 2: Some of the plant expression hosts used for biopharmaceutical production (adapted from Fisher et al., 2004).

Species	*Advantages*	*Disadvantages*
Model plants		
Arabidopsis thaliana	Range of available mutants, accessible genetics, ease of transformation	Not useful for commercial production (low biomass)
Simple plants		
Physcomitrella patens, Chlamydomonas reinhardtii, Lemna minor	Containment, clonal propagation, secretion into medium, regulatory compliance, homologous recombination in Physcomitrella	Scalability
Leafy crops		
Tobacco	High yield, established transformation and expression technology, rapid scale-up, non-food/feed	Low protein stability in harvested material, presence of alkaloids
Alfalfa, clover	High yield, useful for animal vaccines, clonal propagation, homogenous N-glycans (alfalfa)	Low protein stability in harvested material, presence of oxalic acid
Lettuce	Edible, useful for human vaccines	Low protein stability in harvested material
Cereals		
Maize, rice	Protein stability during storage, high yield, easy to transform and manipulate	
Wheat, barley	Protein stability during storage	Low yields, difficult to transform and manipulate
Legumes		
Soybean	Economical, high biomass, expression in seed coat	Low expression levels, difficult to transform and manipulate
Pea, pigeon pea	High protein content	Low expression levels
Fruits and vegetables		
Potato, carrot	Edible, proteins stable in storage tissues	Potato needs to be cooked
Tomato	Edible, containment in greenhouses	More expensive to grow, must be chilled after harvest

Another successful non-food plant used for the expression of high levels of recombinant proteins is represented by *Nicotiana benthamiana* especially used in transient expression systems. Despite its

reduced biomass, this plant is very suitable for large scale heterologous protein production due to its rapid growth and ease of manipulation (Marillonnet et al., 2005) (**Figure 1**).

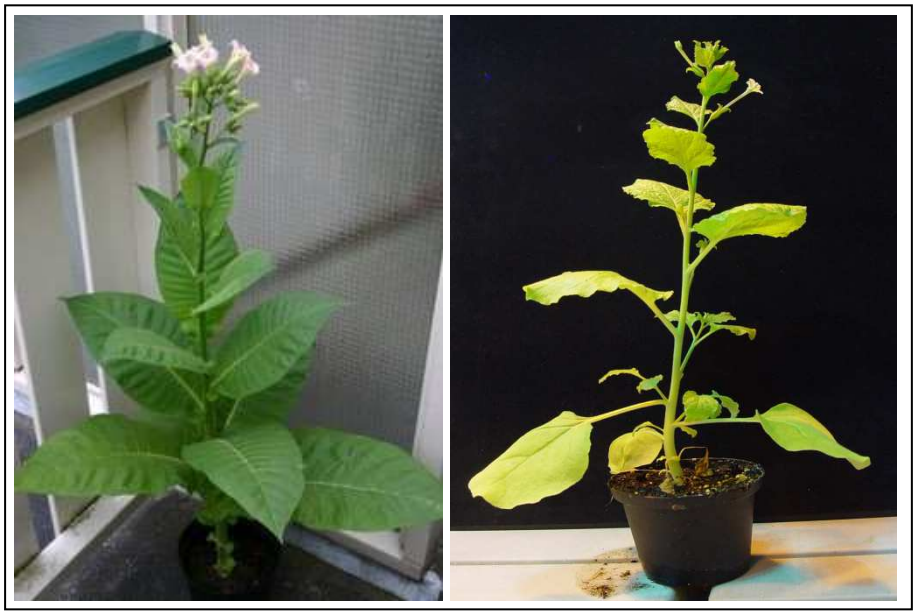


Figure 1: *Nicotiana tabacum* left picture; *Nicotiana benthamiana* right picture

An important advantage in the expression of proteins in plant is represented by the possibility to direct the molecules to a specific tissue or cell compartment using tissue specific promoters and signal peptides respectively. This allows the identification of optimal expression strategies in order to ensure high stability, high yields, and ease of purification.

For a certain class of proteins the Endoplasmic Reticulum (ER) represents the ideal cell compartment. The low concentration of proteases, the presence of numerous chaperones and the oxidizing environment for protein folding and disulfide bond formation, ensures a correct protein conformation (Petruccelli et al., 2006). ER retention, obtained by the addition of a signal peptide to the recombinant protein has been shown to increase the accumulation levels of many recombinant molecules (Pogue et al., 2010). In addition, retention in this cell compartment avoids the addition to the recombinant protein of typical plant sugars, favoring the formation of a “human like” glycosylation profile. In fact, it has been demonstrated that the presence of typical plant sugars attached the recombinant protein could induce, in some cases, adverse effects in human administration, reducing the therapeutic value of the final product (Gomord et al., 2010).

A further advantage of plant expression is related to the safety of the final product. In fact, while the use of mammalian cell cultures involves the risk of contamination by pathogens harmful to human

health, plant expression systems minimize the presence of human pathogens and guarantee high safety in their use (Pogue et al., 2010).

Two main strategies are used to express proteins in plants based either on stable genetic transformation or on transient expression technologies.

2.3 Stable transformation

Stable genetic transformation of plants allows to insert a gene, coding for a protein of interest, in the nuclear or chloroplast genome. The heterologous gene, inserted in the genome of the plant becomes an hereditary character. The stable transformation has been achieved using two main strategies represented by the *Agrobacterium tumefaciens*-mediated transformation and the "gene gun" technology (Pogue et al., 2002).

Attenuated and engineered *A. tumefaciens* is the most generally used organism to transform a variety of dicots and some monocots (Lessard et al., 2002). This soil borne bacterium has the natural capability to integrate a fragment of DNA (T-DNA contained in the Ti plasmid) in the genome of host cells (Escobar and Dandekar, 2003). Exploiting this innate capacity of the bacterium, it is possible to replace the DNA sequence between the left and right border of the T-DNA segment with sequences of interest, without affecting its transformation ability (Lorence and Verpoorte, 2004) (**Figure 2**). In this way the gene coding for a protein of interest can be inserted in the nuclear genome leading to the expression of the protein of interest.

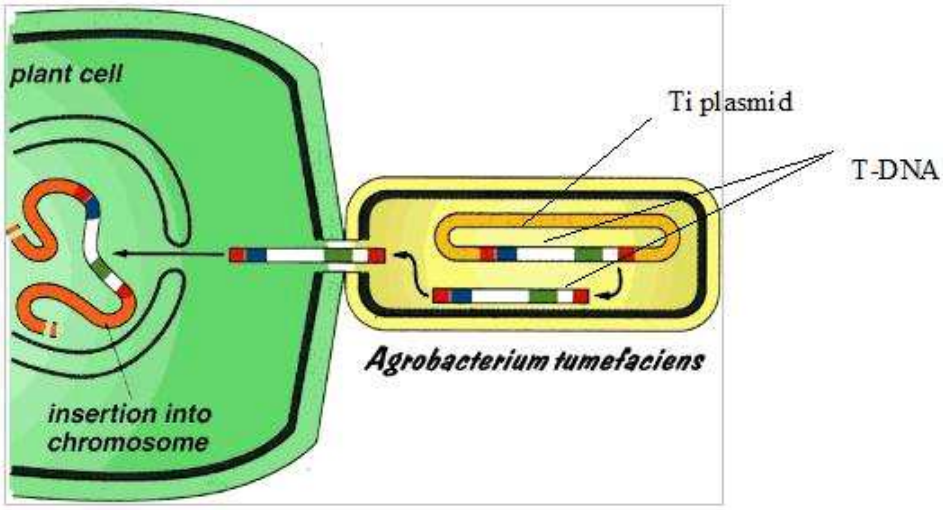


Figure 2: *Agrobacterium tumefaciens* infection mechanism. *A tumefaciens* transfers a copy of T-DNA to the plant nucleus making possible the expression of the protein of interest. (From http://www.plantsci.cam.ac.uk)

This method has excellent advantages such as ease of manipulation, high efficiency of insertion and possibility to transfer long DNA sequences (Krishnamohan et al., 2001). The extensive use of procedures for the *Agrobacterium*-mediated transformation of tobacco leaf disks have made the regeneration of transgenic tobacco lines a routine laboratory procedure (Horsch et al., 1986).

The main limitation of the stable transformation mediated by *A. tumefaciens* is the narrow host spectrum of this plant pathogen that does not include plants of commercial interest such as cereals. For these plants, the transformation of the nuclear genome can be obtained using the "gene gun" technology which involves the bombardment of the tissue to be transformed with microparticles of inert metal (gold or tungsten) to which the DNA sequence of interest is adsorbed. Once in the plant cell, DNA is released from the surface of the microparticles and occasionally transferred and integrated into the genome (Predajňa et al., 2010).

One of the main problems associated with the stable transformation mediated by *A. tumefaciens* or gene gun is related to the randomness with which the transgene integrates into the host genome. In fact, integration could take place in non transcriptional regions of the genome, leading to low expression levels of the heterologous protein.

An alternative method for stable transformation of plants is based on the integration of the exogenous gene in the chloroplast genome (Cardi et al., 2010). The transformation of this organelle can be achieved delivering the heterologous DNA by the use of the gene gun technology. After the integration in the chloroplast genome, due to the polyploid nature of plastids, the primary regenerants are usually in a heteroplasmic state containing a mixed population of wild-type and transformed plastids. The homoplasmic state (in which every chloroplast carries the transgene), with a homogeneous population of transformed plastids, is achieved after a sufficient number of cell divisions under high selective pressure (Scotti et al., 2011). This organelle is particularly attractive for the expression of recombinant proteins, not only because of its ease of manipulation, but also because of the high transcriptional capability due to the large number of copies present in each cell (each plant cell contains from 50 to 100 chloroplasts). In fact, it has been shown that chloroplast transformation allows expression levels of up to 46 % of the total soluble proteins (TSP) in the case of the insecticidal protein Cry B, leading to the folding of cuboidal protein crystals in the chloroplast lumen (De Cosa et al., 2001; Petersen and Bock, 2011) (**Figure 3**).

Figure 3: Electron micrograph showing the presence of the insecticidal protein Cry2Aa2 form *Bacillus thuringiensis* (Bt) Cry folded into cuboidal crystals in the chloroplast lumen (De Cosa et al., 2001).

Table 3: Overview of the immunotherapeutic molecules and enzymes recently produced in plant chloroplasts (From Scotti et al., 2011).

Protein	*Source of transgene*	*Plant species*	*Protein level*	*References*
p24-Nef	HIV-1	Tobacco	40 % TSP	Zhou et al., 2008
Pr55 gag	HIV-1	Tobacco	7–8 % TSP	Scotti et al., 2009
p24	HIV-1	Tobacco (Maryland Mammoth)	4.5 % TSP	McCabe et al., 2008
L1-2xCysM (Mutated L1)	HPV-16	Tobacco	1.5 % TSP	Waheed et al., 2011
E7-CP	HPV-16	Tobacco	0.5 % TSP	Morgenfeld, 2009
F1-V	*Yersinia pestis*	Tobacco	14.8 % TSP	Arlen et al., 2008
VP-βGUS	Footh-and-mouth disease virus	Tobacco	51 % TSP	Lentz et al., 2010
CTB-AMA1	*Vibrio cholerae-Plasmodium falciparum*	Tobacco Lettuce	13.2 % TSP 7.3 % TSP	Davoodi-Semiromi et al., 2010
CTB-MSP1	*Vibrio cholerae-Plasmodium. falciparum*	Tobacco Lettuce	10.1 % TSP 6.1 % TSP	Davoodi-Semiromi et al., 2010
LTB-STa	*Escherichia coli*	Tobacco	2.3 % TSP	Rosales-Mendoza et al., 2009
CTB-proinsulin	Human	Tobacco	47 % TSP	Boyhan and Daniell, 2010
Insulin like growth factor-1	Human	Tobacco	32.7 % TSP	Daniell et al., 2009a
Cardiotrophin-1	Human	Tobacco	4.8 % TSP	Farran et al., 2008
t1:9 Aprotinine	Bovine	Tobacco	0.5 % TSP	Tissot et al., 2008
t1:10 Alpha1-antitrypsine	Human	Tobacco	2 % TSP	Nadai et al., 2009
t1:11 CTB-F.IX (Coagulation factor IX)	Human	Tobacco	3.8 % TSP	Verma et al., 2010
t1:12 Thioredoxin 1	Human	Lettuce	1 % TSP	Lim et al., 2011
Endo-β-1,4-glucanase E1 catalytic domain	*Acidothermus celluloyticus*	Tobacco	12 % TSP	Ziegelhoffer et al., 2009
Endoglucanase Cel6A	*Thermobifida fusca*	Tobacco	10.7 % TSP	Gray et al., 2009
1-Deoxy-D-xylulose reductoisomerase	Synechosystis sp. Strain PCC6803	Tobacco	7.4 % total stroma protein	Hasunuma et al., 2008
Δ9 desaturase	*Solanum commersonii* and *Anacystis nidulans*	Tobacco	NR	Craig et al., 2008
L-aspartate-α-decarboxylase	*Escherichia coli*	Tobacco	NR	Fouad and Altpeter, 2009
Chitinase BjCHl1	*Brassica juncea*	Tobacco	NR	Guan et al., 2008
β-glucosidase BglC	*Termobifida fusca*	Tobacco	12 % TSP	Gray et al., 2011

NR: Non Reported, TSP: Total Soluble Proteins

Despite the advantages in terms of expression levels, the chloroplast lacks enzymes responsible of the post-translational modifications which are necessary for the expression of active mammalian proteins. Furthermore, another drawback of this strategy is represented by the time-consuming processes needed to achieve the plant homoplastomic chloroplast transformation. A summary of immunotherapeutic molecules and enzymes recently produced in plant chloroplasts is reported in **Table 3**.

Another interesting methodology for the expression of recombinant proteins is based on genetic engineering of plant cell suspension cultures to be used in bioreactor based systems. The advantages of this system are both the possibility to guarantee the expression of proteins in sterile conditions coupled with a high-level of containment and the cost-effective downstream processing and purification given by the absence of many plant cell structural elements (Franconi et al., 2010). In addition, this technology is quite fast since stable cell lines can be generated in two-three months (Shaaltiel et al., 2007; Aviezer et al., 2009). Plant as *N. tabacum*, *N. benthamiana*, carrot, rice cell cultures, the aquatic plant *Lemna minor* (duckweed) and the moss *Physcomitrella patens*, have been considered for the expression of several biopharmaceuticals that need contained growth conditions.

Examples of the production of recombinant proteins in bioreactors include the use of tobacco cell cultures to produce the veterinary Newcastle virus vaccine (Miller et al., 2009) and the carrot cells for the expression of the high-value therapeutic protein glucocerebrosidase (Aviezer et al., 2009) which is in fast track for U.S. FDA approval (Xu et al., 2011). Nevertheless, the production of heterologous proteins using this system presents several disadvantages such as difficulties in protein recovery and purification, low accumulation levels (10–250 mg/L) of secreted products, plant typical glycosylation profile of produced molecules, degradation phenomena of proteins secreted in the media (Hellwig et al., 2004) and higher costs in comparison to open-field and green house management. Some examples of proteins expressed in plant cell suspension cultures are reported in **Table 4**.

An alternative to the use of plant cell suspensions is represented by the natural rhizosecretion mechanism of the plant, characterized by the release of proteins from roots (Drake et al., 2009). Plants rhizosecrete several molecules involved in many plant physiological mechanisms such as nodulation, mycorrhizal colonization, growth inhibition of neighbouring plants, acquisition of nutrients from soil and in defense against toxic metals (Gleba et al., 1999).

Table 4: Example of recombinant proteins expressed in seeds, leaves or suspension cultures (Adapted from Wilken et al., 2011).

Platform	*Recombinant protein*	*Application*	*Product concentration*	*Reference*
Corn seed	Aprotinin	Protease inhibitor	0.7 µg/mL TSP	Azzoni et al., 2002
Corn seed	(defatted germ)	Collagen	120 mg/kg DW	Zhang et al., 2009
Corn seed	IgG (2 G12)	Anti-HIV	75 mg/kg DW	Ramessar et al., 2008
Rapeseed	Chymosin	Industrial / food enzyme	4.2 % TSP (47 mg/kg FW)	Van Rooijen et al., 2008
Rice seed	Human lysozyme	Gastrointestinal infections, formula and cell culture	4.4 mg/g FW (3.3 mg/g FW for selected extraction conditions)	Wilken and Nikolov, 2006
Rice seed	Human lactoferrin	Gastrointestinal infections, formula and cell culture	6 mg/g FW (4.2 mg/g FW for selected extraction conditions)	Nandi et al., 2005
Tobacco leaves	Aprotinin	Protease inhibitor	~750 mg/kg FW (greenhouse), ~300 mg/kg FW (open field)	Pogue et al., 2010
Tobacco leaves	β-Glucuronidase	Model protein	1.4 mg/mL TSP	Holler and Zhang, 2008
Tobacco leaves	Influenza virus like particles (H5-VLP)	Avian influenza vaccine (A/H5N1)	NR	D'Aoust et al., 2010
Tobacco leaves	IgG (patient 69)	Idiotype vaccines for non-Hodgkin's lymphoma	1.5 g/kg FW	Bendandi et al., 2010
Tobacco leaves	IgG	Anti WNV	0.8 g/kg FW	Lai et al., 2010
Tobacco leaves	IgG (H10)	anti tenascin C	600 mg/kg FW	Lombardi et al., 2010
Carrot suspension cell culture	Glucocerebrosidase (intracellular)	Gaucher's disease	NR	Shaaltiel et al., 2007
Lemna fronds	IgG1 (anti-CD 30)	Non-Hodgkin's lymphoma and anaplastic large-cell	2 % of TSP	Cox et al., 2006, Nikolov et al., 2009
Lemna fronds	IgG1 (CNTD)	NA	50 % of TSP at pH 4.5; 10 % TSP at pH 7.5	Woodard et al., 2009
Tobacco suspension cell culture	Anti-rabies mAb (intracellular)	Human anti-rabies virus mAb for treatment	3–10 mg/Kg leaf FW	Girard et al., 2006
Tobacco suspension cell culture	Anti-rabies mAb (intracellular)	Human anti-rabies virus mAb for treatment	250 mg/Kg FW	Girard et al., 2006
Tobacco suspension cell culture	GFP-fusion protein (intracellular)	Reporter tag	10.7 mg TSP	Peckham et al., 2006

FW, Fresh Weight; DW, Dry Weight; NR, not reported; TSP, total soluble protein.

Many studies have been carried out showing the high capability of transgenic *N. tabacum* plants to rhyzosecrete different recombinant proteins such as green fluorescent protein (GFP), bacterial xylanase, human placental alkaline phosphatase (SEAP), monoclonal murine IgG1 antibody Guy's 13 and the cyanobacteria anti HIV-1 Microcystin protein (Drake et al., 2003, Drake et al., 2009).

All the secreted proteins showed conserved biological functionality and accumulated in higher amounts in the medium than in the root tissue. One of the advantages offered by this strategy is represented by the possibility to produce the protein of interest in a controlled environment with low levels of plant contaminants and a low concentration of proteases.

The reduced concentration of plant contaminants (e.g. poliphenols, alkaloids) in the culture medium reduces the difficulty of the downstream purification processes, favoring the purification of a high quality final product. Although rhizosecretion remains a promising platform for the expression of recombinant proteins, the main disadvantage of this technology is represented by the relatively low yields of recombinant proteins. Various strategies have been used to increase the production yield, the most successful is represented by the exposure of roots to plant growth regulators which increases secretion of the recombinant product (Drake et al., 2009).

Among other strategies used to increase the production yield in transgenic plants, the targeting and accumulation of heterologous proteins in seeds demonstrated to be a valid approach. There are several examples of molecules successfully produced in plant seeds which include cytokines (Zhu et al., 1994), therapeutic monoclonal antibodies (Petruccelli et al., 2006), industrial enzymes (Hood et al., 2007) and antigens (Streatfield and Howard, 2003; Wu et al., 2007). In 2008 the United States Department of Agriculture (USDA) approved a field release of transgenic seeds expressing human lysozyme, lactoferrin, and serumalbumin in rice, apolipoprotein in safflower, and hepatitis B surface antigen and brazzein in corn (Wilken et al., 2011). This expression system results to be a very useful platform since recombinant proteins can accumulate in a naturally desiccant storage compartment with reduced levels of proteolytic enzymes, ensuring protein stability for long periods of time (**Table 4**) (Fischer et al., 2009; Stoger et al., 2005). Many plant-derived proteins obtained with some of the previously described strategies have been included in clinical trials. A summary of these molecules is reported in **Table 5**.

Table 5: Proteins expressed in different plant systems included in clinical trial or on market (adapted from Obembe et al., 2010).

Product	*Disease*	*Plant*	*Clinical trial status*	*Company*	*Source: URL/academic*
Gastric lipase, Merispase®	Cystic fibrosis	Maize	On market	Therapeutics France Meristem	http://www.meristem-therapeutics.com
α-Galactosidase	Fabry disease	Tobacco	Phase I	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
Lactoferon™ (α-interferon)	Hepatitis B and C	Duckweed	Phase II	Biolex, USA	http://www.biolex.com/
Fibrinolytic drug (thrombolytic drug)	Blood clot	Duckweed	Phase I	Biolex, USA	http://www.biolex.com/
Human glucocerebrosidase (prGCD)	Gaucher ‘s disease	Carrot suspension cells	Awaiting USDA's approval	Protalix Biotherapeutics, Israel	http://www.protalix.com/glucocerebrosidase.html
Insulin	Diabetes	Safflower	Phase III	SemBioSys, Canada	http://www.sembiosys.com/
Apolipoprotein	Cardiovascular	Safflower	Phase I	SemBioSys, Canada	http://www.sembiosys.com/
Nutraceuticals ISOkine™ DERMOkine™	Human growth factor	Barley	On market	ORF Genetics	http://www.orfgenetics.com
Human intrinsic factor	Vitamin B12 deficiency	Arabidopsis	On market	Cobento Biotech AS	http://www.cobento.dk
Human lactoferrin	Anti-infection, anti-inflammatory	Rice	Advanced, on market as fine chemical	Ventria, USA	http://www.ventriabio.com/
Human lysozyme	Anti-infection, anti-inflammatory	Rice	Advanced, on market as fine chemical	Ventria, USA	http://www.ventria.com/
Immunosphere™	Food additive for shrimps	Safflower	Marketing expectedfor 2010	SemBioSys, Canada	http://www.sembiosys.com/

2.3.2 Transient transformation systems for the high yield accumulation of heterologous proteins in plants

Protein production using transient expression systems is a strategy that involves the use of pathogenic agents (bacteria, viruses) to deliver DNA sequences coding for a protein of interest in the genome of plant cells. The gene of interest is not transferred to the offspring of the plant, but the expression is limited to a particular stage of the plant life cycle and confined only in the infected tissues (Fischer et al., 1999). Protein production obtained with transient expression systems is perhaps the most suitable technology for plant molecular farming (Rybicki, 2010). These systems which have been normally used in molecular biology for quick validation of expression constructs are now being used routinely for the production of considerable amounts of proteins within a few

weeks (Villani et al., 2009, Lombardi et al., 2010). The use of transient expression strategies presents many advantages in comparison to stable genetic transformation. In fact, this technology ensures the expression of high levels of heterologous protein in a short time-period, usually within one or two weeks. Moreover, the system offers the possibility to scale up the protein production extending this technology to different plant species. This strategy does not imply vertical or horizontal gene transfer and allows to operate in a contained environment (Santi et al., 2009). Transient expression systems are mainly based on the use of *A. tumefaciens* T-DNA-based expression cassettes or viral vectors. T-DNA expression cassettes vectors are based on DNA modules in which the gene of interest is controlled by strong promoters (e.g. 35S Tobacco mosaic virus (TMV) promoter or beta-phaseolin promoter) and other transcriptional enhancer components (transcriptional enhancer or strong transcription terminators) (Gleba et al., 2007). On the other hand, viral vectors can be classified, based on their construction, in first and second generation vectors. In the case of first generation vectors, constructs consist essentially in whole functional viruses carrying in their genome the gene of interest as an additional ORF ("full virus" strategy) (Pogue et al., 2010). Vectors belonging to the second generation, rather than using complete viral genomes, are based on the construction of an integrated system in which only the viral elements (strong promoters, terminators, enhancers, silencing suppressor genes) required for efficient expression of the sequence of interest are maintained in the expression vector ("deconstructed virus" strategy) (Gleba et al., 2007; Sainsbury et al., 2009).

2.3.2.1 Protein expression using T-DNA vectors delivered by A. tumefaciens

These vectors comprise a basic structure composed of two parts: a T-DNA containing multiple cloning sites, a selectable marker gene for transformed plant cells, a gene of interest and a vector backbone carrying plasmid replication components for *E. coli* and *A. tumefaciens*, selectable marker genes for bacteria and optionally genes encoding plasmid mobilization functions (Lee et al., 2008). These vectors are delivered to the plant cells through the use of a delivery system (Agroinfiltration) exploiting the infective *Agrobacterium tumefaciens*. Agroinfiltration consists in the use of *Agrobacterium* to mediate the transfer of the gene of interest carried in T-DNA vectors to the genome of all plant

Figure 4: Small scale representation of agroinfiltration performed by syringe. The *Agrobacterium* medium is forced to enter the mesophyl through the inferior leaf page after the execution of a little wound.

cells. In this methodology, *Agrobacterium* solution is forced to enter the leaf mesophyl either applying the solution with a needless syringe through the inferior page of the leaf (mechanical injection) (**Figure 4** and **Figure 5 B)** or through the so called vacuum agroinfiltration process in which whole plants are submerged in the *Agrobacterium* solution where vacuum is applied and then suddenly released (**Figure 5** panel **A**). This causes the entrance of the solution in the apoplastic space of the leaves. Depending on the vector used, the host organism and the *Agrobacterium* strain, the expression of the recombinant protein requires from 4 to 10 days and, based on the heterologous gene, it is possible to obtain several grams of recombinant protein per kg of fresh leaf biomass or over 50 % of total soluble protein. In addition, since the vector is composed only by few components it can contain long DNA sequences (up to 2.3 kb inserts or up to 80 kDa proteins) (Desai et al., 2010).

This Agroinfiltration system has been employed to accumulate high levels of several recombinant proteins such as reporter genes (e.g. green fluorescent protein and β-glucuronidase) (Simmons and VanderGheynst, 2007), antigenic proteins (Lombardi et al., 2009) and complex multimeric molecules such as immunoglobulins (Villani et al., 2009, Kathuria et al., 2008). A major drawback of this strategy is represented by post-transcriptional gene silencing (PTGS) response that may take place in the infiltrated plant tissue, in the presence of an high number of transcripts coming from the heterologous gene expression (Johansen et al., 2001). To overcome this limitation and enhance transient expression levels, the protein of interest has been co-agroinfiltrated with a second *Agrobacterium* harbouring a gene coding for a viral protein suppressor of gene silencing. An example is represented by the use of the P19 gene silencing suppressor protein from Artichoke mottled crinckle virus and Tobacco bushy stunt virus able to block the silencing mechanism and enhance expression yields of a range of proteins (Voinnet et al., 2003; Lombardi et al., 2009; Circelli et al., 2010; Sainsbury et al., 2010).

2.3.2.2 Protein expression using viral vectors

Viral vectors based on the use of the whole viral genome (first generation vectors), or deconstructed genome elements able to enhance the transcription and translation mechanisms (second generation vectors), are widely used to obtain high levels of recombinant proteins in plants. In the case of the first generation vectors, in order to obtain high expression levels, the heterologous gene is put under the control of a sub-genomic promoter, in place of the gene coding for the coat protein or as a fusion with the coat protein itself (e.g. for the expression of small protein fragments such as immunogenic epitopes). Among the viral genomes, RNA viruses are generally preferred for their

higher plasticity and stability. Recombinant expression vectors containing complete viral genomes allowed the transcription and translation of heterologous genes with high effectiveness (Awram et al., 2002; Gleba et al., 2008; Lico et al., 2008). These viral vectors are inoculated as virus particles or viral RNA and utilize the natural ability of cell-to-cell and systemic movement of the original virus to spread within the whole plant. Several plant viruses have been used to achieve this strategy among which Potato Virus X (PVX), Tobacco mosaic virus (TMV), Cauliflower mosaic virus (CaMV), Cowpea mosaic virus (CPMV) (Pogue et al., 2002; Lico et al., 2008). The first protein that has been expressed by transient expression using plant viruses is the enzyme dihydrofolate reductase (DHFR). In this case, the DHFR gene was inserted in the CaMV DNA genome as additional ORF under the control of a sub-genomic promoter (Brisson et al., 1984). These systems offer rapid and efficient expression characteristics, new genes can be tested for expression and test quantities of the recombinant protein can be obtained in as little as 4–8 weeks (Pogue et al., 2002).
One of the drawbacks of this approach is the impossibility to insert long DNA sequences (larger than 1 kb), which may cause problems of virus assembly and systemic movement. Moreover, many vectors cannot infect all harvestable parts of the plant and the spread of the virus is asynchronous in different leaves. In addition, in some cases the vector is usually unstable, thus not all infected tissues express the protein of interest (Lico et al., 2008). Besides, the use of whole infective virus particles includes also several bio-safety problems. In fact, the direct use of whole transgenic viruses presents the risks of their spreading in the environment with dramatic consequences in terms of transgene vertical transfer, recombinant protein accumulation in the ecosystem and contamination of food and feed chains. Therefore, the employ of this system requires an appropriate green-house containment level leading to the increase of the management costs (Pogue et al., 2002).
In the last decade, in order to solve the problems related to the limitations offered by first generation vectors, a second generation of viral vector was introduced. In this case, the expression vector consists in an integrated system of viral components able to ensure high expression of the protein of interest without the formation of infective virus particles. These vectors are unable to perform plant cell-to-cell or systemic movement in plant and require a system that ensures their delivery in all plant tissues and this is generally obtained using the Agroinfiltration process. The most known second generation viral vector-based transient expression system is the MagnICON® technology. This technology is based on the use of a TMV vector obtained with the “deconstructed virus technology”. This strategy is based on the efficient assembly of DNA modules (pro-vectors) obtained by recombination in plant cells (Komarova et al., 2010). Moreover, this system offers enhanced versatility and efficiency due to the possibility to vary the gene combinations, the

targeting signals and tags that can be tested without engineering each individual construct (Engler et al., 2009). This pro-viral expression platform proved to be very efficient for high-yield production of different proteins (> 10 % TSP) and is widely used in many laboratories.

A significant aspect of this technology is the possibility to express complex multimeric proteins by simultaneous infection of the same tissues with several strains of *A. tumefaciens*, each harbouring a different cassette with genes coding for the single monomers. An example of complex protein assembly is represented by monoclonal antibodies (Ma et al., 2005) as the full size A5 mAb (Giritch et al., 2006) and the *Yersinia pestis* antigens fusion protein F1–V (Rosales-Mendoza et al., 2010). Another interesting strategy used for the expression of multiple polypeptides is represented by the CPMV-HT system in which the expression cassettes coding for different monomers and the sequence encoding P19 gene silencing suppressor protein have been incorporated into a single T-DNA, allowing the high-level expression obtained through agroinfiltration of a single construct. The high production speed offered by this system makes CPMV-based expression vector an attractive alternative for biopharmaceutical development and production (Sainsbuty et al., 2010).

Some examples of immunotherapeutic proteins expressed through the transient expression system based on viral vectors are reported in **Table 6**.

Table 6: Yield of recombinant molecules of immunological interest obtained by plant virus-mediated transient expression systems.

Virus vector	***Type of inoculation***	***Pharmaceutical protein***	***VLP***	***Highest yield***
TMV	TMV-30B-based vector-directed RNA transcript inoculation of spinach leaves	HIV-1 Tat protein (86 aa)	No	0.3–0.5 mg/g FW
TMV	TMV-30B-based vector-directed RNA transcript inoculation of *N. benthamiana* leaves	HPV-16 L1 protein (531 aa)	Yes	~0.03 x 10-3 mg/g FW
TMV	ToMV-TocJ-based vector directed RNA transcript inoculation of *N. benthamiana* leaves	The dengue virus envelope protein (102 aa fragment)	No	~0.1 mg/g FW
TMV	*N. benthamiana* leaf agroinfiltration	*Mycobacterium tuberculosis* Ag85B and ESAT6 proteins (342 aa)	No	0.8–1.0 mg/g FW
TMV	TMV TTO1A vector-directed RNA transcript inoculation of *N. benthamiana* leaves	Human scFv proteins (~30 kDa) derived from human tumor immunoglobulin	No	100–800 μg/ml of leaf interstitial fluid

		genes of non-Hodgkin's lymphoma patients		
TMV	TMV launch vector pBID4 agroinfiltration of *N. benthamiana* leaves	hGH (~30 kDa)	No	0.7 mg/g FW
TMV and PVX	*N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anticancer full-size mAb (~160 kDa)	No	100–300 μg/g FW, affinity purified
TMV and PVX	MagnICON: *N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anticancer full-size mAb (~160 kDa)	No	0.3–0.5 mg/g FW, affinity purified
TMV and PVX	MagnICON: *N. benthamiana* leaf agroinfiltration with two populations of recombinant *Agrobacterium* encoding the mAb light and heavy chains	Anti-West Nile virus mAb (~160 kDa)	No	0.8 mg/g FW affinity purified
TMV	MagnICON: *N. benthamiana* leaf agroinfiltration	*Yersinia pestis* F1 (362 aa) and V (150 aa) antigens	No	0.3–0.5 mg/g FW; 1.2 mg/g FW (purified product)
TMV	MagnICON: *N. benthamiana* leaf agroinfiltration	VV B5 (275 aa) antigenic domain	No	0.1 mg/g FW
TMV	MagnICON: *N. benthamiana* leaf agroinjection	*Plasmodium* antigen PyMSP4/5 (33 kDa)	No	1–2 mg/g FW
PVX	Inoculation of *N. benthamiana* leaves with pPVX201 vector-based cDNA construct	HBV nucleocapsid protein (21 kDa)	Yes	0.005–0.01 mg/g FW
PVX	Agroinfiltration of *N. benthamiana* leaves with pPVX201-based vector cDNA construct	*M. tuberculosis* ESAT6 antigen fusion with PVX CP (31 kDa)	No	0.5–1 % of the TSP
PVX and TMV	*N. benthamiana* leaf agroinfiltration	GFP fused with CAV VP1, VP2 and VP3 (40–51 kDa)	No	1.2–5.4 % of the TSP
BeYDV	*N. benthamiana* leaf agroinfiltration	HBc (21 kDa) and NV CP (58 kDa)	Yes	HBc: 0.80 mg/g FW; NV CP: 0.34 mg/g FW

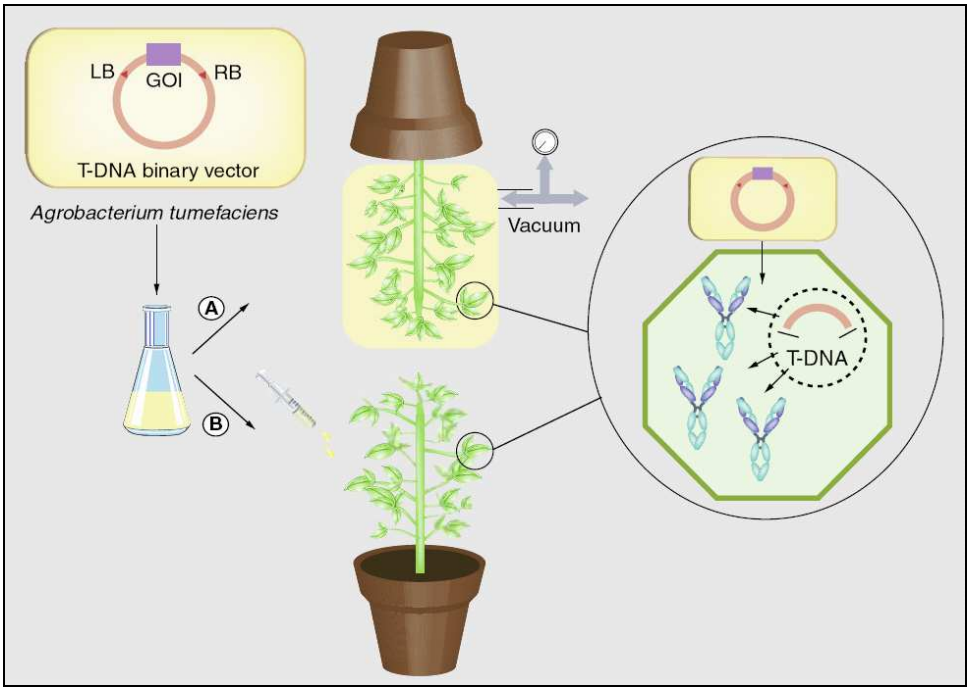


Figure 5: Schematic representation of plant Agro-infiltration system used for recombinant antibody transient expression. *Agrobacterium tumefaciens* strain transformed with the T-DNA binary vector bearing the gene of interest (GOI) is used to infiltrate plant leaf tissue Agrobacteria are forced in the intercellular space of plant tissue. Leaves of greenhouse-grown plants may be infiltrated either by a needleless syringe (**a**) or by vacuum infiltration (**b**). Antibody expression can be directed in different sub-cellular compartment. LB: T-DNA left border; RB: T-DNA right border.

All the expression systems described before rely on the use of the plant agroinfilitration process. This technique, as previously introduced, can be performed both on small scale (**Figure 4, Figure 5 B**) or at large scale by vacuum agroinfiltration (**Figure 5 A**). In the last years the efforts of several companies operating in molecular farming allowed to scale up the vacuum agroinfiltration strategy in order to perform the infiltration of kilograms of plants per hour and lead to the accumulation of 25–75 g of antibody per greenhouse batch using the MagnICON vectors (**Figure 6**, Pogue et al., 2010). The facility was calculated to be able to process up to 750 kg of plant biomass in an 8 hours production cycle, depending to the plant growth conditions rendering plant transient expression very competitive to traditional bio-fermentor based systems. An alternative to the agroinfiltration method has been recently presented by Azhakanandam et al., (2007). This system provides an optional *Agrobacterium* delivery in plants of *N. benthamiana*, via 'agrospray' method. This strategy is obtained spraying an *Agrobacterium* solution containing surfactants agents directly on the plant leaf surface, in order to enhance the entrance of the bacterium through the abrasions caused by surfactants. The use of this methodology should reduce the agroinfiltration costs ensuring comparable expression levels of the recombinant protein.

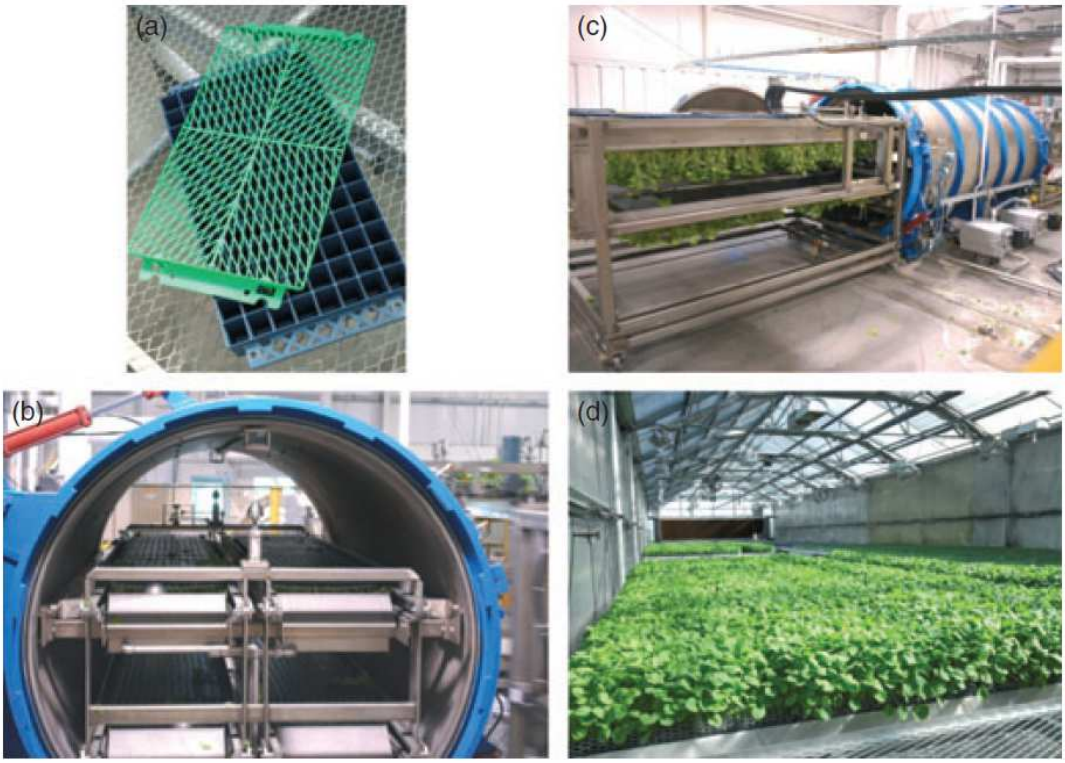


Figure 6: Representation of the "at scale" agroinfiltration system set up by KBP company. (a) Trays used for the seeding designed with special lids that favorite the growth providing a barrier for soil and root components. After growing, plant trays are loaded on conveyors in order to enter the vacuum chamber (b). After a rotation of 180°, conveyors enter the chamber (c) and plants are submerged in *Agrobacterium*-containing solution. At this stage vacuum is applied and suddenly released. After vacuum agroinfiltration plants are transferred in a green house in order to favor the expression of the recombinant protein accumulation (d) (from Pogue et al., 2010).

2.4 Expression of Antibodies in Plants

2.4.1 Advantages and drawbacks of plant expression systems for antibody production

Monoclonal antibodies (mAbs) are useful tools in medicine, biology and biochemistry because of their binding specificity and stability both *in vivo* and *in vitro*. Antibodies generated by hybridoma technology are widely used in medical research and disease diagnosis. Mammalian cell cultures are still the favored systems for the production of commercial mAbs, even if the increasing demand and the high costs are encouraging the development of alternative expression platforms. In fact, the facilities required for large scale production using mammalian cells necessitate long time to be set and their building and maintenance costs are extremely high, while validation under Good Manufacturing Practice (GMP) still requires an average of 3 years following construction (Vézina et al., 2009). Among alternative expression systems, plants represent ideal bioreactors for the large

scale over-expression of antibodies. In fact, plants offer a cost effective expression platform, (plant-derived immunoglobulins estimated cost are 0.1–1 % of the production cost in mammalian cell culture and 2–10 % in bacteria systems (Chen et al., 2005), that can assemble such complex multimeric molecules with high quality (Conrad and Fiedler, 1994). Many antibody formats have been successfully produced in plants so far such as Fab fragments, scFvs, bispecific Fvs, diabodies, minibodies, single variable domains, antibody fusion proteins, large single-chain antibodies and camelid heavy-chain antibodies (**Figure 7**). These recombinant antibodies have been expressed in plants for different purposes such as modulators of plant cell processes (Owen et al., 1992), to confer virus resistance (Villani et al., 2005), for phytoremediation (Longstaff et. al, 1998; Drake et al., 2002) and for the production of valuable immunotherapeutic agents (Ma et al., 2003; Hull et al., 2005; Ko and Koprowski, 2005; Ramessar et al., 2008; Villani et al., 2009).

Another advantage of the production of antibodies in plants is represented by the possibility to direct the protein expression in specific cell compartments. Most antibodies have been directed to the ER which is their natural cell compartment due to the low concentration of proteases, the presence of numerous chaperones and the oxidizing environment (Petruccelli et al., 2006). In fact, these molecules find in the plant cell an oxidant environment that ensures a correct assembly of individual chains and the formation of inter and intra-chain disulfide bonds (Ma et al., 2003). Furthermore, ER retention increases expression levels and prevents the transfer of proteins through the Golgi, where the addition of typical plant sugar residues occurs (α 1,6-fucose and sialic acid instead of β 1,2 xylose and α 1,3-fucose).

Antibody expression has been directed also to specific cell storage compartments such as seeds. Seed-specific production of recombinant proteins is an appealing alternative strategy for the production of complex molecules in transgenic plants (Boothe et al., 2010). Seeds proved to be versatile targets for recombinant protein or peptides accumulation, including short and long polypeptides as well as complex proteins like antibodies. Several interesting properties of seeds can be exploited in numerous molecular farming applications, mainly when it is desirable to accumulate in a naturally designed protein storage compartment high amounts of a recombinant molecule. Many factors could be controlled to ensure high accumulation levels of heterologous proteins in seed, such as, promoters and enhancers, which regulate transcript abundance (Wilken et al., 2011).

In a recent study, the potential of *A. thaliana* seeds to produce mAbs was explored together with the determination of their intracellular trafficking and deposition (Loos et al., 2011). To this aim, two model antiviral mAbs, 2G12 (anti-HIV) (Trkola et al., 1996) and HA78 (anti-Hepatitis A Virus) (Cao et al., 2004) were expressed in Arabidopsis seeds in order to determine expression levels and

the subcellular localization. Depending on the mAb and the plant line, maximal expression levels of 2–10 µg/mg dry seeds were observed, corresponding to 0.2 %–1 % of dry seed material.

Using the same expression system in *A. thaliana* seeds, even higher expression levels have been achieved: 74 µg/mg seeds (De Jaeger et al., 2002) and 26 µg/mg seeds (Van Droogenbroeck et al., 2007) for different formats of single-chain antibody fragments. An important result has been recently obtained with the HIV-specific monoclonal 2G12 antibody produced in maize seeds, expressed at levels of 75 µg/g dry weight and the purified product resulted fully functional as its CHO derived counterpart (Ramessar et al., 2008). Nevertheless, expression of antibodies in plants still presents several drawbacks related to the extraction procedures, purification steps, quality of the final product and regulatory issues.

Extraction procedures represent an important step, since these can influence the quality of the final product even after purification. In fact, recombinant protein extraction is often conducted in the presence of unwanted plant proteins and compounds such as proteolytic enzymes, pigments, alkaloids, phenolics, polysaccharides and DNA that can reduce the efficiency of protein extraction and the final quality. Another limitation is represented by the high costs relative to the purification process based on protein A affinity chromatography. This purification methodology represents the most selective strategy to achieve high efficiency recovery of antibody in a single step (Hober et al., 2007). However, susceptibility of protein A column to chemical and biological degradation caused by the aggressive characteristics of plant extracts, decreases column life with higher costs on large scale application (Platis et al., 2008). In addition, the possible leaching of protein A during the purification process could contaminate the eluted purified antibody causing immunogenic responses after its administration in humans (Terman et al., 1985).

Another important concern to be addressed in the expression of immunoglobulins in plants is related to the intrinsic stability of immunoglobulins in the plant cell environment and their susceptibility to degradation (Gomord et al., 2005). In fact, unintended proteolytic mechanisms triggered by different plant cell processes can involve the degradation of plant derived antibodies causing a dramatic reduction of intact IgG recovery.

Another important issue is represented by the strict parameters imposed by regulatory bodies that control the introduction of plant derived immunoglobulins in the market. Requirements for downstream processing techniques that meet the terms of the GMP involve extensive quality controls and assurance programs, including, in the case of transgenic plants, the establishment of master plant banking procedures (Schillberg et al., 2005).

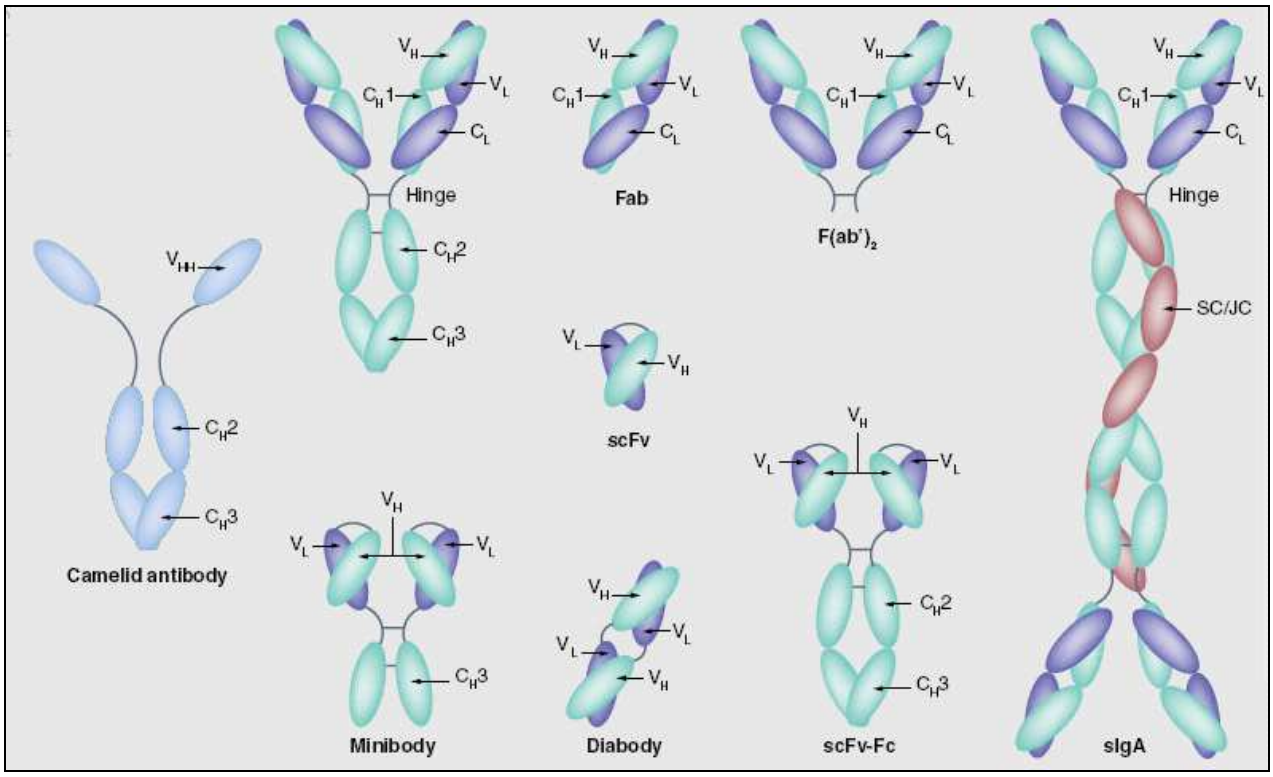


Figure 7: Several immunoglobulin types have been expressed successfully in plants, including several classes of IgG , IgA and a chimeric IgA/G. A variation on the classical IgG tetrameric structure is the camelid heavy-chain antibody, which lacks a light-chain component and can be expressed as a single transgene. Another example of antibody format is represented by secretory IgA composed by dimers of the typical IgG including two extra components: the secretory component and the joining chain. Other forms are represented by smaller engineered antibody derivatives as minibodies, diabodies, large single chains, single-chain Fv fragments (scFvs) and single variable regions, which have both the heavy and light chain variable regions on the same polypeptide chain. More specialized derivatives include bispecific scFvs,which contain the variable regions from two parent immunoglobulins and recognize two unrelated antigens, and scFv fusion proteins in which the scFv is genetically fused to a toxin, cytokine or enzyme (from Capodicasa et al., 2011).

2.4.2 Transgenic plants *versus* transient expression systems

The production of antibodies in plants has been achieved using different expression strategies, including stable transformation of the nuclear genome and transient expression systems using viral or *Agrobacterium* T-DNA based expression vectors (Vaquero et al., 1999; Kathuria et al., 2002; Rodríguez et al., 2005; Sainsbury et al., 2008; Lombardi et al., 2010).

In the case of stable genetic transformation the expression of complete IgG was traditionally obtained transforming plants separately with the genes coding for heavy or light chain (HC, LC); plants were then cross-pollinated in order to select, in the next generations, transgenic plants co-expressing both chains. This approach allowed the production of correctly assembled IgG molecules with expression yields ranging from 0.1 to 5 % of total soluble protein (Hiatt et al., 1989; Ma et al., 1995). The first example of complex secretory antibodies expressed in tobacco plants,

achieved by sequential crossing of four lines carrying individual components, is represented by the *CaroRx* SIgA/G that specifically binds *Streptococcus mutans* adhesion molecules. This immunoglobulin was efficiently expressed in *N. tabacum* reaching levels of up to 5 % to 8 % of total soluble leaf protein (Ma et al., 1995). However, this strategy has been recently replaced by the use of binary vectors bearing both heavy and light chain coding sequences in the same T-DNA which allows to obtain transgenic plants expressing full IgGs in a single transformation event (van Engelen et al., 1994). In some cases this strategy revealed to be more efficient both in terms of rapidity and production yield. For example, the anti-cutinase antibody 21C5 produced in transgenic tobacco plants using a traditional cross-pollination approach showed significantly lower levels compared to tobacco plants obtained using T-DNA bearing both genes arranged in a divergent orientation on the same expression cassette (De Muynck et al., 2010).

Several species have been used for stable transformation among which *Nicotiana tabacum*, *Nicotiana benthamiana, Zea mays, Arabidopsis thaliana, Lactuca sativa, Medicago sativa, Solanum tuberosum*. The best results in terms of production yield have been recently obtained by expressing the neutralizing anti-HIV-1 2G12 antibody in seeds of transgenic *Z. mays* plants. In this case, the extractable yield of 2G12 was 75 mg/Kg of dry seed weight, 75 % of which could be recovered (Ramasser et al., 2008). Most antibodies reported in literature have been expressed at good levels in *N. tabacum* leaves as the monoclonal mouse IgG1 MGR48 expressed at levels of 30-60 mg/Kg under strictly controlled growth conditions (Stevens et al., 2000) and the mouse IgG2 BR-55-2 (31 mg/Kg FW) (Brodzik et al., 2006). A summary of the most representative antibodies expressed in transgenic plants is listed in **Table 7**. There are many successful examples of antibody expression in plants using transient expression systems. *Agroinfiltration* was used for the production of mAb 2G12 cloned in a T-DNA expression cassette harboring HC and LC in tandem obtaining a final yield of 110 mg/Kg FW (Schähs et al., 2007). A recent study demonstrated that the combined effect of a strong viral promoter (alfalfa plastocyanin promoter), together with the enhancing activity of the HcPro silencing suppressor in the same vector, could lead to high yield production in agroinfiltrated *N. benthamiana* plants of both the secreted form of the anti-human globulin (AHG) C5-1 antibody (558 mg/kg FW) and of the ER-retained variant (757 mg/kg FW).

Table 7: Antibodies expressed in transgenic plants (adapted from De Muynck et al., 2010).

Antibody	*Isotype*	*Promoter H*	*Promoter L*	*Host species*	*Organ*	*Expression Level*	*Reference*
21C5κ Anti-cutinase	mIgG1κ	CaMV 35S	mas1'2	*N. tabacum*	Roots	0.6 % TSP	van Engelen et al., 1994
CaroRx *Streptococcus mutans*-specific	SIgA/G	CaMV 35S	CaMV 35S	*N. tabacum*	Leaves	5-8 % TSP	Ma et al., 1995
mAb24 TMV-specific	mIgG2bκ	CaMV 35S	Single Native	none	*N. tabacum*	Leaves F0: 0.04–0.16 % TSP; F1: 0.07–0.3 % TSP	Voss et al., 1995
						Suspension Cells 15–16 μg⁄g wet cell weight or 0.3 %TSP	Fischer et al., 1999
C5-1 anti-human globulin	mIgGκ	CaMV 35S	Native	none	*Medicago sativa* Leaves	0.13–1.0 % TSP	Khoudi et al., 1999
rAb29 TMV-specific	mIgG1κ	CaMV 35S	None	none	*N. tabacum* Leaves	8.5 μg⁄g FW, 0.14 % TSP	Schillberg et al., 1999
MGR48 anti-*Globodera rostochiensis*	mIgG1κ	CaMV 35S	mas1'2'	none	*N. tabacum* Leaves	30–60 μg⁄g FW (top leaves) 5–15 μg⁄g FW (bottom leaves)	Stevens et al., 2000
CB-Hep.1 anti-HBsAg	mIgG2bκ	CaMV 35S	Sweet potato sporamin KDEL	none	*N. tabacum* Leaves	60 μg⁄g FW or 0.5 % TSP Seedlings 17 μg/g DW	Ramirez et al., 2003 Triguero et al., 2005)
SO57 rabies virus	hIgG1κ	CaMV 35S	Native⁄ KDEL	Native ⁄none	*N. tabacum* Leaves Suspension cells 10–30 μg⁄ g DW	3 μg⁄g FW or 0.07 % TSP	Ko et al., 2003 Girard et al., 2006

14D9 hydrolysis of enol ethers	mIgG1	CaMV 35S	Murine/ none	none	*N. tabacum* Leaves	2.9 % TSP	Petruccelli et al., 2006
BR55-2 anti- LeY Cancer Cells	mIgG2κ	CaMV 35S	Native/ KDEL	Native /none Single	*N. tabacum* Leaves	31 μg/g FW	Brodzik et al., 2006
A5 tumor specific	hIgG1λ	CaMV 35S	Native/none	none	*N. benthamiana* Leaves	10–15 μg/g FW	Giritch et al., 2006
2G12 anti-HIV-1	hIgG1	gt-1	Native/none		*Zea mays* Seeds	75 μg/g DW	Ramessar et al., 2008
		CaMV 35S			*A. thaliana* Leaves	0.2 % TSP	Schahs et al., 2007
		gt-1	Native/ SEKDEL		*Zea mays* Seeds	F1: max 30 μg/g DW; F3: 40–60 μg/g DW	Rademacher et al., 2008
2F5 anti-HIV-1	hIgG1	CaMV 35S	Native/ SEKDEL		*N. tabacum* Suspension cells	6.4 μg/g FW	Sack et al., 2007
		CaMV 35S	Native/c-myc, KDEL	Native/ c-myc, KDEL	cross-pollination Leaves	0.1 % TSP 0.3 % TSP	Floss et al., 2008

FW, Fresh Weight; DW, Dry Weight; TSP, total soluble protein. hIgG: human IgG; mIgG: mouse IgG; κ: kappa light chain; λ: lambda light chain

Viral vectors have been used for the production of the CCR5 mAb, specific for the ligand-binding region of the human chemokine receptor and HIV-1 co-receptor CCR5. The production of this humanized mAb was performed using the previously described MagnICON expression system. In this methodology, anti-CCR5 mAb HC and LC encoding genes were cloned into two different non-competing viral vectors obtained from Turnip vein-clearing virus (TVCV) and PVX. Expression was performed in *N. benthamiana* plants processing batches of 40-60 Kg of plant material. The combination of these two non-competing viral vectors led to a final yield of 250 mg/kg of fully assembled IgG showing a high purity (Pogue et al., 2010). Another efficient viral-derived expression system is based on the Cowpea mosaic virus (CPMV). This virus has a bipartite RNA which has been used to develop two types of deconstructed expression vectors able to ensure the production of complex multimeric proteins in plants such as antibodies (Sainsbury et al., 2007). Vectors were obtained engineering the viral RNA-2, using either the full-length RNA-2 or a deleted version (delRNA-2). Replication of the modified RNA-2 in plant cells is achieved by co-

agroinfiltation with RNA-1. An application of this system was described for the production of the blood group-typing antibody C5-1 in which HC and LC coding genes were inserted into two separate RNA-2 and then agroinfiltrated in the presence of RNA-1. The results showed the accumulation of high antibody levels using both full-length and deleted RNA-2 vectors, even if the latter gave the best results in terms of antibody yield. This work represents the first example in which a bipartite virus has been used as platform for high production level of immunoglobulins that does not rely on full virus replication (Sainsbury et al., 2008). A development of this vector is represented by the "hyper-translatable" Cowpea mosaic virus based protein expression system ('CPMV-HT'). This vector system, differently from the previously described expression vectors, does not require co-agroinfiltration with RNA-1 for its replication. Furthermore, the sequences of interest and the gene encoding the P19 gene silencing suppressor protein have been incorporated into the same T-DNA, allowing the expression of complex multimeric proteins through agroinfiltration of a single construct. An example of application of this system is the expression of the 2G12 antibody in *N. benthamiana* plants leading to extremely high accumulation levels. The plant derived 2G12 produced with this system, resulted fully functional and equivalent to its mammalian counterpart.

Both transgenic plants and transient expression system are valuable platforms for the production of biopharmaceuticals. However, recent results obtained using the MagnICON and the "hyper-translatable" technologies showed that viral based expression systems have high potential in terms of yield, rapidity and possibility of contained GMP production. Even if the first antibody in Europe entering clinical trials is represented by the 2G12 mAb produced in stable transgenic tobacco plants, in the near future, viral based expression systems will be the preferential platform for the expression of biopharmaceuticals production in plants.

2.4.3 Plant-made antibodies and immunotherapy of infectious diseases

Anti-infective antibodies have the capacity to target viral, bacterial and fungal infections and are a valid alternative to traditional antibodies to combat infectious diseases, especially in cases of multi-drug resistance pathogens. In the last years, there have been several examples of plant derived antibodies exhibiting a high neutralizing potential against several infectious pathogens.

One of the first examples of a plant derived antibody developed for the treatment of infectious diseases is the secretory antibody directed against an exposed antigen of *Streptococcus mutans*, found to be as effective as the original mouse derived IgG (Guy's 13) in protecting against *S.*

mutans colonization on teeth (Ma et al., 1994). The immunoglobulin has been further transformed by a biotech company (Planet Biotechnology) into a biopharmaceutical product to be used for clinical purposes with the name of CaroRX™, which has recently gained EU approval to be employed as medical device to prevent oral bacterial infection that causes dental caries (Kaiser, 2008). Moreover, promising results have been described about the over expression of anti-HIV neutralizing antibodies. For example, two mAbs, the monoclonal antibody 2G12 previously described, specific for the glycans exposed on the HIV-1 envelope protein gp120 (Trkola et al., 1996) and the 2F5 anti-gp41 (Conley et al., 1994), showing a strong HIV neutralizing activity, have been widely characterized for plant expression. In particular, an important result has been recently obtained with the monoclonal 2G12 antibody, produced in maize seeds. This plant produced antibody was found to be functional in terms of antigen-binding activity as its CHO derived counterpart (Ramessar et al., 2008). The same antibody derived from stable tobacco plants, produced by the 'Pharmaplanta' Consortium, a program aiming to develop a production pipeline for pharmaceutical proteins made in plants, has obtained the permission to enter into a human clinical trial phase, the first under the new cGMP guidelines (Scotti et al., 2010).

In the case of the 2F5 monoclonal antibody produced in transgenic tobacco plants, it has been described that the addition of an elastin-like peptide (ELP) repeat to the heavy and light chain of the immunoglobulin increased the production yield by improving protein stability. Besides, this strategy resulted also useful to simplify the recovery and purification of the protein product (Floss et al., 2008). Another example of therapeutic antibody described by Zeitlin et al., (1998) is the anti-herpes humanized mAb produced at high levels in soybean. The biological activity of this antibody resulted perfectly comparable to the same molecule produced using mammalian cell culture. In fact, this antibody was able to protect mice against *herpes simplex virus-2* with high efficiency (Horn et al., 2004). Plant transient expression systems were successfully used for the production of a neutralizing humanized mAb against the West Nile virus to be used in post-exposure prophylaxis (Hu-E16). This antibody was expressed using the MagnICON system and led to an accumulation of 8.1 g⁄Kg FW with a yield of purified antibody of 0,8 g/Kg. Furthermore, the antibody showed a strong neutralizing activity and an effective pre- and post-exposure protection in mice, similarly to what observed using the mammalian cell derived antibody (Lai et al., 2010). A recent example of wide-spectrum antifungal immunotherapeutic molecules are the chimeric mAb 2G8 and its recombinant scFv-Fc derivative produced in *N. benthamiana* plants by agroinfiltration (Capodicasa et al., 2010). These plant derived antibodies showed the capability to confer significant protection against different opportunistic pathogens such as *Candida albicans*, *Aspergillus fumigatus* and

Cryptococcus neoformans in animal models (Torosantucci et al., 2009). A successful example of protective plant-derived antibody is the production of the glycosylated and non-glycosylated versions of an antibody directed against protective antigen (PA) of *Bacillus anthracis* using agroinfiltration in *N. benthamiana* plants. It has been shown that these antibodies were able to neutralize anthrax lethal toxin activity *in vitro* and protected mice against an intraperitoneal challenge with spores of *B. anthracis* Sterne strain. A single 180 µg intraperitoneal dose of glycosylated mAb or non glycosylated mAb provided 90 % and 100 % survival, respectively. A summary of the anti infectious Abs transiently expressed in plants and their relative yield is presented in **Table 9**.

Table 9: Summary of the anti infectious Abs transiently expressed in plants (Adapted from Capodicasa et al., 2011).

Target/mAb name	*Host plant*	*Expression method*	*Yields*	*Antibody format*
HIV (2G12)	*N. benthamiana*	Agroinfiltration	50 mg/Kg	Human IgG1
HIV (2G12)	*N. benthamiana*	Cowpea mosaic virus	100 mg/Kg	Human IgG1
HIV (4E10)	*N. benthamiana*	Agroinfiltration	Not reported	Human IgG1
West Nile Virus (Hu-E16)	*N. benthamiana*	MagnICON system	30 mg/kg	Human IgG1
***B. anthracis* PA (pp-mAb^{PA}, pp-mAb^{PANG})**	*N. benthamiana*	Agroinfiltration	250 mg/Kg	Human IgG
		(TMV)-based vectors + Agroinfiltration	120 mg/kg	
***Candida albicans* (Hu2G8)**	*N. benthamiana*	Agroinfiltration	50 mg/Kg	Humanized murine IgG1; scFv-Fc
***Ebola* Virus GP1 (6D8)**	*N. benthamiana*	BeYDV-derived DNA replicon	Not reported	Humanized murine IgG1

2.4.4 Anti-cancer immunotherapeutic antibodies

In the last decades, several monoclonal antibodies targeting different types of cancer have been efficiently expressed in plant systems. One of the first examples of plant production of a recombinant anti-cancer antibody is represented by the carcinoembryonic antigen-specific diabody produced in tobacco (Vaquero et al., 2002). Another example is represented by the TheraCIM recombinant humanized antibody targeting the Epidermal Growth Factor receptor (EGF-R), produced in tobacco leaves. The antibody gene constructs were transiently-expressed in an aglycosylated format by vacuum agroinfiltration resulting as active as the cognate mammalian product (Rodriguez et al., 2005). Recently, another humanized monoclonal antibody, which recognizes HER2/neu on the surface of a human mammary gland adenocarcinoma cell line SK-BR-

3, has been produced at high levels in *N. benthamiana* leaves using the MagnICON viral-based transient expression system. It has been demonstrated that this immunoglobulin is able to specifically bind the Her2/neu oncoprotein exposed by adenocarcinoma cells (SK-BR-3) showing a high capability to inhibit cell proliferation *in vitro*. Furthermore, intraperitoneal administration of the antibody in mice, showed to efficiently retard the growth of xenografted Her2+ human ovarian tumors (Komarova et al., 2011). A particular application of plant-produced antibodies was developed for the treatment of non-Hodgkin's lymphoma by individualized idiotype vaccines created from patient's own cancer cells (Bendandi et al., 2011). In this cancer, several clonal immunoglobulin idiotypes are displayed on the surface of most malignant B cells, and these can be exploited for therapeutic vaccination. Thanks to the rapid and high level expression offered by plants, it was possible to proceed from biopsy to vaccine in less than 12 weeks. Several antibodies directed against different types of cancer have been expressed in plants and some examples of plant-derived anti-cancer immunoglobulins are reported in **Table 8**.

Table 8: Examples of plant derived anti-cancer immunoglobulins (Adapted from Capodicasa et al., 2011).

Target	***Host plant***	***Expression method***	***Yields***	***Antibody format***
HER2/neu (Trastuzumab)	*N. benthamiana*	TMV and PVX vectors	200/300 mg/Kg	Humanized murine IgG1
HER2/neu (Trastuzumab)	*N. benthamiana*	MagniICON viral-based	45 mg/Kg	Humanized murine IgG1
Human tumor specific mAbA5	*N. benthamiana*	TMV and PVX vectors	500 mg/Kg	Human IgG1
EGF-R (TheraCIM)	Tobacco	Vacuum agroinfiltration	Not reported	Humanized antibody
Tenascin-C (H10)	*N. benthamiana*	Vacuum agroinfiltration	50-100 mg/Kg	Human IgG1
CEA (T84.66/GS8)	Tobacco	Agroinfiltration	1-5 mg/Kg	Diabody
Individualized idiotype vaccines for non-Hodgkin's lymphoma	Tobacco *N. benthamiana*	TMV vector Magninfection	5-800 μg/ml 0.5-4.8 g/Kg	scFv IgG

Transient expression systems have proved their potential in producing large quantities of recombinant antibodies in plants. Therefore antibodies will be among the first clinical grade biopharmaceuticals for human treatment to be produced in plants. Currently, five different plant-produced monoclonal antibodies have been included in clinical trials confirming this trend (**Table 10)** (Obembe et al., 2010).

Table 10 Examples of antibodies included in clinical trials.

Product	*Disease*	*Plant*	*Clinical trial status*	*Company*	*URL/academic*
CaroRX	Dental caries	Tobacco	EU approved as medical advice	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
DoxoRX	Side-effects of cancer therapy	Tobacco	Phase I completed	Planet Biotechnology, USA	http://www.planetbiotechnology.com/
RhinoRX	Common cold	Tobacco	Phase I completed	Planet Biotechnology, USA	http://www.planetbiotechnology.com
Fv antibodies	Non-Hodgkin's lymphoma	Tobacco	Phase I	Large Scale Biology, USA	http://www.lsbc.coma
IgG (ICAM1)	Common cold	Tobacco	Phase I	Planet Biotechnology, USA	http://www.planetbiotechnology.com/

2.4.5 The tumour-targeting anti tenascin-C mAb H10

In this work we describe the expression and characterization of the human recombinant mAb H10 antibody specific for the large isoform of tenascin-C (TNC-C). TNC is a glycoprotein of the extracellular matrix that comprises several alternatively spliced domains (domains A1, A2, A3, A4, B, C, D). It is over-expressed in adult tissues undergoing remodelling and neoplasia (Borsi et al., 1992), but remains undetectable in healthy adult tissues. The C domain of TNC exhibits a restricted pattern of expression (Carnemolla et al., 1999), and therefore represents a preferential tumor-associated antigen target for both therapy and diagnosis. Tumor targeting by anti-tenascin antibodies has been already achieved, but most antibodies are either of murine origin or human–mouse chimeras, and may therefore be unsuitable for repeated administration and for the generation of therapeutic derivatives (Rizzieri et al., 2004). We previously described the expression of the human tumor targeting monoclonal antibody H10 both by vacuum co-agroinfiltration of *N. benthamiana* plants with a T-DNA 35S based *Agrobacterium* expression vector and by stable transformation of *N. tabacum* plants (Villani et al., 2009). mAb H10 was obtained fusing the variable regions of an anti-TNC scFv(H10) selected from a human phage display library against the C domain of TNC, to the constant regions of the human γ chain subgroup III and human λ chain subgroup IV domains. Both genes coding HC and LC were then cloned into the plant binary vector (pBI-Ω) (Marusic et al., 2007) under the control of the Cauliflower mosaic virus (CaMV) 35S

promoter and the omega (Ω) translational enhancer sequence from Tobacco mosaic virus (TMV) (**Figure 8**).

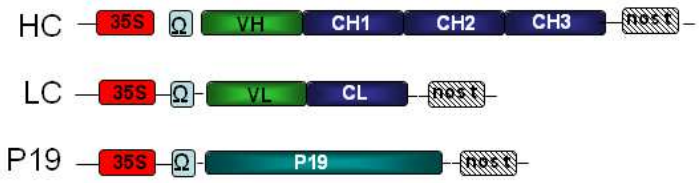


Figure 8: Schematic representation of the constructs used for plant expression. Both heavy (HC) and light (LC) chain and p19 gene silencing suppressor coding sequences were put under the control of the CaMV 35S promoter (35S) and Ω translational enhancer sequence of the TMV.

Over 100 transgenic T_0 plants were screened by both PCR and quantitative enzyme-linked immunosorbent assay (ELISA) for the expression of individual chains, and the lines with the highest expression levels were selected and cross-pollinated. After the germination of T_1 seeds on appropriate selection medium, the plants were screened for antibody expression by ELISA. Tobacco lines showing the highest expression levels are reported in figure (**Figure 9**).

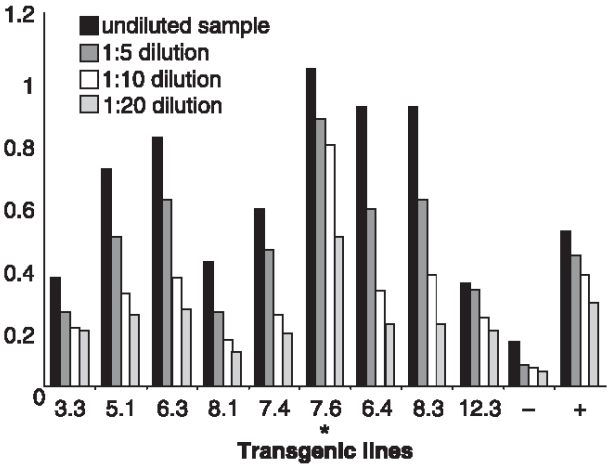


Figure 9: Functional ELISA for monoclonal antibody (mAb) H10 expressed in 9 transgenic *N. tabacum* lines. Plant extracts were normalized for total soluble protein (TSP) content and were assayed at different dilutions for their binding to mouse tenascin-C. Dilutions are reported from the undiluted to the 1:20. As positive control (+), 100ng of purified mAb H10 were used, negative control (-) was obtained using a WT plant (from Villani et al., 2009).

Transgenic lines exhibiting the highest expression levels of mAb H10 evidenced a final antibody yield comprised in a range of 0.6–1.1 mg/kg FW.

In the case of the transient expression strategy, a vacuum-agroinfiltration system was used for the antibody production. Furthermore, in order to enhance the yield of recombinant product in the co-agroinfiltration method, the gene silencing suppressor protein p19 from Artichoke mottled crinckle

virus (AMCV) has been used (Lombardi et al., 2009). This strategy led to an increase of at least 10-fold of mAb H10 expression levels in comparison to the expression in absence of the p19 protein.

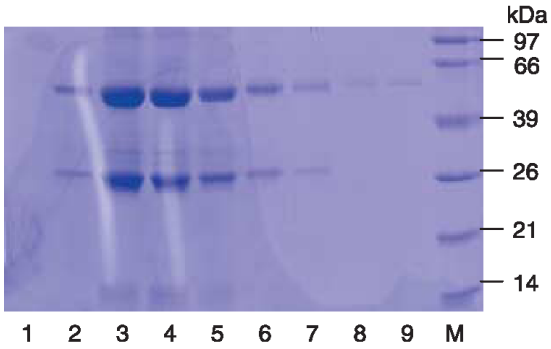


Figure 30: Typical sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of eluted fractions (1–9) obtained after Protein A affinity chromatography. Lane 1, 1 µg of a reference human IgG; lanes 2 and 3, 1 µg of two different batches of purified H10; lane 4, 1 µg of a reference human IgG; lanes 5 and 6, 2 µg of two different batches of purified H10; M, molecular mass marker (from Villani et al., 2009).

Purification of the antibody was performed using a Protein A column, and the average yield obtained after purification was typically 50–100 mg/kg FW. A typical SDS-PAGE analysis of the eluted fractions after affinity chromatography is shown in **Figure 10**. These expression results are comparable to those obtained using virus-based systems for either the production of the blood group-typing IgG C5-1 (75 mg/kg fresh plant tissue) (Sainsbury et al., 2008) or the human tumor-specific mAb A5 (500 mg/kg fresh plant tissue) (Giritch et al., 2006). The functionality of the antibody purified from agroinfiltrated leaves was assessed by surface plasmon resonance, and the dissociation constant calculated for mouse TNC was 14 nM. The purified antibody also retained full biological activity, as demonstrated by immunohistochemical analysis, which showed specific accumulation of H10 around tumour blood vessels.

2.4.6 Optimization of the purification of antibodies in plants

Many efforts have been made in the last years to increase the expression levels of heterologous proteins in plants by developing novel efficient expression systems. Promising results have been obtained from the use of production systems based on viral vectors, and in many cases recombinant protein yield is not a limitation any more. Therefore, the interest is gradually shifting from optimization of expression systems to the improvement of the extraction/purification strategies (Gottschalk, 2008). Based on the product and its application, downstream processing can represent

a significant percentage of the total managing costs for product manufacturing. For this reason, the development of efficient and selective extraction and purification processes, able to ensure high recovery and final product quality, is a crucial point to be addressed (Basaran and Rodriguez-Cerezo, 2008). The efficiency of downstream steps is influenced by the recombinant protein concentration, the plant extracts composition and the purification method used. Moreover, recombinant protein extraction is often conducted in the presence of unwanted plant proteins and compounds such as DNA, pigments, alkaloids, phenolics, polysaccharides, and proteases that could interact with the recombinant protein affecting final quality and yield or limiting the efficiency of chromatographic steps. For this reason, extraction needs to be optimized evaluating different parameters such as tissue homogenization, buffer composition, and subcellular protein localization (Hassan et al., 2008; Woodard et al., 2009; Zhang et al., 2005, 2010). The control of these conditions can improve the purification processes by reducing the impact of contamination of plant compounds on the final recovery steps.

A detailed study has focused on the initial extraction steps from plant leaves, evidencing that optimal procedures are mainly influenced by the antibody expression strategy (secretory, ER retained or membrane bound) (Hassan et al., 2008). In this study, three different transgenic plant lines were analyzed in order to set up the parameters to improve extraction procedures of a monoclonal antibody addressed to different cell compartments (apoplastic space, plasma membrane or ER). Seven methodologies for physical extraction were evaluated for each transgenic line. It has been shown that the optimal extraction strategy depends on the recombinant protein localization within the cell and, in some cases, the addition of a detergent in the extraction buffer may improve the yield. It has also been shown that recovery of secretory antibodies from leaf disks of transgenic tobacco plants can be improved using mild extraction techniques (passive elution and freeze-thaw) reducing plant contaminant levels in the extract. In fact, contaminants as polyphenols and alkaloids (especially in the case of tobacco) represent a major issue in downstream purification processes. Homogenization of the plant material is the common methodology used for plant protein extraction and is usually followed by clarification using centrifugation and/or microfiltration. An example of this methodology is represented by large-scale purification (50–200 kg of leaves) of the anti-HBsAg antibody from a partially clarified transgenic tobacco leaves extract using protein A affinity chromatography. This purification strategy showed high efficiency in terms of elution capacity, antigen purity, purification cycles and ligand leakage, but plant pigments and phenols were still present in the elution fractions (Valde´s et al., 2003). Recent strategies were focused on the removal of polyphenols/alkaloids content before the protein A affinity chromatography step for both safety

and economical reasons. In fact, plant contaminants may have a negative impact on protein A resin reducing column performances. An example of purification strategy aiming to reduce plant contaminants was efficiently used for the purification on lab scale (5 g of leaf tissue) of the anti-HIV monoclonal antibody 2G12 (mAb 2G12) and 4E10 (mAb 4E10) from unclarified transgenic tobacco crude extract. In this study, antibodies were purified by the use of an aqueous two-phase partitioning system followed by cation-exchange chromatography and immobilized metal affinity chromatography. This strategy revealed an high antibody recovery with over 96 % purity grade and absence of polyphenol and alkaloid contaminants (Platis et al., 2008; Platis and Labrou, 2009). A similar study was carried out by Lee and Forciniti (2010) that evaluated the use of the aqueous two-phase partitioning as a single recovery and purification strategy of an aglycosylated IgG expressed in corn seeds. They controlled pH and ionic strength in order to partially purify the antibody in a process composed of three steps, with the aim to deliver the antibody in a clarified solution. The first two steps were based on the delivery of the antibody in the aqueous salt bottom phase keeping plant impurities in the top (PEG) phase. These two-extraction stages resulted in a poor mAb purification of 1.3- and 1.4-fold, respectively. The third step consisted in the accumulation of the antibody at the two-phase interface resulting in a purification of 10-fold. At the end, the three-stage processes led the accumulation of a 72 % pure mAb with 49 % of final yield. Clearly, additional adsorption steps, most likely affinity chromatography, would be needed to purify the antibody for biopharmaceutical applications. In a recent study, using a similar approach a 94 % removal of tobacco proteins from the acidic recombinant protein β-glucuronidase extracts was reported (Ross and Zhang, 2010). Another example of IgG purification from tobacco extracts is represented by the anti-WNV monoclonal antibody Hu-E16 (Lai et al., 2010). Leaf extracts after clarification were first treated with 25 % ammonium sulfate to remove impurities and then concentrated by precipitation with 50 % ammonium sulfate. After solubilization, pellet enriched with Hu-E16 IgG was processed by protein A affinity chromatography followed by a weak anion-exchange column. This methodology was applied at 5 kg of plant leaves and IgG purity was > 95 % with 49 % yield, in spite of the three column steps.

An interesting purification strategy has been used in the case of the 2F5 monoclonal antibody produced in transgenic tobacco plants, in which an elastin-like peptide (ELP) repeat was fused to the heavy and light chain C-terminus. ELP consists in a protein tag obtained repeating the pentapeptide VPGVG. This peptide can undergo a reversible phase transition from water-soluble forms into aggregates after a temperature transition. Therefore, after protein extraction from plant leaves expressing the 2F5-ELP antibody, a temperature increase (up to 40°C) was operated in order

to induce the precipitation of antibody aggregates. Although the final recovery of 2F5-ELP antibody was quite low, the system offered a valuable strategy to improve a rapid and cost-effective purification process (Floss et al., 2008).

2.4.7 Modulation of IgG glycosylation in plants

Post-translational modifications occurring in antibodies have been extensively studied and it has been demonstrated that glycosylation is crucial for many fundamental biological processes, including antibody dependent cellular cytotoxicity (ADCC), complement activation and Fcγ receptor binding ability (Gomord et al., 2005). These modifications are often essential for the stability and biological activity of a protein especially in the case of antibodies (Gomord et al., 2010). In fact, it has been evaluated that in these molecules, different glycosylation patterns could affect protein half-life in blood and, in some cases, the capability to interact with the components responsible of the stimulation of the immune system (Jefferis, 2005). In eukaryotes, oligosaccharides are added on specific sequences of secreted proteins before entry into the ER in a process called N-glycosylation. An example of plant N-glycosylation is shown in **Figure 11**.

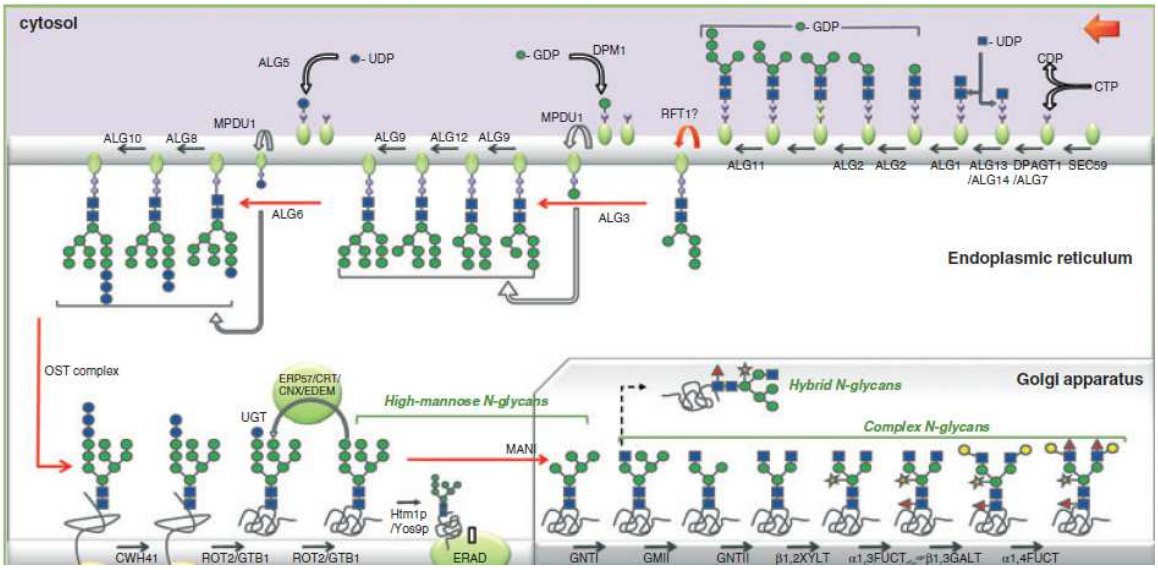


Figure 11: Reference pathway for protein N-glycosylation in plants. Assembly of the lipid-linked oligosaccharide precursor begins on the cytoplasmic side of the endoplasmic reticulum (ER) membrane. The resulting Man5GlcNAc2-PP-Dol then flips into the ER lumen, where four further Man residues and three glucose residues are added to produce the complete Glc3Man9GlcNAc2-PP-Dol.
After transfer of the oligosaccharide precursor from the lipid carrier onto the nascent protein by the action of the multisubunit oligosaccharyltransferase complex, the newly formed N-glycoprotein enters the calnexin-calreticulin (CNX–CRT) cycle. The alternate action of glucosidase II and UDP-glucose:glycoprotein glucosyltransferase drives the glycoprotein through this cycle until it is correctly folded and exported from the ER to the GA (From Gomord et al., 2010).

The addition of the N-glycan chain contributes to the folding process of the protein and the following sugar modifications occurring in the ER have a signaling function in the protein-folding quality-control mechanism. Biosynthesis of N-glycans can be divided into two steps which occur sequentially in the ER and downstream in the Golgi. In the ER, glycosylation between mammals and plants is highly similar showing minimal differences in N-glycans found on mature glycoproteins (Gomord et al., 2010). The major differences are found in the Golgi apparatus were plant specific complex-type glycans are specifically added by the enzymes β 1,2-xylosyltransferase and α 1,3-fucosyltransferase instead absent in mammalian cells (Lerouge et al., 1998) (**Figure 11**). Furthermore, plant glycoproteins differ from their mammalian counterpart for the lack of β 1,4-galactose, sialic acid complex-type glycans, core α 1, 6-fucose and the homologous of the mammalian N-acetylglucosaminyltransferases involved in further branching of the bi-antennary N-glycans (**Figure 12**).

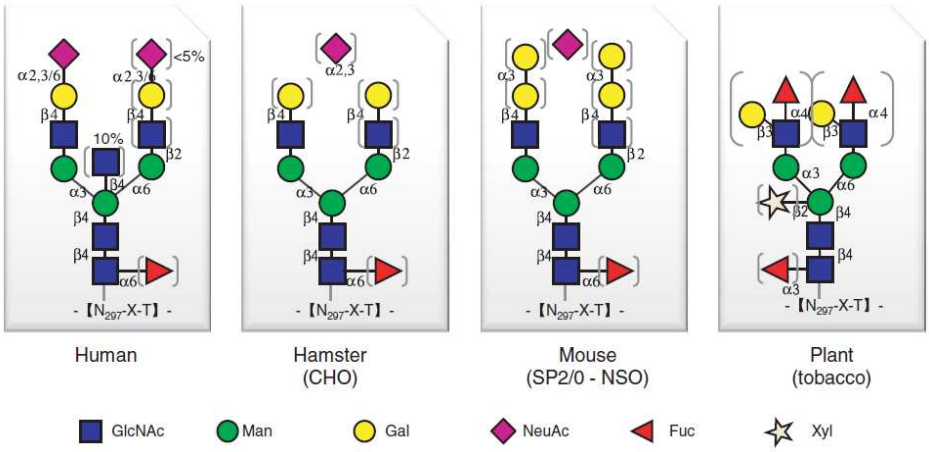


Figure 12: N-glycans of human native antibodies (a) and recombinant antibodies produced in hamster CHO cells (b), murine cells (SP2/0 or NSO cell lines) (c), or tobacco plants (c) (Adapted from Gomord et al., 2010)

Among differences, the lack of core α 1,6-fucose in plant produced IgGs is an advantage since it has been demonstrated that the elimination of these sugar moieties on the Fc portion of immunoglobulins drastically increases antibody-dependent cellular cytotoxicity (ADCC) (Shields et al., 2002). On the contrary, plant typical oligo-mannosidic IgG glycosylation profiles could induce the rapid clearance of the IgG from the blood stream exhibiting lower complement-dependent cytotoxicity (CDC) generated by binding to the antibody Fcγ (Raju, 2008; Jefferis, 2009). The main limitation of plant expression of recombinant protein, is represented by the presence of the typical plant sugars β 1,2-xylose and α 1,3-fucose, normally absent in mammalian proteins. As described in

literature, proteins harbouring these plant sugars could induce adverse side effects when used in human therapy. In fact, the immunogenicity of these N -glycan epitopes is well documented and their role in allergy has not yet been clarified (van Ree et al., 2000; Bardor et al., 2003; Gomord et al., 2005; Altmann, 2007). Based on this concern, new strategies have been adopted to engineer antibody glycosylation profiles in order to reduce problems related to the presence of typical plant sugars. Modulation of the N-glycosylation profile of plant produced antibodies, is largely described in literature and known as "glycoengineering". Several strategies have been exploited until now to modulate the glycan structure such as retention of immunoglobulins in the endoplasmic reticulum (ER) using C-terminal KDEL tags (Floss et al., 2008; Kajiura et al., 2009; Sainsbury et al., 2010; Loos et al., 2011) production of aglycosylated antibodies through mutation of the HC specific N-glycosylation site (Rodriguez et al., 2005; Nuttal et al., 2005) co-expression of antibodies with mammalian glyco-enzymes (Vézina et al., 2009) and expression of antibodies in plants in which genes coding for glycotransferase are inactivated or silenced by expression of siRNA (Strasser et al., 2008; Schahs et al., 2007; Gomord et al., 2010). Specific alterations of Fc glycosylation can improve antibody biological activity as recently shown by Forthal et al., (2010). Such alterations may result in better immunotherapeutic reagents in the case of antiviral activity, because complex glycan structures influence the binding to Fcγ receptors. In this study, five different glycoforms of the mAb 2G12 in wild-type and glycoengineered plants and CHO cells were produced. As a consequence, two 2G12 glycoforms produced in glycoengineered plants lacking β 1,2-xylose and α 1,3-fucose and harbouring a uniform human-like glycosylation profile, exhibited considerably improved binding to FcγRIIIa in comparison to the 2G12 expressed in CHO cells or wild-type plants (Forthal et al., 2010). This work demonstrates that plant-produced antibodies may be endowed with improved biological functions compared to their mammalian derivatives (bio-betters). The expression of antibodies harbouring the KDEL signal peptide at their C-terminus generally leads to accumulation of mAbs with high mannose-type N-glycans lacking β 1,2-xylose and α 1,3-fucose residues (Petruccelli et al., 2006; Floss et al., 2009; Sainsbury et al., 2010; Loos et al., 2011). However, in some cases, ER retention in plant cells seems not to be complete since KDEL tagged antibodies with a percentage of complex type plant typical sugars in the glycan chain have been documented. This event could be likely ascribed to a partial escape from the ER retrieval system during intracellular trafficking or to a partial proteolytic cleavage of the KDEL tag (Tekoah et al., 2004; Triguero et al., 2005; Floss et al., 2008; Kajiura et al., 2009).

In order to avoid the potential immunogenicity deriving from the presence of typical plant sugars, aglycosylated mAbs have also been produced in plants. These immunoglobulins without N-linked

glycans are correctly assembled showing an antigen-binding capability comparable to their mammalian counterpart (Nuttal et al., 2005). Nevertheless, aglycosylated antibodies have some disadvantages such as shorter half-life *in vivo* (Saint-Jore-Dupas et al., 2007) or impaired binding to Fc receptors. Moreover, recent studies demonstrated that aglycosylated IgGs opportunely engineered are still able to interact with FcγRs both *in vitro* and *in vivo*, demonstrating that Fc glycosylation is not necessarily required for the activation of immune cells by IgGs (Jung et al., 2010).

Gene knock-out or silencing represents another alternative strategy used to decrease or eliminate the activity of plant-specific glycoltransferases. Plants in which the glycotransferases responsible for the addition of β 1,2-xylose or α 1,3-fucose have been down-regulated allowing the synthesis of proteins devoid of plant-specific glycoepitopes harbouring a "human-like" sugar profile. The *N*-glycan composition was modulated using RNA interfering (RNAi) constructs to obtain a targeted down regulation of the expression of endogenous *FucT* and *XylT* genes in both *A. thaliana* and *N. benthamiana*. The absence of β 1,2-xylose and α1,3-fucose in the final purified antibodies was confirmed by Mass Spectrometry analysis (MS) and immunoblotting, demonstrating the possibility to use these engineered plants as an efficient expression platform for the production of human mAbs without detectable plant-specific N-glycan residues (Schahs et al., 2007; Strasser et al., 2008; Loos et al., 2011).

Another glyco-engineering strategy was based on the co-expression of specific human glycosyltransferases in wild type *N. benthamiana* plants, in order to generate recombinant antibodies with β 1,4-galactose or sialic acid residues (Vézina et al., 2009; Strasser et al., 2009; Fujiyama et al., 2007). A very significant result in the production of a "human like" glycosylation pattern was obtained by Castilho et al., (2010). They demonstrated the possibility to incorporate the entire biosynthetic pathway for protein sialylation in a plant host naturally lacking sialylated glyco-conjugates, for the expression of mAb 2G12. This result was accomplished expressing, by agroinfiltration, the six enzymes responsible of the complete mammalian syalylation pathway, for the biosynthesis, activation, transport, and transfer of Neu5Ac to terminal galactose in *N. benthamiana* plants. The co-expression of these glycoenzymes with the HC and LC of the 2G12 monoclonal antibody, resulted in quantitative sialylation of the Fc domain with the lack of plant-specific N-glycan residues. This strategy showed the possibility to obtain immunoglobulins harbouring a N-glycosylation pattern similar to that obtained in mammalian cells, representing a model system for the manipulation of complex plant metabolic pathways.

2.4.8 Antibody proteolysis in plants

A major issue for the production of recombinant proteins in plants is represented by degradation phenomena taking place in the cell or during downstream processes. In fact, unintended proteolysis processes could cause a dramatic reduction of the final yield of intact heterologous proteins. Moreover, quality and yield of the recombinant protein is significantly influenced by the relative intrinsic stability of polypeptide chains expressed in a heterologous cell environment (Faye et al., 2005). Within cells, several proteolytic processes, crucial to guarantee fundamental metabolic functions, but in some cases these processes could represent a drawback for the production of biologically active recombinant products (Goulet et al., 2008). Proteases contribute to the regulation of several metabolic pathways by directing the activation of proteins involved in essential regulatory processes, by eliminating misfolded or mistranslated proteins and by recycling essential amino acids from short-lived proteins (van der Hoorn, 2008). Hundreds of genes encoding enzymes involved in proteolytic pathways have been identified in plants (Rawlings et al., 2008); for instance, about 800 genes have been discovered in *Arabidopsis thaliana* directly or indirectly involved in the hydrolysis of peptide bonds (Smalle and Vierstra, 2004). Moreover, the presence of proteases is very abundant in some subcellular compartments such as the vacuole and tonoplast or in the apoplast, the final destination of antibodies targeted to the secretory pathway (De Wilde et al., 1998; van der Hoorn, 2008). According to this data, it is important to consider that the plant cell environment presents a complex net of proteolytic processes and a multitude of possible proteases could heavily affect accumulation of the recombinant product (Beers et al., 2004).

In the case of antibodies, unintended proteolysis driven by this complex proteolytic machinery could dramatically affect the final yield of intact IgG and in some cases could lead to an almost complete degradation (more than 90 %) of the final product (Villani et al., 2009). Many studies of antibodies expressed in plant leaves reported strong antibody degradation phenomena visible in SDS-PAGE analysis for the presence of additional smaller gel fragments between 20 and 150 kDa under non-reducing conditions (de Neve et al., 1993; Ma et al., 1994; Stevens et al., 2000; Sharp and Doran, 2001; Villani et al., 2009). Proteases may reduce the integrity of heterologous proteins in different manners, both *in planta* during protein synthesis and intracellular trafficking (Villani et al., 2009) and *ex planta* during the extraction procedure and subsequent purification phases (Sharp and Doran, 2000; Rivard et al., 2006). In fact, antibody fragments in plants have been interpreted as results of three phenomena: i) antibody assembly intermediates; ii) extracellular peptidase activity after secretion; iii) activity of peptidases released during sample homogenization (Sharp and Doran,

2001) **(Table 11).** As shown by a pioneering study, MAK33 antibody presented a stronger degradation when produced in *N. tabacum* than in *A. thaliana* confirming the hypothesis that proteolysis could be also a species-dependent event (De Neve et al., 1993). Besides, it is possible that degradation fragments resulting from a first "opening cleavage" might be further processed into smaller fragments by plant proteases (De Muynck et al., 2010). In addition, it is important to point out that the detection of IgGs by Western blotting analysis may not help to identify all generated degradation fragments, since antibodies used for detection, could bind proteolytic fragments with different affinity. Consequently, it is possible that degradation is largely underestimated in some cases (De Muynck et al., 2010). Recent studies have been focused on the identification of the degradation fragments obtained from *in planta* proteolysis. These fragments were analyzed by either MS (Ramessar et al., 2008; Villani et al., 2009) or N-terminal sequencing with Edman degradation (De Muynck et al., 2009) in order to localize the cleavage sites in the antibody aminoacid sequence. In the case of mass analysis, two major degradation products derived from an hypothetical cleavage occurring close to the hinge region of the H10 antibody were identified (Villani et al., 2009). Instead the study performed by De Muynck et al., (2009) thorough Edman Degradation led to the identification of the N-terminal sequence of a HC degradation product of the chimeric rat/human Lo-BM2 antibody localized in the hinge region. Data presented in these studies has been obtained expressing different antibody sequences in different host species, using different production systems and extraction procedures, therefore, the possibility to find a common degradation mechanism for all these antibodies is very difficult.

Different strategies have been used to overcome unintended *in planta* proteolysis, some are based on the subcellular localization of the protein in specific tissues or cellular organelles. Antibody delivery in cellular compartments exhibiting low levels of total proteolytic activity could reduce the unintended antibody proteolysis, increasing the accumulation of the final product (Vitale and Pedrazzini, 2005; Stoeger et al., 2005). An example is represented by the production of a scFv antibody expressed in the ER of potato tubers cells. In this case, the antibody remained detectable and functional even after 1.5 years of tuber storage at 4 °C (Artsaenko et al., 1998). It has been proposed that ER retained antibodies, due to their protracted interactions with ER resident chaperones, can increase their folding efficiency, ensuring higher protection from plant proteolytic degradation and increasing accumulation levels (Ko et al., 2005; Petruccelli et al., 2006). However, a recent study performed by Loos and colleagues demonstrated that the secretory form anti-HIV-1 2G12 antibody produced in *N. tabacum* seeds, accumulated at higher levels than the ER retained antibody (Loos et al., 2011).

Table 11: Putative degradation products noted on SDS-PAGE of plant-produced antibodies (es), size estimation from authors' SDS-PAGE; (p), purified antibody (Adapted from De Muynck et al., 2010).

Antibody	*Host species*	*Organ / ER retention signal (X)*	*Additional bands under non-reducing conditions*	*Additional bands under reducing conditions*	*References*
MAK33	*N. tabacum*	Callus	35 kDa 40 kDa 44 kDa 65 kDa 88 kDa 110 kDa (es) 126 kDa (es) 139 kDa (es) 144 kDa (es)	No	De Neve et al., 1993
	A. thaliana	Callus	35 kDa (es) 88 kDa (es) 126 kDa (es) 139 kDa (es) 144 kDa (es)	45 kDa (es)	
	A. thaliana	Leaves	2 bands > 110 kDa (p)	Not mentioned	De Wilde et al., 1996
	Solanum tuberosum	Tubers (X)	Not mentioned	28 kDa (p) 34 kDa	De Wilde et al., 2002
Guy's 13	*N. tabacum*	Leaves	40 kDa (es) 72 kDa (es) 116 kDa (es) 44 kDa (es)	40 kDa	Ma et al., 1994
		Hairy root cultures	40 kDa (es) (biomass) 50 kDa (es) (biomass) 60 kDa (es) (biomass) 90 kDa (es) (biomass) 115 kDa (es) (biomass) 140 kDa (es) (biomass)	40 kDa (es) (biomass)	Wongsamuth and Doran, 1997
		Hairy root cultures	40 kDa (es) (biomass) 45 kDa (es) (biomass) 50 kDa (biomass and medium) 90 kDa (biomass and medium) 120 kDa (biomass and medium) 140 kDa (biomass and medium)	40 kDa	Sharp and Doran, 1999
		Suspension Cells	135 kDa (es) (biomass) 120 kDa (es) (biomass) 80 kDa (biomass and medium) 50 kDa (biomass and medium)	Not mentioned	
		Roots (rhizosecretion)	20 kDa 60 kDa 110 kDa 125 kDa 140 kDa	Not mentioned	

C5-1	*N. benthamiana*	Leaves (X)	35 kDa 50 kDa 60 kDa 75 kDa 95 kDa 130 kDa	Not mentioned	Sainsbury et al., 2008
MGR48	*N. tabacum*	Leaves	44 kDa (p) 65 kDa (p) 125 kDa (p) 160 kDa (p)	Not mentioned	Stevens et al., 2000
SO57 (X)	*N. tabacum*	Leaves Suspension cells (X)	Not mentioned Not mentioned	22 kDa (es) 60 kDa (biomass) 75 kDa (biomass)	Ko et al., 2003 Girard et al., 2006
CL4	*N. tabacum*	Suspension Cells	Not mentioned	33 kDa (p) (es) 37 kDa (p) (es) 39 kDa (p) (es) 41 kDa (p) (es)	Yano et al., 2004
2G12	*Zea mays*	Seeds	Not mentioned	40 kDa (p)	Ramessar et al., 2008
	A. thaliana	Leaves	Not mentioned	30 kDa (es) 40 kDa (es)	Schahs et al., 2007

Similarly, the SEKDEL-tagged version of 2G12, expressed in maize seeds, accumulated to a lower amount compared to its secretory counterpart, showing an higher degradation (Rademacher et al., 2008; Ramessar et al., 2008). These results indicate that ER retention of antibodies is not always an effective strategy to increase the accumulation levels of intact immunoglobulins in plants.

Another interesting strategy to minimize *in planta* hydrolysis of recombinant proteins is to use plants expressing specific protease inhibitors. An example of this application has been described by Rivard et al., (2006). In this study, transgenic lines of potato expressing tomato cathepsin D inhibitor, active against trypsin and chymotrypsin, were obtained by *A. tumefaciens* mediated nuclear transformation. Protein extracts obtained from leaf exhibited decreased levels of Cathepsin D protease activity *in vitro* against ribulose 1,5-bisphosphate, even if a decreased proteolytic activity *in vivo* was not verified on recombinant proteins.

Production of protease knock down organisms proved to be a successful strategy in bacteria and yeasts, but its application to complex multicellular organisms such as higher plants is very difficult since transgenic plants lacking specific proteases would not be viable (Schaller, 2004). For this reason, transient co-expression of recombinant proteins with protease inhibitors was recently described (Rivard et al., 2006). A study presenting a similar approach was reported by Komarnytsky et al., (2006). In this work, transient co-expression of a Bowman-Birk serine protease inhibitor, together with different mAbs in *N. tabacum* decreased breakdown of immunoglobulins favoring a sensible increase of the final IgG yield.

In a recent study, genomic data for *Arabidopsis*, rice and some *Nicotiana* species were assessed in order to evaluate the presence of different proteases in the cell secretory pathway. The analysis of plant protease families present in *N. benthamiana* was performed using the MEROPS database (http://merops.sanger.ac.uk), a catalogue that contains the classification and nomenclature of peptidases, including their inhibitors and substrates. From this study the relative distribution of secreted protease-encoding genes among protease families in *Arabidopsis*, rice and *Nicotiana* species were recorded. In addition, an *in vitro* protease assay was carried out to confirm the presence and activity of *N. benthamiana* proteases predicted from the MEROPS analysis. Current genomic data suggested the presence of several protease families in *N. benthamiana* apoplast, including asparagine proteases, cysteine proteases and serine proteases. Subsequently, the inhibitors of the protease classes found (asparagines, serine and cystein proteases) both by the *in silico* and *in vitro* analysis were tested by transient expression in *N. benthamiana*, in order to verify the efficacy of protease inhibitors in stabilizing proteins targeted to the apoplast. As first result, transient expression and secretion of a tomato cathepsin D inhibitor (cystein proteases inhibitor) increased of 45 % the total amount of the leaf apoplast protein favouring the accumulation of a murine diagnostic antibody C5-1, co-secreted in the apoplast. The same effect of stabilization on the same antibody was observed when this immunoglobulin was co-expressed in presence of a SlCYS9 (Cysteine protease inhibitor) (Goulet et al., 2011). These preliminary data show that the use of proteases inhibitors could increase the *in situ* protection of recombinant proteins along the plant cell secretory pathway.

At the moment, no general conclusions about proteases involved in the specific degradation of plant derived antibodies can be made. For this reason, an effective strategy to better investigate the mechanisms of plant proteolysis, aiming to increase the yield of intact plant derived recombinant proteins, should be focused on the analysis of several factors such as plant species and relative typical protease families, expression system used for the immunoglobulin production, and susceptibility of the specific antibody sequence to the proteolytic attack.

2.5 Thesis Scope of investigation

This PhD work intends to give a contribution to knowledge in the field of plant-made biopharmaceuticals by providing new data on the transient over-expression of a tumour targeting antibody (mAb H10) with a particular focus on the optimization of plant extraction procedures, optimization of glycosylation profile and characterization of plant proteolysis. In this work a transient expression system based on vacuum-agroinfiltation of *N. benthamiana* plants, using the viral gene silencing suppressor protein P19 from Artichocke mottled crinckle virus (AMCV, Lombardi et al., 2009) allowed high protein expression yields, demonstrating to be a rapid and cost-effective strategy for the production of the fully human anti-tenascin-C mAb H10 (Villani et al., 2009). The aim of this PhD thesis was to optimize antibody extraction/purification methodologies, modulate the glycosylation profile and characterize *in planta* proteolysis.

The optimization of the extraction/purification steps was carried out evaluating different strategies in terms of antibody yield, degradation and level of contaminants in the final purified product. In addition, the glycosylation pattern of the plant produced mAb H10 was modulated evaluating the effects of the different glycan moyeties on antibody quality and final yield. Results also suggest that, among all factors contributing to protein stability, glycosylation might play an important role. Finally, we characterised the degradation profile of the tumour targeting H10 antibody in the attempt to identify specific proteolytic cleavage sites of the plant produced immunoglobulin. Overall data indicate that possible ‘fragile’ sites in the H10 antibody reside in the hinge region, that is particularly prone to cleavage by the plant protease machinery. The identification of these cleavage sequences is a first step towards the development of new strategies to reduce antibody degradation in plants and increase the yield of intact IgG molecules.

3. MATERIALS AND METHODS

3.1 Bacterial Strains

Escherichia coli XL1-Blue: supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac -.

Agrobacterium tumefaciens LBA 440 (Ach5 pTiAch5) Sm/Sp(R)

3.2 Plant Material

Seedling and germination of *N. benthamiana* plants used for transient expression and *N. tabacum* used for protoplast recovery were performed under artificial illumination (16 hour light /dark cycle) at a constant temperature of 26 °C. After 2 weeks from seedling, plants were transferred in single pots and grown under the same conditions.

3.3 Vectors

pGEM-Nos. Cloning vector derived from the pGEM ®-4Z (Promega). The pGEM-4Z (Figure M1) contains a polylinker riarranged from pUC18 plasmid, in the region of Lac-Z gene coding for the α peptide of β-galactosidase, required for the α-complementation assay. Polymarese promoters SP6 and T7, are localized respectively upstream and downstream of the multiple cloning site; the gene used for the antibiotic resistence is the *β-lactamase* that confers resistance to ampicillin. The pGEM-Nos was obtained by cloning the terminator sequence (253 bp) gene of the enzyme nopaline synthase (NOS) derived from *Agrobacterium tumefaciens*.

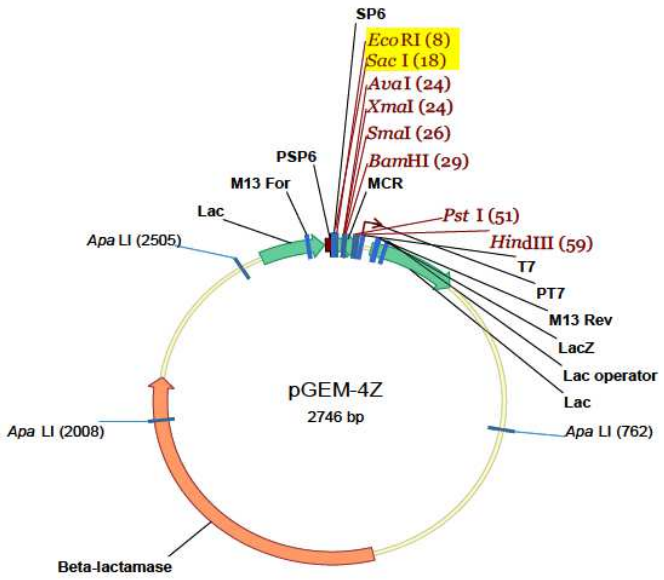
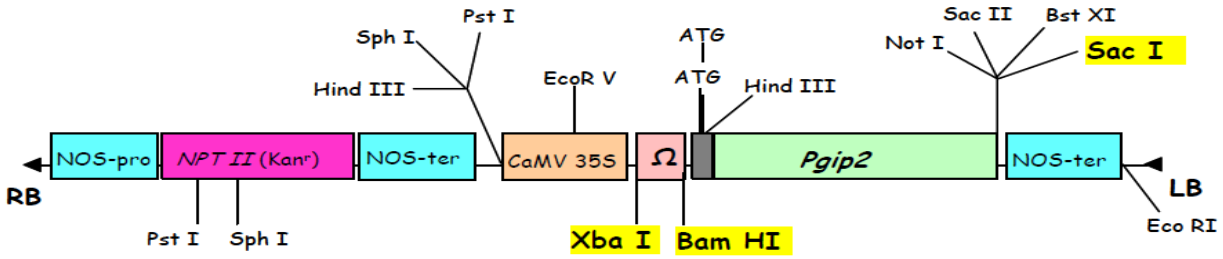


Figure M1: Vector pGEM-4Z. SacI-EcoRI restriction sites used to insert the terminator sequence of nopaline synthase gene (Nos) are highlighted in yellow.

pBIΩ. Binary vector derived from plasmid pBI121. The pBI121 contains an expression cassette (T-DNA) harbouring the *GusA* gene controlled by the strong constitutive promoter of the Cauliflower Mosaic Virus CaMV-35S, between the "right" and the "left" border, the gene coding for the *GusA* gene, the *nptII* gene encoding neomycin phosphotransferase, which confers resistance to kanamycin, and the NOS terminator sequence of nopaline synthase gene. The pBIΩ was obtained by cloning the nucleotide sequence of "Ω translational enhancer" (Gallie et al., 1992) derived from the Tobacco mosaic virus TMV downstream the 35S-CaMV promoter, in the XbaI-BamHI sites, and replacing the *pgip2* with the *GusA* gene in the BamHI-SACI site (**Figure M2**).



FiguraM2. Schematic representation of the pBIΩ. Expression cassette**.** In yellow are reported the XbaI-BamHI site used for the insertion of the Ω translational enhancer and the Bam HI-SacI site used to replace the *pgip2* with the *GusA* gene.

3.4 Manipulation of *Escherichia coli* and *Agrobacterium tumefaciens*

3.4.1 Preparation of elettrocompetent *Escherichia coli* XL-1 Blue

Two millilitres of Luria Bertani (LB) medium (10 g/l bacto-tryptone; 5 g/l yeast extract; 10 g/l sodium chloride) was inoculated with 10 µl of XL-1 Blue (in 50 mM $CaCl_2$; 20 % (v/v) glycerol), and incubated overnight at 37 °C shaking at 250 rpm. One millilitre of this culture was then used to inoculate 100 ml of LB sterilised in a 1l conical flask. The cells were incubated at 37 °C shaking at 250 rpm until the culture had reached an $O.D._{600}$ of 0.6. The culture was then cooled on ice for 30 minutes, and transferred into two sterile 50 ml Falcon tubes. Cells were harvested by centrifugation at 3000 × *g* (Beckman centrifuge, Avanti JA-25) at 4 °C for 10 minutes. Centrifugation was repeated 3 times. The supernatant coming from the last centrifugation was discarded, and the pellets resuspended in 25 ml cold water each, by swirling. The resuspended cells were kept on ice for 30 minutes and centrifuged again as previously described. The supernatant was discarded, and the

pellet was gently resuspended in 3 ml cold 10 % (v/v) glycerol. Finally, a further 3 ml cold 10 % (v/v) glycerol was added and, after mixing, cells were transferred to pre-cooled 1.5 ml eppendorfs in 50 µl aliquots. These aliquots were frozen immediately in liquid nitrogen, and stored at −80 °C.

3.4.2 Preparation of electro-competent *Agrobacterium tumefaciens* LBA 4404

Several colonies from a stock agar plate were transferred to 100 ml LB media supplemented with 50 mg/ml rifampicin in a large conical flask. The culture was incubated at 28 °C shaking at 250 rpm overnight. Fifty millilitres of the overnight culture was used to inoculate a similar, 500 ml culture which was allowed to grow under the same conditions until $O.D._{600}$ of 0,6. The resulting culture incubated on ice for 20 minutes before centrifugation at 4,000 *g* for 15 minutes at 4 °C (Beckman centrifuge, Avanti JA-25). This step was repeated 4 times. The supernatant coming from the last centrifugation was discarded and the pellet resuspended in 25 ml ice-cold ddH2O. After the sixth centrifugation, the cell pellet was resuspended in 5 ml sterile ice-cold 10 % glycerol and combined into two 50 ml centrifuge tubes. Following a final centrifugation at 3,000 *g* for 10 minutes at 4 °C, the cell pellets were resuspended in 1 ml ice-cold 10 % glycerol and divided into pre-cooled Eppendorf tubes in 50 µl aliquots. Cells were either used immediately or flash-frozen in liquid nitrogen before storage at -80 °C for up to 1 year.

3.4.3 Transformation of electro-competent *Agrobacterium tumefaciens* LBA 4404 and *Escherichia coli* XL-1 Blue

Fifty nanograms DNA in 1 µl ddH2O was mixed with 50 µl electro-competent cells in a 1.5 ml eppendorf tube and immediately transferred to a pre-cooled electroporation cuvette (Biorad). The mixture was subjected to a 2,5 kV discharge with a capacitance of 25 mF and a resistance of 200 Ω (Biorad Gene Pulser). The newly-transformed cells were mixed with 1 ml LB broth and incubated horizontally at 28 °C in the case of LBA 4404 and 37 °C for XL1-Blue, shaking at 250 rpm for 1 hour. Cells were harvested by centrifugation at 3000 rpm (bench microfuge) for 2 minutes, 800 µl supernatant was removed, and the pellet was resuspended in the remaining broth and plated on LB-agar plates (LB, 2 % (w/v) bacto agar) containing the appropriate selective antibiotics. Plates were incubated inverted overnight at the specific temperature

3.5 Nucleic acid techniques

3.5.1 Preparation of plasmid DNA from *Escherichia coli* XL-1 Blue

Mini-preparation of plasmid DNA was performed using the QIAprep Spin Plasmid Kit. An overnight culture of the plasmid-containing *E. coli* was prepared by inoculating 3 ml LB, containing the appropriate selective antibiotic, with the colony of interest and incubating at 37 °C with 250 rpm shaking. The following day, 1.5 ml of this culture was transferred to a 1.5 ml eppendorf and cells were harvested by centrifugation at 13000 rpm (bench microfuge) for 1 minute. The supernatant was discarded, and the resulting cell pellet was used according to the manufacturer's protocol. The DNA was recovered in 50 µl of ddH_2O, and either used immediately or stored at −20 °C.

3.5.2 Restriction digestion of DNA

Restriction endonucleases were used to digest DNA constructs in order to prepare them for ligation. Typically, 1-2 µg DNA was incubated with the appropriate NEB (New England Biolabs) buffer to a final concentration of 1 X in the presence of 1 µl enzyme (5-10 U/µl) in a total volume of 20 µl at 37 °C for 1-2 hour. The resulting DNA fragments were analysed by agarose gel electrophoresis. When more than one enzyme was used on the same DNA, and these enzymes did not have compatible buffers, the cut DNA was isolated and purified between each digestion. When plasmid DNA was cut in preparation for the ligation of an insert, it was dephosphorylated by incubation with 1 µl (20 U/µl) Antarctic Phosphatase (AP, NEB) for 1 hour at 37 °C, before being isolated and purified.

3.5.3 Agarose DNA gel electrophoresis

Agarose gels were prepared with 1-2 % (w/v) agarose dissolved in 1 X TAE buffer (2 M Tris-Acetate, 0.05M EDTA, pH 8.3) containing 0.05 % (w/v) ethidium bromide, to stain the DNA. DNA samples were mixed with 5X DNA sample buffer (40 % (w/v) sucrose; 0.25 % (w/v) bromophenol blue, 10 % glycerol) to a final concentration of 1 X buffer, and the mixture loaded into the wells. DNA was subjected to electrophoresis at 80 V. The DNA was visualized by placing the gels on a short wave ultra-violet transilluminator.

3.5.4 Isolation and purification of DNA from agarose gels

DNA digests were carried out and run on agarose gels. Fragments were excised from the gel under short wave ultra-violet illumination, keeping exposure time to a minimum, using clean scalpel blades and transferred to eppendorf tubes. DNA was then either stored in the gel fragment at −20 °C, or purified immediately, according to the manufacturer's protocol, using the QIAprep Gel Extraction Kits. DNA was typically recovered in 30 µl ddH_2O, and either used immediately or stored at −20 °C.

3.5.5 Ligation of DNA fragments

DNA ligations were performed using T4 DNA ligase, either blunt-ended or following restriction endonuclease digestion and subsequent generation of cohesive DNA ends. DNA fragments, typically in a 5:1 molar ratio of insert to vector, were mixed with 2 µl of 5 X T4 DNA ligase buffer (250 mM Tris-HCl, pH 7.6; 50 mM $MgCl_2$; 5 mM ATP; 5m M DTT; 25 % (w/v) polyethylene-glycol 8000) and 1 µl T4 DNA ligase (1 U/µl), and made up to a total volume of 10 µl with ddH_2O. The mixture was normally incubated for 1.5 hours at 37 °C. Typically, 5 µl of the ligation reaction was used to transform competent *E. coli* cells.

3.5.6 DNA mutagenesis

Point mutations performed to obtain H10 Mut, H10 CH_1-VH, H10 CH_1-CH_2, H10 VH-CH_1-CH_2 were generated using *Pfu* DNA polymerase from the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Mutagenic primers were designed to contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. The primers were built in order to have a minimum G/C content of 40 %, a melting temperature (Tm) ~10 °C above the extension temperature of 68 °C, and with standard purity. 30 ng of dsDNA template was mixed with 125 ng of each oligonucleotide primer, 1 µl of 10 mM dNTP mix (2.5 mM of each NTP), and 5 µl of 10X reaction buffer, in a total reaction volume brought to 50 µl with ddH_20. 1 µl *Pfu* DNA polymerase (2.5 U/µl) was added, mixed, and the reaction subjected PCR (polymerase chain reaction) according to the manufacturer's protocol. Once at 37 °C, 1 µl Dpn1 (10 U/µl) restriction enzyme (10 U/µl) was added, and the reaction mixed and incubated for 1 hour at 37 °C to digest the parental, non-mutated DNA. 5 µl of the DNA left after this digestion, containing the nicked, mutated vector, was then used to transform competent cells.

3.5.7 Gene construction

All constructs encoding sequences were cloned into the pBI-Ω plant expression vector (Marusic et al., 2007) under the control of the constitutive CaMV 35S promoter and the nopaline synthase (NOS) terminator sequence. To enhance translational efficiency, the Tobacco mosaic virus (TMV) 5' leader sequence Ω (Gallie and Kado, 1989) was fused in frame with the protein sequences.

The H10 antibody was obtained starting from the anti-TNC scFv(H10) selected from the human ETH-2 phage display library (Viti et al., 2000) against hC-TNC, as described in Villani et al., (2009). Sequences encoding VH and VL, derived from human germ-line segments DP47 H and DPL16 L, were amplified from the pDNEK scFv(H10) plasmid using the following primers: SP-VH1for (5′-TCTTCCTCCTGTCAGGAGCTGCAGGTGGTACCTCGGAGGTGCAGCTGGTGGAGTCTGG-3′), VH-CH1rev (5′-CCTTGGTCGACGCTCTCGACACGGTGACCAGGGTTCC- 3′) and SP-VL1for (5′-GTCAGGAGCTGCAGGTGGTACCTCGTCTGAGCTGACTCAGGACCCTGC- 3′), VL-CL rev (5′-GCAGCCTTGGGCTGGCCTAGGACGGTCAGCTTGGTCC- 3′), respectively. The signal sequence of an embryonic mouse IgG HC gene was subsequently added at 5′ of both VH and VL sequences, amplifying with the forward primer SP2. For the generation of (H10) heavy chain and (H10) light chain constructs, the amplified VH and VL segments were fused to the heavy γ1 and light λ constant regions of a human IgG1 (provided as cDNA by Philogen S.p.A., Monteriggioni, Italy) by SOE-PCR (Ho et al., 1989). Briefly, a set of primers was used to generate two DNA fragments having overlapping ends using PCR with *Pfu* polymerase (Stratagene, La Jolla, CA, USA). PCR-generated DNA fragments were first purified from agarose gel and then used in a subsequent overlap extension reaction to obtain the resulting fusion products. The IgGγ1 constant region was amplified using the primers VH-CH1for (5′-CACCGTGTCGAGAGCGTCGACCAAGGGCCCATCGG- 3′) and CH3rev (5′-TCTTACCCGGGTCATTATTTACCCGGAGACAGGGAGAGGC- 3′), and the λ constant region was amplified using the primers VL-CLfor (5′-CCGTCCTAGGCCAGCCCAAGGCTGCCCCCTCG-3′) and CLrev (5′-CATTATGAACATTCTGTAGGGGCCACTGTCTTC-3′).

The H10-Mut was obtained mutating the Asn 297 to Ala by using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, USA). An oligonucleotide containing the site-specific mutation was used for the mutagenesis reaction following the manufacturer indications. H10-SEKDEL was constructed fusing the DNA sequence coding for the SEKDEL to the 3'-end of both HC and LC chain antibody coding genes. Fusion was conducted using specific primers (LCSEKDEL: 5'

TTGAGCTCTCATTACAGCTCATCCTTCTCTGATGAACATTCTGTAGGGGCCACTGTC 3'; HCSEKDEL: 5' TTGAGCTCTCATTACAGCTCATCCTTCTCTGATTTACCCGGAGACAGGGAGAGGCTCTT C 3') through an overlap extension PCR. For transient expression in *N. benthamiana* plants all constructs were cloned into the binary vector pBI-Ω, confirmed by DNA sequencing and introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The mAb H10 forms obtained with the agroinfiltration of this vector are indicated in **Table M1**:

Table M1: Description of all the H10 constructs used for transient expression in N. benthamiana plants of different H10 formats. An indication of the property of every antibody format is enlisted.

H10 FORM	***COSTRUCTS CO-EXPRESSED***	***ANTIBODY PROPERTY***
H10	p35:HC, p35:LC, p35:P19	Secretory form
H10 SEKDEL	p35:HC-SEKDEL, p35:LC-SEKDEL, p35:P19	ER retained form
H10 Mut	p35:HC-Mut, p35:LC, p35:P19	Aglycosylated form
$H10_{VH\text{-}CH1}$	p35:$HC_{VH\text{-}CH1}$, p35:LC, p35:p19	Mutated in the VH-CH1 region
$H10_{CH1\text{-}CH2}$	p35:$HC_{CH1\text{-}CH2}$, p35:LC, p35:p19	Mutated in the "hinge region"
$H10_{VH\text{-}CH1\text{-}CH2}$	p35:$HC_{VH\text{-}CH1\text{-}CH2}$, p35:LC, p35:p19	Mutated in both VH-CH1 and "hinge region"
$H10^{XylT/FucT}$	p35:HC, p35:LC (expressed in XylT/FucT RNAi-silenced *N. benthamiana* plants)	Antibody harbouring a "human like" glycosylation profile

For transient expression in tobacco protoplasts, 35S expression cassettes of all constructs were cloned in a pGEM-nos plasmid vector. The H10 forms obtained with this vector are enlisted in **Table M2**:

Table M2: Description of all the H10 constructs used for transient expression in tobacco protoplasts of different H10 formats. An indication of the property of every antibody format is indicated.

H10 FORM	***COSTRUCTS EXPRESSEDIN PROTOPLASTS***	***ANTIBODY PROPERTY***
H10	pGem:HC, pGem:LC	Secretory form
H10 SEKDEL	pGem:HC-SEKDEL, pGem LC-SEKDEL	ER retained form
H10 Mut	pGem:HC-Mut, pGem:LC	Aglycosylated form

3.6 Manipulation of *N. benthamiana* and *N. tabacum* plants

3.6.1 Agroinfiltration of *Nicotiana benthamiana* leaf cells by needleless syringe

Five millilitres overnight cultures of *A. tumefaciens* transformed with the different constructs (p35: HC, p35:LC, p35:p19) were grown separately to $O.D._{600}$ of 0.4–1.5 at 28 °C shaking at 250 rpm. The culture was centrifuged at 5000 *g* in a Eppendorf 5810R centrifuge for 5 minutes. The pellet was resuspended in a volume of infiltration medium (10 mM MES, 10 mM $MgCl_2$, pH 5.5) to reach a final $O.D._{600}$ of 0.5-0.6 for each Agrobacterium. The cells were pressure injected into the lower epidermis of the leaf through an 1 ml disposable syringe. Multiple transformation was achieved by mixing relevant cultures prior to infiltration. The plant was then incubated under a 16 hr /8 hr light /dark regime at room temperature for 2-6 days before protein extraction.

3.6.2 Vacuum agroinfiltration of *Nicotiana benthamiana* plants

Large scale transient expression in *N. benthamiana* was performed by vacuum agroinfiltration. 300 ml of each *A. tumefaciens* clone harbouring the specific constructs (p35: HC, p35:LC, p35:p19) were grown separately, and bacteria were pelleted by centrifugation at 4000 *g*, resuspended in infiltration buffer (10 mM 2-(*N*- morpholino) ethanesulphonic acid (MES), 10 mM $MgSO_4$, pH 5.8) and mixed together in order to reach a final $O.D._{600}$ value of 0.5-0.6 for each construct. Six-week-old *N. benthamiana* plants (at the six- to seven-leaf stage) were infiltrated by completely submerging each plant in the *Agrobacterium*-containing solution inside a vacuum chamber. Vacuum was then applied, reaching approximately 10 mmHg, and then quickly released. Infiltration was confirmed visually, observing infiltrated areas as translucent. The $H10^{XylT/FucT}$ antibody was obtained by agroinfiltrating plants of the XylT/FucT *Nicotiana benthamiana* transgenic line (Strasser et al., 2008) with a suspension of *A. tumefaciens* clones harbouring HC and LC encoding sequences exactly as previously described. For antibody purification, batches of 40 g of agroinfiltrated leaves were collected and directly processed.

3.6.3 Preparation of protoplasts from *N. tabacum* SR1 leaves

An enzyme mix (Macerozyme Onozuka R-10, 2 % (w/v); Cellulase Onozuka R-10, 4 % (w/v) in K3 medium (Gamborg's B5 basal medium with minimal organics, 3.78 g/l; $CaCl_2.2H_2O$, 750 mg/l; NH_4NO_3, 250 mg/l; sucrose, 136.2 g/l; xylose, 250 mg/l; 6-BAP, 1 mg/l; a-naphtalenacetic acid

(NAA), 1 mg/l) was poured into 10 cm Petri dishes in 7 ml aliquots. 4-6 week old green leaves were cut from a sterile plant and carefully scarified on their underside (operating quickly in order to avoid excess dehydration of the leaf) and incubated overnight in the dark at 26 °C. The digestion mix was carefully removed using a sterile Pasteur pipette and discarded, leaving protoplasts still attached to the leaves. 3 ml of K3 was added drop wise over the leaves, and the plates gently agitated in order to release the protoplasts. The resulting protoplast solution was removed, using a fresh sterile Pasteur, and filtered through a 100 μm brass sieve, flamed and wet with K3 medium before use. The harvested, filtered protoplasts were centrifuged at 100 *g* (600 rpm, Beckman GPR bench centrifuge, GM3.7 rotor) for 20 minutes at room temperature. The pellet of broken protoplasts and most of the K3 medium in excess were removed, using a Pasteur pipette connected to a 25 ml pipette. The remaining 5 ml of medium contained the floating layer of viable protoplasts. After mixing gently to redistribute this floating layer, 4 volumes of W5 (NaCl, 9 g/l; KCl, 0.37 g/l; $CaCl_2.2H_2O$, 18.37 g/l; glucose, 0.9 g/l) was added drop-wise down the wall of the tube. Protoplasts were pelleted by centrifugation at 100 *g* (600 rpm, Beckman GPR bench centrifuge, GM3.7 rotor) for 10 minutes at room temperature, and the supernatant aspirated off and discarded. Protoplasts were pelleted again and the supernatant discarded. Finally, protoplasts were carefully resuspended in 10 ml of W5 and incubated in the dark for 30 minutes at room temperature, and of 50 μl of sample was removed for cell count.

3.6.4 Pulse-chase analysis

Forty micrograms of each plasmid (*pGemHC, pGemLC, pGemHC-Mut, pGemHC-SEKDEL, pGemLC-SEKDEL*) were used to transform 10^6 protoplasts in a volume of 1 ml. Pulse-chase and immunoprecipitation were performed essentially as described before (Hadlington et al., 2003). Briefly radioactive labeling was performed by incubation 1 h at 26 °C in the dark in K3 medium (Gamborg's B5 basal medium with minimal organics, 3.78 g/L; , supplemented with 750 mg/L $CaCl_2$~$2H_2O$, 250 mg/L NH3NO, 136.2 g/L sucrose, 250 mg/L xylose, 1 mg/L 6-benzylaminopurine, and 1 mg/L a-naphthalenacetic acid, pH 5.5) supplemented with 150 μg/mL of BSA and 7 μCi/mL of PRO-MIX (^{35}S-methionine and cysteine; Amersham). Unlabeled methionine and cysteine (10 mM and 5 mM, respectively) were used for the chasing. The zero time-point was taken by removing protoplasts, immediately after mixing. The protoplasts were pelleted by centrifugation at 100 g at 4 °C for 10 minutes. The supernatant was centrifuged as before, in order to precipitate any protoplasts carried over from the pellet. The subsequent time-points (2.5 and 5 hours) were taken in the same manner, and samples were stored at −80 °C until use. Harvesting was

performed at 0, 2.5 h, 5 h, after the chasing. Protoplasts and incubation media were frozen and homogenized by adding two volumes of ice-cold homogenization buffer (150 mM Tris-HCl, 150 mM NaCl, 1.5 mM EDTA, and 1.5 % (wt/vol) Triton X-100, pH 7.5). Immunoprecipitation of expressed polypeptides was performed using a 1:1 mix of Protein-A-Sepharose® (P3391 Sigma Aldrich) and Protein G Sepharose®, (P3296 Sigma Aldrich). Separation was performed by SDS-15 % (w/v) PAGE. Gels were vacuum dried at 80 °C, using a gel drier (Bio-Rad 583), before being exposed to X-ray film (Kodak BioMax MR, UK) in a light-proof cassette stored at −80 °C for an appropriate length of time. Films were developed under darkroom conditions using an automatic developer (Agfa Curix 60).

3.7 Protein extraction, separation and detection

3.7.1 Protein extraction procedures: Total leaf extract (small scale), Pilot-Scale extraction, isolation of leaf Intercellular Fluids

Batches of fresh leaves (40 g) were homogenised in 80 ml of extraction buffer (1 X Phosphate Buffered Saline (1 X PBS) containing protease inhibitors (Complete™, Roche, Germany), using an Ultra-Turrax homogeniser T25 equipped with a S25N-18G disperser (IKA, Staufen Germany). After homogenisation (three pulses of 1 min at 17,500 rpm in ice), the slurry was filtered through Miracloth (Sigma-Aldrich, USA) and centrifuged twice at 8,000 *g* for 20 minutes at 4 °C. For purification, after microfiltration through 0.45 μm and 0.20 μm Minisart filters (Sartorius, Germany), the extract was directly loaded on a HiTrap™ Protein-A HP column (GE Healthcare, Uppsala, Sweden). Total protein and antibody concentration were determined by Bradford and ELISA, respectively.

For pilot-scale purifications, batches of 250 g of leaves (stored at -80 °C) were homogenised using a blade blender in extraction buffer (PBS pH 7.4, EDTA 5 mM, and protease inhibitor cocktail 1 % (v/v) to a final volume of about 750 ml. The extract was then centrifuged in a Beckmann JA-10 rotor for 25 min at 8,000 rpm at 4 °C. The pH of the recovered supernatant was adjusted at pH 7.5 with 5 M NaOH and centrifuged again as described above. The supernatant was pre-filtered through a 0.8 μl filter and subsequently through a 0.22 μl Stericup filter (Millipore, MA, USA). Total protein and antibody concentration were determined by Bradford and ELISA, respectively.

Intercellular fluids, from agroinfiltrated *N. benthamiana* leaves, were collected through an infiltration-centrifugation method according to a previous work with minor modifications (Lohaus et al., 2001). In order to obtain intercellular fluids for affinity chromatography purification, 80 g of

full-size leaves were collected from 15 plants of *N. benthamiana* at 6 d.p.i. and immediately infiltrated in 1 l of room temperature infiltration buffer (PBS, 10 mM phenylmethnesulphonyl fluoride PMSF) using a vacuum-infiltration system. After vacuum release, leaves were gently paper-dried (15 leaves at a time) and put in 50 ml tubes previously perforated at the bottom to allow liquid flow through. Tubes were placed in Beckman 250 ml tubes and centrifuged at 800 g for 10 minutes in an eppendorf centrifuge 5810 R (A-4-62 rotor). From this step, 80 ml of clear fluid (about 1 ml of IFs per g of fresh leaves) were collected. After microfiltration through 0.45 and 0.20 ml Minisart filters, the fluid was directly loaded on affinity chromatography.

3.7.2 Isolation and purification of protein bands from polyacrylamide gels

Purified antibodies obtained from protein A chromatography were run by SDS-PAGE in non reducing conditions in order to obtain the separation of antibody fragment. Fragments were excised from the gel, using clean scalpel blades and transferred to eppendorf tubes. Proteins were immediately ground with a pestel in presence of 50-70 µl of PBS 1 X and subsequently centrifuged at 15000 *g* at 4 °C for 5 minutes. Supernatants containing the protein fragments were recovered and subjected to Western Blot (25 µl of sample per lane), ELISA (70 µl of sample per well).

3.7.3 Western blotting of SDS-polyacrylamide gels

Protein samples obtained by leaf extracts separated by SDS-PAGE were transferred from gels onto PVDF membrane (prepared by soaking briefly in MeOH and then for 2 minutes in transfer buffer (25 mM Tris; 192 mM glycine; 20 % MeOH) in transfer buffer using Sigma-Aldrich techware Semi-dry blot apparatus under a constant current of 40 mA/gel for 1 hour. The membrane was then blocked in phosphate-buffered saline (PBS, 137 mM NaCl; 0.27 mM KCl; 10.4 mM disodium hydrogen phosphate; 1.8 mM potassium dihydrogen phosphate) containing 5 % (w/v) dried skimmed milk powder and 0.2 % Tween-20 with agitation over night. The block was discarded and the membrane was washed 3 times, 10 minutes with PBST 1 X (PBS 1 X, 0.05 % Tween 20) and 2 times 10 minutes with PBS 1 X, than incubated, with continual agitation, in 20 ml PBST 1 X containing the primary antibody or the concanavalin A for 2 hour. In the case of non-conjugated primary antibodies, the membrane was washed, as described above, and incubated in 20 ml PBST 1 X containing the horseradish peroxidase-conjugated secondary antibody for 1 hour. The membrane was then washed as before, and detection of immunoreactive bands performed using the ECL kit

(Enhanced chemiluminescence, Amersham, UK), according to the manufacturer's instructions, and recorded by exposing to X-ray film (Kodak BioMax MR, UK) in a light-proof cassette for an appropriate time. The proteins and antibodies used in the Western blot experiments are listed in **Table M3**.

Purified IgGs from agro-infiltrated plants were analysed by reducing and non-reducing SDS-12 % (w/v) PAGE (Tavladoraki et al., 1999) and stained with Coomassie or silver nitrate (Merril et al. 1981). Plant extracts were also analysed by Western blot as previously described. Glycans were analysed using biotin-conjugated Concanavalin A from *Canavalia ensiformis* (C2272 Sigma) diluted 1:10,000 in 1 % BSA, 1 X PBS and peroxidise conjugated streptavidin (S5512, Sigma) diluted 1:12,000 in 0.1 % BSA, 1 X PBS. Proteins were detected by enhanced chemioluminescence (ECL, Plus, GE Healthcare, UK).

In the case of band shift analysis of H10-SEKDEL and H10-Mut heavy chain, reducing SDS-9 % (w/v) PAGE was performed running gels for a long time period (2.5 h).

Table M3: Reagents used in Western Blot and ELISA experiments.

Antibody/protein	Antigen	Dilution	Used as	Obtained in	Supplier
Antibody A8419	Human γ-Chain	1:5000	Primary Ab	Goat	**Sigma-Aldrich**
Antibody A5175	Human λ-Chain	1:5000	Primary Ab	Goat	**Sigma-Aldrich**
***Canavalia Ensiformis* Type IV C2272**	Plant Lectins	1:12000	Primary ligand (Biotinylated)	Rabbit	**Sigma-Aldrich**
Streptavidin-PO S5512	Biotin	1:10000	Secondary ligand		**Sigma-Aldrich**

3.7.4 ELISA assays

For the quantification of HC and LC expression, agroinfiltrated leaves from *N. benthamiana* were analyzed by double-antibody-sandwich ELISA. Eighty milligrams of leaf tissue were ground in liquid nitrogen and homogenized in 500 µl of PBS containing protease inhibitors (Complete™, Roche, Mannheim, Germany). After centrifugation at 15000 g for 30 min at 4 °C, the supernatant was recovered and quantified for total soluble protein (TSP) using the Bradford colorimetric assay, as specified by the manufacturer (Bio-Rad, Hercules, CA, USA). The capture antibody (anti-human γ chain (8419, Sigma-Aldrich) or anti-human λ chain (5175, Sigma-Aldrich) at a concentration of 2

μg/mL in 1 X PBS) was coated directly on to Nunc-Immuno Maxisorp wells (Nunc-Immuno, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then blocked with 2 % milk (w/v) in PBS at 37 °C for 2 h. After washing, different dilutions of leaf extracts, normalized for TSP concentration, were added to the wells (100 μl) and incubated at 37 °C for 2 h. After washing, the anti-human γ chain horseradish peroxidase (HRP)-conjugated (8419, Sigma-Aldrich) or anti-human λ chain HRP-conjugated (5175, Sigma-Aldrich) antibodies were added at a dilution of 1:5000 in 2 % milk (w/v) in PBS and incubated for 1 h at 37 °C. Enzymatic activity was measured after 30 minutes at 405 nm on a microtitre plate reader (TECAN-Sunrise, Groedig, Austria) using 2,2-azino-di-3- ethylbenz-thiazoline sulphonate (ABTS, KPL, Gaithersburg, MD, USA). As a positive control, human IgG1-γ (I5029, Sigma-Aldrich) at different dilutions (ranging from 10 to 100 ng) was used.

Quantitative ELISA on agroinfiltrated leaf extracts was performed by coating the recombinant BCD domains of mouse TNC (mTN-C BCD) (provided by Philogen S.p.A.) at a concentration of 20 μg/ml in PBS 1 X onto Nunc-Immuno Maxisorp plates (Nunc-Immuno, Roskilde, Denmark) and overnight incubation at 4 °C. Plates were blocked and serial dilutions of leaf extracts (100 μl per well), normalised for TSP content using the Bradford colorimetric assay (Bio-Rad, Germany), were incubated as described before. After washing, the anti-human λ-chain HRP-conjugated (1: 5,000 in PBS 1 X) was added to detect bound mAb H10 and enzymatic activity was measured after 30 minutes at 405 nm on a microtitre plate reader as previously described. As an internal standard, we used mAb H10 previously purified by protein-A and cation-exchange chromatography from agroinfiltrated *N. benthamiana* leaves at known concentrations (ranging from 1 to 100 ng) spiked in wild type *N. benthamiana* extract. Each sample was assayed in triplicate and concentrations were interpolated in the linear portion of the standard curve.

3.7.5 2-D Gel elettrophoresis analysis

For 2-DE analysis, solubilized protein samples were supplemented with 350 μL of isoelectrofocusing (IEF) rehydration buffer (7 M urea, 2 M thiourea, 13 mM DTT, 2 % w/v ASB-14, 1 % IPG buffer) and incubated with IPG-strips 3-11NL/18 cm (GE Healthcare, Uppsala, Sweden) O/N at room temperature. IEF was performed on an IPGphor unit (GE Healthcare, San. Francisco, CA, USA) at 20 °C, using a 50 μA current limit per strip and the following program settings: 10 h at 30 V, 1 h at 200 V, 30 min at 3500 V gradient and 3 h at 3500 V step and hold, 2 h and 30 min at 8000 V gradient and 8 h at 8000 V step and hold. After focusing, the IPG strips were

incubated in 10 mL of equilibration buffer (6 M urea, 30 % w/v glycerol, 2 % w/v SDS, 0.002 % w/v bromophenol blue, 50 mM Tris pH 8.8) containing 1 % w/v DTT for 15 min, and subsequently in 10 mL of the same buffer containing 2.5 % w/v iodacetamide for 15 min. The amount of protein loaded onto an IPG-strip for analytical and preparative runs was 100 μg and 1 mg, respectively. Second dimension was run on 12.5 % (w/v) polyacrylamide gels (18 cm × 20 cm × 1 mm) in 250 mM Tris-HCl, pH 8.3, 1.92 M glycine, 1 % w/v SDS, at 15 °C, applying 2 W/gel for 30 min and 20 W/gel for the remaining 4−5 h, using an Ettan DALT twelve unit (GE Healthcare, San Francisco, CA, USA).

3.7.6 N-glycan analysis

N-Glycan analysis was performed as previously reported (Kajiura et al., 2010) with some modifications. In brief, N-glycans from purified H10 variants were released by hydrazinolysis at 100 ºC for 10 h. The hydrazinolysates were lyophilized and N-acetylated with saturated sodium bicarbonate and acetic anhydrade, followed by desalting with Dowex 50 x 2 (Muromachi Kagaku Kogyo Kaisha, Tokyo, Japan). Pyridylamination of the oligosaccharides obtained was described previously (Misaki et al., 2001) and purified by size fractionation-high-performance liquid chromatography (HPLC) using Shodex Asahipak NH2P-50 4E (4.6 mmID x 250 mm, SHOWA DENKO Co. Ltd., Tokyo, Japan). Pyridylaminated (PA) sugar chains were detected by reverse phase (RP)-HPLC. The mobile phase composed of 0.02 % trifluoroacetic acid (solvent A) and 20 % acetonitrile/0.02 % trifluoroacetic acid (solvent B). RP-HPLC was performed using a Cosmosil 5C18-AR-II column (4.6 x 250 mm, Nacalai tesque, Kyoto, Japan) with HITACHI LaChrom by linearly increasing solvent B concentration from 0 to 20 % over 35 minutes at a flow rate of 1.2 mL/min. The eluted fractions were monitored by measuring fluorescence intensity using excitation and emission wavelengths of 310 and 380 nm, respectively.

The molecular masses of the collected PA-sugar chains and the number of their sugar moieties were estimated by LC-MS(/MS) using Agilent Technologies 1200 series (Agilent Technologies, Santa Clara, CA) equipped with a HCT plus (BRUKER DALTONICS, Bremen, Germany). In LC part, the mobile phase was composed of acetonitrile/acetic acid (solvent A: 98/2, v/v) and water/acetic acid/triethylamine (solvent B: 92/5/3, v/v/v). The PA-sugar chain was separated using Shodex Asahipak NH2P-50 2D (2.0 mmID x 150 mm) by linearly increasing solvent B concentration from 20 % to 55 % over 35 minutes at a flow rate of 0.2 mL/min. MS/MS parameters were as follows: scan range m/z = 350 – 2750, nebulizer flow of 5.0 psi, dry gas flow rate of 3.0 L/min, dry temperature of 300 ºC, target count of 200,000, MS/MS Frag. Ampl. of 1.0 V in the positive-ion

mode. N-Glycan structures were speculated by their elution positions comparing with glucose-oligomer units in RP-HPLC (Tomiya et al., 1991) and the intensities of product ions detected in LC-MS/MS comparing with in-house PA sugar chain libraries.

Analysis of glycans was also performed by Western blot using Concanavalin A biotin conjugate from *Canavalia ensiformis* (Jack bean), Type IV (Sigma C 2272). Samples were normalized for TSP and separated by SDS 9 % (w/v) PAGE under reducing conditions, and subsequently blotted on a PVDF membrane. Blocking was performed in PBS 1 X, 2,5 % BSA over night at 4 °C. The immunoblotting was performed with Concanavalin A biotin conjugate at a concentration of 1:12000 in PBS 1 X, 0,1 % BSA, 1h at room temperature followed by incubation with Streptavidin-PO (S 5512, Sigma-Aldrich) diluted 1:10000 in PBS 1 X, 0,1 % BSA, for 20 minutes at room temperature. Proteins were detected as described before.

3.7.7 IgG purification

For small scale mAb purifications, 40g of agroinfiltrated leaf fresh weight were used for affinity chromatography purification. Clarified supernatants from mechanical homogenisation extraction procedure and intercellular fluids were purified using separate affinity chromatography columns (1 ml HiTrap™ Protein-A HP, 1 ml HiTrap™ Protein-G HP and agarose conjugated anti-Fc (109-035-003, Jackson laboratories) column.

All columns were previously equilibrated with extraction buffer, at a flow rate of 1 ml/min. The column was washed with 10 ml of 1 X PBS (10 column volumes) and the antibody was eluted with 100 mM citrate, pH 3.0 and buffered with 1:5 (v/v) 1 M Tris-HCl, pH 8.0. Total protein concentration was determined by the colorimetric Bradford assay. Antibody yield was evaluated as the amount of antibody eluted from the chromatographic column/antibody loaded into the column. Antibody containing fractions were pooled, dialyzed against 1 X PBS using a PD10 column (GE Healthcare, Germany) following manufacturer instructions and concentrated in Centricon YM30 (Amicon, Bedford, MA, USA). Antibody concentration was also determined spectrophotometrically at 280nm absorbance (Gill and von Hippel, 1989).

3.7.8 Gel filtration Analysis

Purified H10 antibody variants were analysed by size-exclusion chromatography carried out at 20 °C in 1 X PBS on a calibrated Superdex^{TM} 200 5/150 GL column (GE Healthcare, Sweden) (at 0.3

ml/min flow rate) using an ÄKTA FPLC P920 instrument (GE Healthcare, Sweden). Protein absorbance expressed as Absorption Units (mAU) was measured at 280 nm. Column calibration was performed using gel filtration calibration kits (Low and High Molecular Weight, GE Healthcare) following manufacturer instructions. A purified human IgG_1-λ (I5029, Sigma-Aldrich) was also used as a positive control. Peaks representing different protein fractions were collected using a fraction collector FRAC-920 (GE Healthcare, Sweden). For a better interpretation of results, the chromatograms are presented over Kav = (elution volume -void volume)/(column volume - void volume) instead of elution volume or retention time.

3.7.9 Surface plasmon resonance analysis

Binding properties of plant purified H10 from pilot scale purifications were evaluated by surface Plasmon resonance (SPR) using a BIAcore biosensor system instrument (GE Healthcare) as previously described (Villani et al., 2009). Approximately 3400 resonance units (RU) of purified H10 (20 ng/ml in 10 mM acetate buffer, pH 5.5) were coupled to the dextran matrix of a CM5 sensor chip using the Amine coupling kit (GE Healthcare) at 5 ml/min flow rate.

To estimate the apparent equilibrium constants (KD), concentrations of recombinant BCD domains of mouse TNC (mTN-C BCD) ranging from 500 ng to 3 μg in PBS were injected. All binding experiments were performed at 25 °C in PBS at 20 ml/min flow rate and pulses of 10 mM NaOH were used to regenerate the sensor chip. Rate constants were calculated on the basis of a single site model using the BIAevaluation 3.0 software (GE Healthcare).

3.7.10 N-terminal sequencing

The N-terminal sequencing analysis was performed on protein fragments immobilized on a PVDF membrane using a filter device (ProSorb from Perkin Elmer) which allows buffers to be washed out. Each degradation band was excised from the membrane and analyzed by Edman Degradation reaction (Edman, 1950), using an ABI Procise 494HT Protein Sequencer. The analysis was restricted to five residues per band.

The analysis was performed at PNAC Facility (Protein & Nucleic Acid Chemistry Facility) Department of Biochemistry, University of Cambridge.

3.8 Plant contaminants detection

3.8.1 Analysis of polyphenols and alkaloids

Determination of total phenolics was essentially carried out as described before (Platis et al., 2008). To 1 ml of sample 500 μl of Folin–Ciocalteau reagent (Sigma–Aldrich) were added. The samples were prepared by diluting 100, 50, 20 and 10 μl of *N. benthamiana* extracts, intercellular fluids or purified fractions eluted from protein A or cation exchange chromatography, in bi-distilled water to a final volume of 1 ml. A Na_2CO_3 solution (20 % w/v, 2 ml) was then added and incubated at room temperature before reading the absorbance at 725 nm. As concentration standard serial dilutions of gallic acid (Sigma–Aldrich) dissolved in bi-distilled water was used (0–450 μg/ml). Total alkaloids were semi-quantitatively determined from the samples described above using Dragendorff's reagent (Sigma-Aldrich) as previously described (Platis et al., 2008).

3.8.2 Endotoxin removal and analysis

Endotoxin removal was performed by affinity chromatography using an Endo Trap Red column (Hyglos GmbH, Germany) following manufacturer instructions. Briefly, column was regenerated with 3 column volumes of regeneration buffer (provided by manufacturer) and then equilibrated with 3 column volumes of equilibration buffer (10 mM Na_2HPO_4, 80 mM NaCl, pH 7.4). Three millilitres of purified mAb H10 (0.5 mg/ml) were added to the column and the flow through was collected. Protein concentration was determined spectrophotometrically (280nm absorbance). This process was repeated twice. Endotoxin levels in the plant purified antibody samples were determined using the Multi Test Lymulus Amebocyte Lysate (LAL; sensitivity limit of 0.25 endotoxin units (EU)/ml) Pyrogent (CapeCod, MA, USA). Endotoxin-free water, pipette tips and test tubes were used (ACILA, Germany). To determine the sensitivity of the LAL reaction and obtain a reference standard, 4 dilutions starting from 1 EU/ml up to 0.06 EU/ml of lipopolysaccharide (LPS) from E. coli 0113:H10 (CapeCod, MA, USA) were prepared and tested in apyrogen certified tubes using manufacturer instructions to determine the lowest concentrations of LPS giving a positive LAL reaction.

The presence of LPS was confirmed by gelification of the sample mixed with the amebocyte lysate after 1 h incubation at 37 °C. Tests were performed on plant purified mAb H10 before and after two cycles of Endo Trap Red affinity chromatography by mixing with the lyophilised amebocyte lysate

100 µl of the undiluted sample as well as 100 µl of eight different dilutions (1:2; 1:4; 1:8; 1:16; 1:64; 1:128; 1:512; 1:1,024) and incubating 1 h at 37 °C without shaking.

3.9 Bioinformatic analysis

To identify Ab residues potentially responsible for cleavage by *N. benthamiana* peptidases, we searched the MEROPS database (Rawlings et al, 2010; http://merops.sanger.ac.uk/), release 9.5, for all the peptidases encoded by the genomes of *N. benthamiana* or other plants belonging to the *Nicotiana* genus. For each of the peptidases assigned to these organisms we gathered information about their substrate specificity.

Blast (Altschul et al., 1997) has been used to search the NCBI sequence database of proteins whose three-dimensional (3D) structures have been experimentally determined for Ab domains with high sequence identity with H10. The structures of these Abs have been downloaded with the Protein Data Bank (Rose et al., 2011). InsightII (Accelrys Inc.) has been used for structure visualization and analyses, DSSP for secondary structure assignment (Hooft et al., 1996) and Naccess (Hubbard and Thornton, 1993) for accessible surface area calculations. Ab structural domains have been defined as in the Structural Classification Of Proteins database (SCOP) (Andreeva et al., 2008).

4. RESULTS AND DISCUSSION

4.1 OPTIMIZATION OF PLANT EXTRACTION AND PURIFICATION OF THE SECRETORY mAb H10

In the first part of the thesis we analyzed different steps of protein extraction from agroinfiltrated *N. benthamiana* leaves in order to optimize the purification process of the secretory mAb H10. The aim of the work was to offer additional information about the strategies able to improve antibody extraction/purification on large-scale. The extraction procedures analyzed were the mechanical homogenization and recovery of intercellular fluids (IFs) in order to compare the purified immunoglobulins, in terms of antibody yields, proteolytic degradation and total phenolic compounds content. Final data emphasized that the application of different extraction methodologies significantly affected the integrity and yield of antibody purified from agroinfiltrated *N. benthamiana* leaves. According to these data, we optimized a pilot-scale purification method, using a two-step purification protocol from 250 g of fresh agroinfiltrated leaves leading to the purification of milligrams of fully assembled and functional antibody, free of phenolic and alkaloid compounds and with low endotoxin levels.

4.1.1 Plant Material

Transient expression of the secretory mAb H10 was performed by co-agroinfiltrating *N. benthamiana* plants with recombinant *Agrobacterium* strains, containing the plant expression vectors harbouring specific 35S T-DNA cassettes.

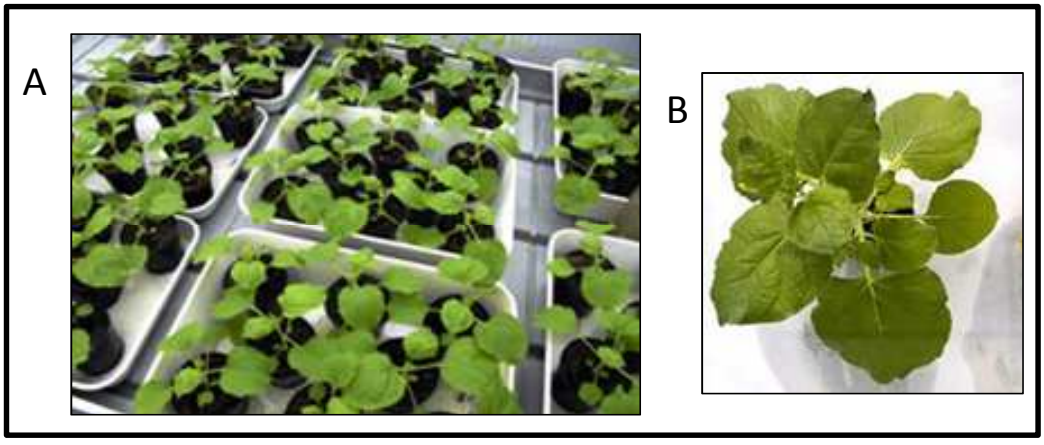


Figure R1: A) Wild type *N. benthamiana* plants placed in 4-inch pots and organized in vessels to facilitate watering. B) 6-8 leaves stage plant.

In order to prepare the plant material to be used for the agroinfiltration process, wild type *N. benthamiana* plants were typically grown from more than 100 seeds per batch in soiled-filled seed trays. Seedlings were transplanted into individual pots with the aim to optimize growth conditions (**Figure R1**, panel **A**).

All plants were grown in a containment greenhouse (16 hour light /dark cycle) (**Figure R2**) at a constant temperature of 26 °C. 4–6 weeks old *N. benthamiana* plants, at a 6-8 leaves stage (**Figure R1**, panel **B**), were used for agroinfiltration.

Preparation of *Agrobacterium* cultures was carried out growing the specific strains in LB medium in 1L flasks. Bacterial cultures were incubated over night at 28 °C and, after centrifugation, bacterial pellet was resuspended in agroinfiltration buffer.

Figure R2: Containment greenhouse (P_2 level) at ENEA Casaccia Research Center, Rome.

4.1.2 H10 transient expression based on vacuum agroinfiltration

As previously described in Materials and Methods section, transient expression system used for the production of H10 was performed by vacuum agroinfiltration, a strategy able to ensure infiltration of several plants with high efficiency. *A. tumefaciens* clones containing the H10 Heavy Chain (p35:HC), Light Chain (p35:LC) and P19 (p35:p19) expression cassettes were used for the co-agroinfiltration strategy. The use of the P19 gene silencing suppressor protein from Artichoke mottled crinkle virus (AMCV) allowed to enhance the yield of recombinant product. All the vectors harbouring these expression cassettes were electroporated into *A. tumefaciens* strain LBA4404 and used in co-agroinfiltration experiments. A schematic representation of the expression cassettes used to achieve the expression of the secretory mAb H10 is reported in **Figure R3**.

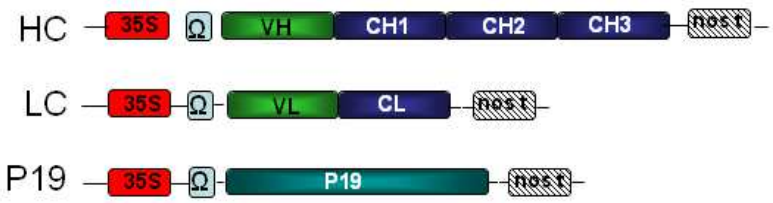


Figure R3: Schematic representation of the constructs used in the agroinfiltration experiments performed to obtain the secretory mAb H10. A detailed description of the construction of every expression cassette is presented in Material and Methods section.

All genes were cloned in the pBI-Ω plant expression vector under the control of the CaMV 35S promoter, containing the TMV Ω translational enhancer and the NOS terminator sequence (Marusic et al., 2007).

For vacuum infiltration, *Agrobacterium* suspensions harbouring the different vectors were mixed in equal volumes to obtain a final optical density value ($O.D._{600}$) of 0.6 for each suspension in the infiltration buffer. The agroinfiltration was conducted by completely submerging each plant in the *Agrobacterium*-containing solution inside a vacuum chamber (**Figure R4**, panel **A**).

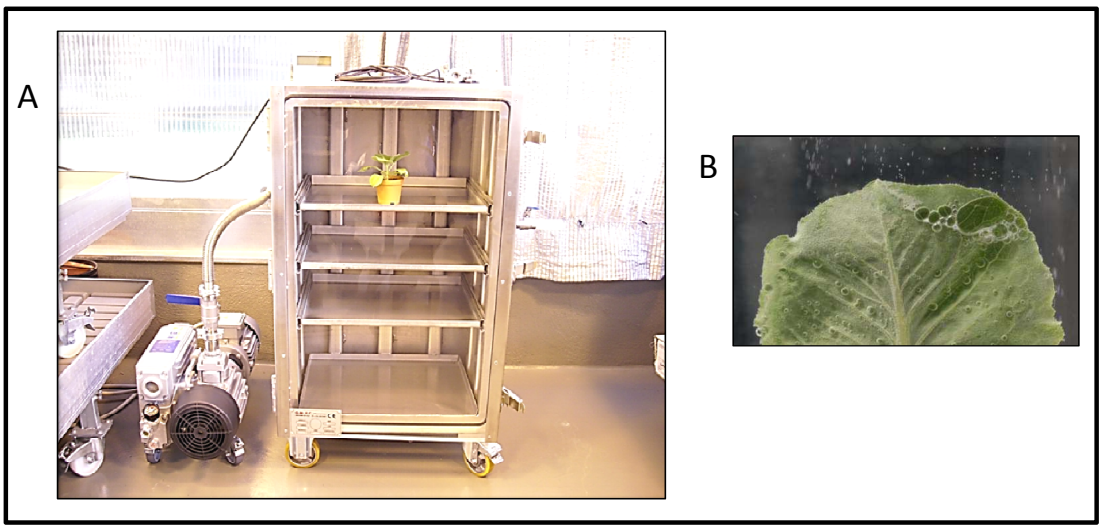


Figure R4: A) Vacuum chamber used for the agroinfiltration of *N. benthamiana* plants. B) Detail of a submerged plant leaf subjected to vacuum

Vacuum was then applied and quickly released in order to drive the entrance of the agrobacterium solution into the leaves apoplast (**Figure R4**, panel **B**). After agroinfiltration (**Figure R5**, panels **A** to **D**), plants were grown for six days in the greenhouse before the extraction/purification process. The extraction process was typically performed starting from batches of 40 g of fresh agroinfiltrated leaves and homogenization in extraction buffer was achieved using an Ultra-Turrax homogenizer.

After homogenisation, the slurry was filtered through Miracloth paper and centrifuged. For purification, the clarified extract was directly loaded on a HiTrap Protein-A HP column.

For large scale purifications, extraction was performed starting form batches of 250 g of vacuum agroinfiltrated leaf material obtained by vacuum agroinfiltration of high number of plants. This system allowed the purification of milligrams of fully assembled, functional and pure antibody free of phenolic and alkaloid compounds. A better description of the system is present in **Paragraph 4.1.10.**

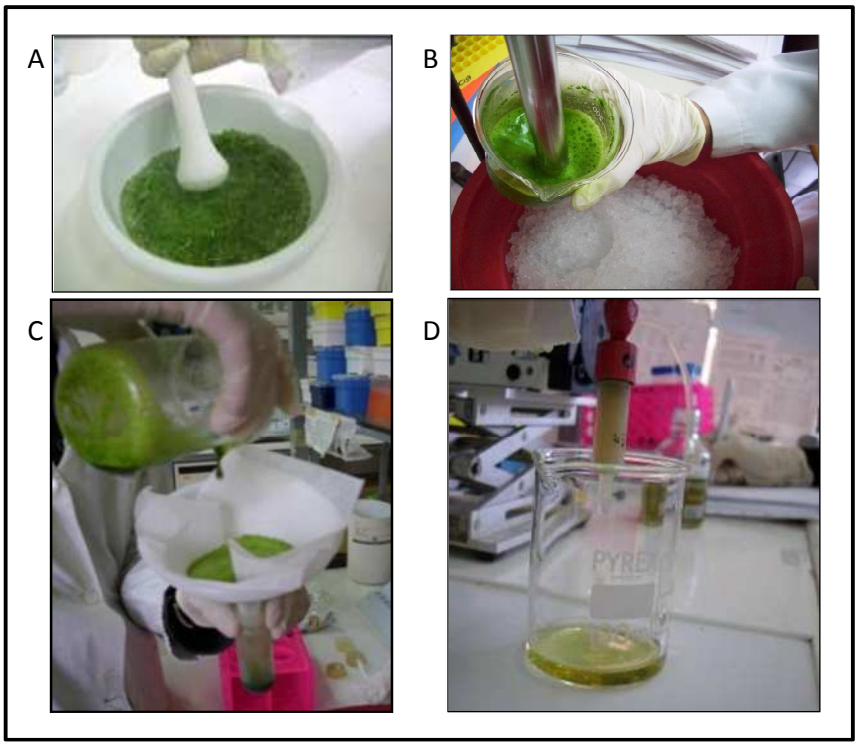


Figure R5: Stages of a typical lab-scale extraction and purification process of the mAb H10 from agroinfiltrated *N. benthamiana* plants. A) Leaves disruption was performed with pestle and mortar in presence of liquid nitrogen obtaining a fine powder. B) The powder was placed in a beaker with agroinfiltration buffer and homogenized with an Ultraturrax homogenizer. C) The slurry was filtered in a double layer of miraclot paper. C) After centrifugation the clarified plant extract was processed with HiTrap protein-A column in order to purify the mAb H10.

4.1.3 Antibody expression analysis using the P19 gene silencing suppressor protein

In order to evaluate the optimal sampling time point, leaves were collected on days 2, 3, 5 and 6 post-infiltration (p.i.), and expression was assayed by Western blot analysis on reducing SDS-PAGE (**Figure R6**) and by ELISA (**Figure R7**). In the case of Western Blot, the analysis was performed on the leaves sampled on days 2, 3, 5 and 6. Significant differences were observed in the expression levels of both HC and LC between plants infiltrated with and without p19 construct

(**Figure R6**). Maximum expression levels were typically observed at 6 days p.i. in the presence of p19 gene silencing suppressor.

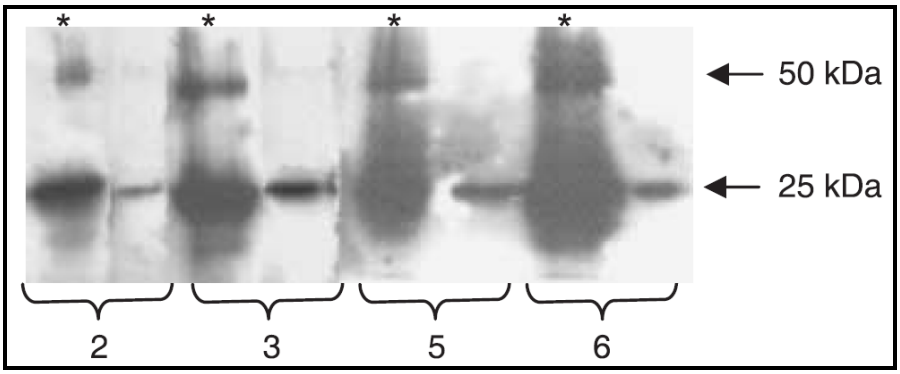


Figure R6: Western blot analysis of leaf extracts from *N. benthamiana* plants co-agroinfiltrated with *Agrobacterium tumefaciens* strains harbouring the heavy chain (HC), light chain (LC) and p19 silencing suppressor from Artichoke mottled crinkle virus (AMCV). (a) Both anti-γ and anti-λ chain antibodies were used for detection. Lanes indicated by an asterisk were loaded with extracts obtained from plants co-agroinfiltrated with HC, LC and p19; the other lanes refer to plants co-agroinfiltrated with HC and LC only. Numbers indicate days post-infiltration. Plant extracts were normalized for total soluble protein (TSP) content (20 μg of TSP were loaded on to each well).

This result was confirmed also by a sandwich ELISA assay performed using an anti-γ antibody for the coating and anti-λ antibody for detection (**Figure R7**). In this case, the analysis was performed directly on crude extracts from plants agroinfiltrated with HC and LC in presence of the p19 gene silencing suppressor. Sampling was performed at 2, 4 and 6 days p.i. ELISA results showed a gradual increase of the expression levels of the protein that reached the highest concentration at the 6th day p.i.. Further time points were not assayed since an occurrence of leaf necrosis from the 7th day p.i. was observed.

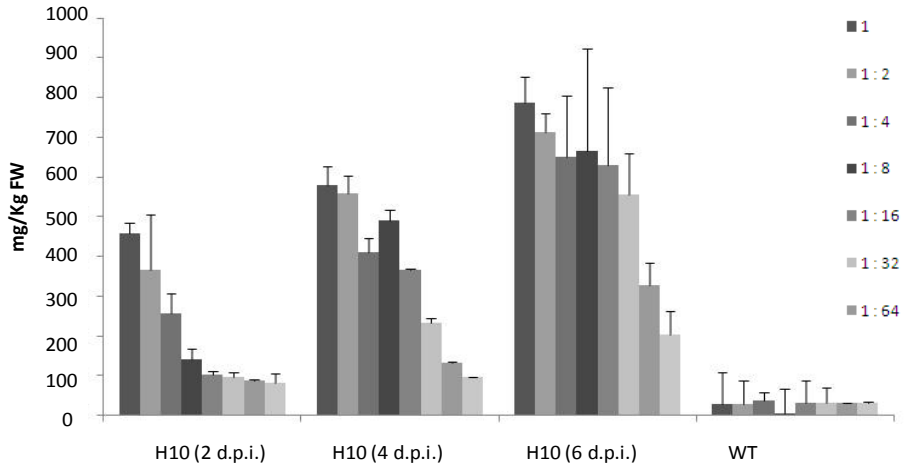


Figure R7: Sandwich ELISA (anti-γ/anti-λ-HRP) assay performed on crude extracts of *N. benthamiana* plants agroinfiltrated with HC, LC and p19 expression cassettes. Every value is presented in mg/Kg FW and represents the mean ± standard error of the mean of triplicate samples. Dilutions are reported on a side from 1 to 1:64.

4.1.4 Extraction of H10 from fresh agroinfiltrated leaves, lyophilized leaves and intercellular fluids

The strategies used to obtain plant extracts from agroinfiltrated leaves have been based on several extraction techniques such as, total extract from fresh or lyophilized leaves (stored at room temperature), intercellular fluid recovery (IFs), total extract form leaves depleted of intercellular fluids (total extract – IFs) (**Figure R8**).

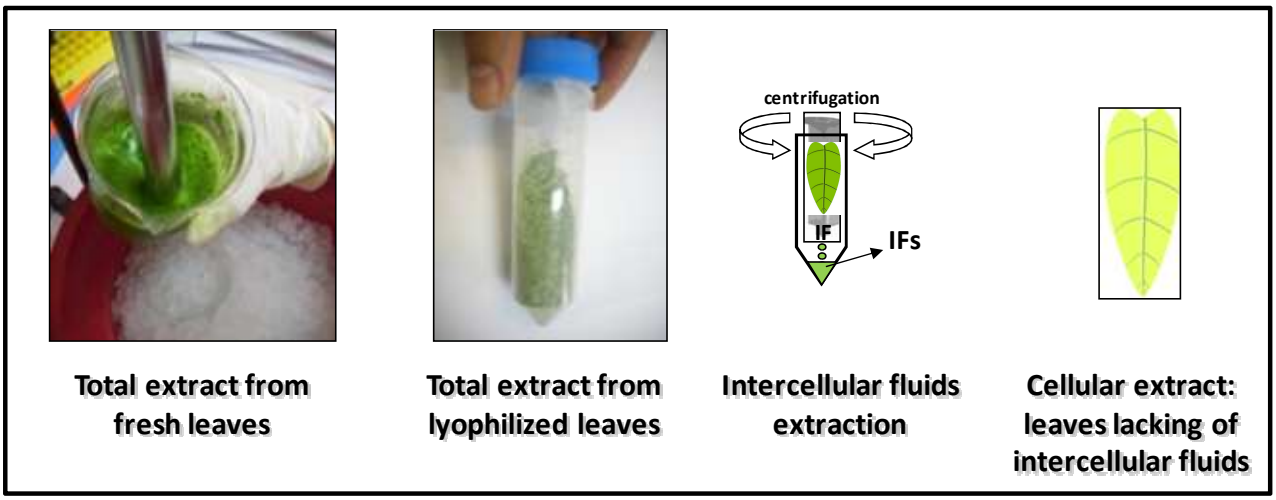


Figure R8: Schematic representation of the extraction strategies used for mAb H10 purification.

In the first strategy, agroinfiltrated leaves were directly processed by mechanical homogenization (total extract), or lyophilized and then treated with the same extraction methodology used for fresh leaves. The recovery of Intercellular Fluids from fresh agroinfiltrated *N. benthamiana* leaves was achieved using an infiltration-centrifugation method (as described in Materials and Methods section). The technique was performed applying a mild and immediate centrifugation of the leaves after vacuum infiltration with the extraction buffer, allowing the recovery of the intercellular fluids from the apoplast. Leaves after IFs extraction were also homogenized in order to obtain a total extract from leaves depleted of IFs. For every extraction procedure, agroinfiltrated leaves were collected six days post infiltration (d.p.i.), since maximum expression levels of mAb H10 were typically observed at this time point (**Figure R6** and **R7**). In the case of the analysis of lyophilized material, lyophilization was performed on batches of 40 g of fresh agroinfiltrated leaves immediately after plant sampling. This process led to the complete dehydration of the plant leaves that, were kept 8 months at room temperature and therefore analyzed (**Figure R9**). Also for this leaf

material, the extraction process was performed using the standard mechanical homogenization methodology.

Figure R9: Falcon tube containing plant leaf material subjected to lyophilization.

4.1.5 ELISA assay of different plant extracts

To compare mAb H10 expression levels, a quantitative ELISA was performed on the extracts obtained with different strategies. Before the analysis, all extracts were normalized for TSP content using the Bradford colorimetric assay. In the case of IFs, a TSP value of 0.6 mg/ml was obtained, resulting significantly lower if compared to total extracts (1.5 - 2 mg/ml).

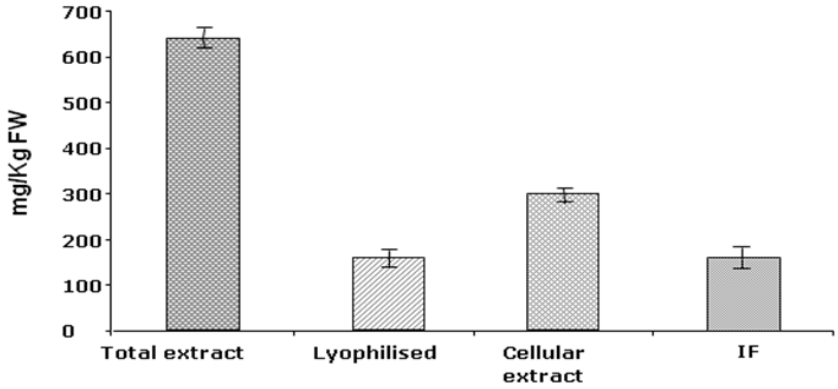


Figure R10: Quantitative ELISA assay performed on the crude extracts obtained with different extraction strategies. Every value is presented in mg/Kg FW and represents the mean ± standard error of the mean of triplicate samples. The experiment has been performed by coating the plate with an anti γ chain antibody and detecting with an anti λ chain HRP antibody.

The results showed an average IgG expression level of 160 mg/Kg FW for IFs, 300 mg/Kg for lyophilized leaves and 640 mg/Kg in the case of total leaf extract (**Figure R10** and **Table R1**). Antibody expression levels, reported as percentage of Total Soluble Proteins (% TSP), revealed values of 16 % in the case of total extract and 4 and 10 % for lyophilized leaves and total extract-

IFs, respectively. Furthermore, the IFs analysis revealed a surprisingly high value of about 27 % TSP (**Table R1**).

Table R1: Comparison of different extraction procedures in terms of antibody yield, antibody degradation and plant contaminants in the final purified product.

Extraction procedure	[a] **TSP content in the extract (mg/ml)**	[b] **Yield (mg/Kg)**	[b] **IgG levels (% TSP)**[a]	[c] **IgG purification yield (mg/Kg FW)**	[d] **Percentage of intact IgG**	[e] **Total phenolics in the extract (µg/g FW)**	[e] **Phenolics after protein A purification (% of total loaded phenolics)**
Total extract (fresh leaves)	2.0± 0.16	640± 20	16	75	75	420± 40	1.2
Total extract (lyophilized leaves)	2.0± 0.24	160± 15	4	45	10	370± 30	0.8
Intercellular fluids (IFs)	0.6±0.1	160± 11	26.6	17	37	21± 10	n.d.
Total extract – IFs	1.5± 0.15	300± 10	10	30	30	195± 45	1.0

[a] Total soluble protein (TSP) content in the extract was determined by Bradford colorimetric assay. Values are the mean ± standard error of the mean (SEM) of triplicate samples.
[b] IgG concentration in the extracts was calculated by quantitative ELISA as described in Materials and Methods and values are the mean ± standard error of the mean (SEM) of triplicate samples.
[c] The purification yield is the mean value obtained from three purifications of different batches (40 g) of agroinfiltrated leaves using protein A affinity chromatography.
[d] Percentage of intact mAb H10 was calculated from the size-exclusion chromatography analysis.
[e] Total phenol concentration was determined using Folin-Ciocolteau reagent. Values are the mean ± standard error of the mean (SEM) of triplicate samples.
n.d.: not detected.

4.1.6 Western blot analysis of different plant extracts

The evaluation of the expression and degradation pattern of the H10 in all extracts, was performed by Western blot analysis. The samples analyzed were normalized for TSP content. The Western blot analysis performed with an anti-γ antibody revealed the presence of a major band around 50 kDa corresponding to the heavy chain (HC) in all extracts (**Figure R11** panel **a**) and by a specific degradation band at 20 kDa. Furthermore, in the case of IFs, two additional degradation band around 35 kDa and 12 kDa, barely visible in the other extracts, were observed. Western blot analysis, performed using an anti-λ antibody, showed in all extracts only the expected band at 25 kDa corresponding to the intact light chain (LC); in this case, degradation bands at lower molecular weight were not detected (**Figure R11** panel **b**).

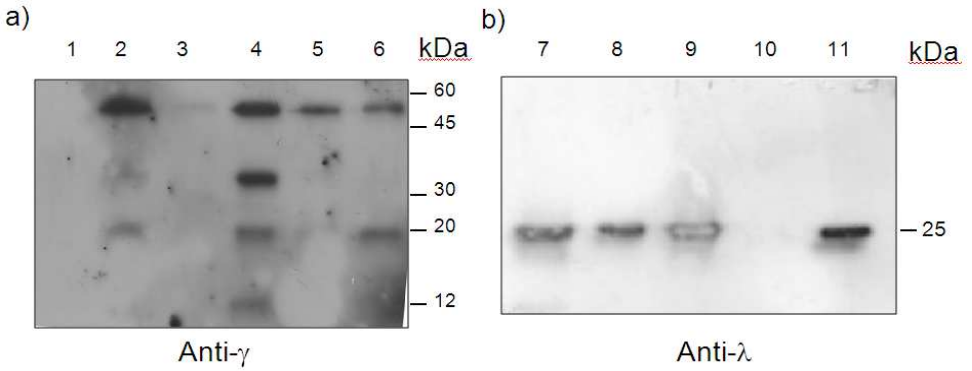


Figure R11: Western blot analysis of the different plant extracts on reducing SDS-12 % (w/v) PAGE. Immunoblotting with a peroxidase-conjugated anti-human γ chain (a) or anti-human λ chain (b). Plant extracts were normalized for TSP content and a total of 500 ng TSP were loaded on each well in both gels. Lanes 1 and 10: extracts from plants agroinfiltrated only with the p19 silencing suppressor used as a negative control; 2 and 7: total extracts from fresh agroinfiltrated leaves; 3: 10 ng of commercially purified human IgG used as a reference; 5 and 11: 20 ng of commercially purified human IgG used as a reference; 4 and 8: Intercellular Fluids (IFs) recovered from agroinfiltrated plants; 6 and 9: extracts obtained from homogenization of lyophilized agroinfiltrated leaves.

4.1.7 Purification of H10 from total leaf extracts or intercellular fluids of agroinfiltrated plants

The purification procedure used for mAb H10 was carried out starting from batches of 40 g (total leaf extracts) or 80 g (IFs) of agroinfiltrated *N. benthamiana* leaves. Protein-A affinity chromatography was used for all extracts using 80 ml of plant extract in 1 X phosphate saline buffer pH 7.0 (1 X PBS), and the average purification yields obtained from three independent experiments are reported in **Table R1**. The values obtained showed that the mechanical homogenization from total leaf extracts presented the highest yield of 75 mg/Kg FW, whereas purification from IFs yielded an average value of 17 mg/Kg FW. The functionality of the purified H10 obtained from different extracts was confirmed by antigen-binding ELISA on mouse TNC-BCD (**Figure R12**).

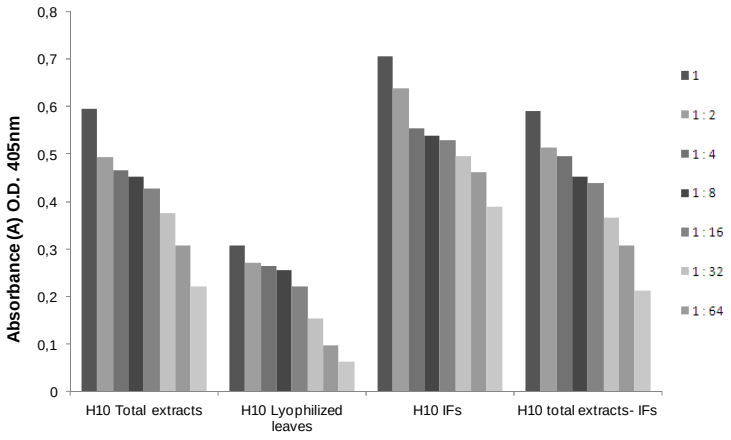


Figure R12: ELISA assay against the antigen (TNC) performed on the purified H10 obtained with different extraction strategies. Every value is presented in Optical Density absorbance at 405 nm. Dilutions are reported on a side from 1 to 1:64.

Protein-A purified antibodies obtained from all extraction strategies were further analyzed by non-reducing SDS-PAGE. A band at about 150 kDa, corresponding to the intact antibody, and a major degradation band at about 45-50 kDa (indicated by an arrow) was observed for all antibodies (**Figure R13**). Moreover, two additional degradation bands at 75 and 100 kDa (indicated by asterisks) were also present, most abundantly in the H10 purified from lyophilized leaves and from total extracts - IFs.

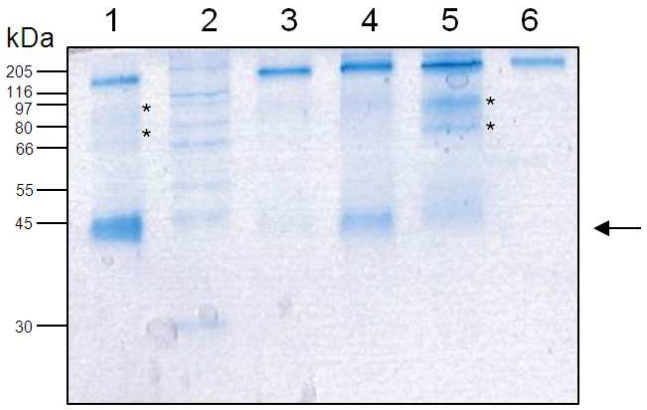


Figure R13: Analysis of mAb H10 purified from different extracts of vacuum-agroinfiltrated plants. Analysis was performed loading 1 μg of antibody purified by protein A affinity chromatography from batches of 40 g of vacuum-agroinfiltrated leaves onto non reducing SDS-10 % (w/v) PAGE stained with Coomassie blue (a). Lane 1: antibody purified from extracts of lyophilized leaves; 2: Molecular weight marker; 3: H10 purified from total extracts of fresh agroinfiltrated leaves; 4: antibody purified from intercellular fluids (IFs); 5: Antibody purified from leaves from which IFs were previously extracted (Total extract-IFs); 6: commercially purified human IgG1 used as a control. The arrow indicates a major band observed at *45 kDa while asterisks indicate additional bands observed at about 75 and 100 kDa.

4.1.8 Analysis of the phenol contaminants in plant extracts and purified antibody preparations

The next analysis was focused on the evaluation of phenolic contaminants in the extracts. In the case of fresh or lyophilized leaf extracts, a similar value of 420 and 370 μg/g FW respectively was observed, whereas the analysis of IFs exhibited a strong reduction (10-fold lower) in phenolic compounds (21 μg/g FW) (**Table R1**). Also pigments in IFs were greatly reduced compared to total extracts as illustrated in **Figure R14**. After purification, also protein-A eluted fractions were subjected to the analysis of phenolic compounds. This assay revealed a level of about 1 % of total loaded phenolics both in the eluted fractions obtained from total and lyophilized extracts, while in the case of the purified fractions from IFs no plant phenols were detected (**Table R1**). As expected, such a low level of phenolics and pigments observed in the IFs extract revealed to have a positive impact on the maintenance of protein A column performances. In fact, due to the reduced

contamination of the resin, the same column could be reused up to 5 times without showing significant activity loss. Protein A columns after 3 cycles of purification either from total extracts or from IFs are shown in **Figure R14** indicating a visible difference in resin pigmentation.

Figure R14: Visual comparison between intercellular fluids (IFs) (1) or clarified total extract from fresh agroinfiltrated leaves obtained by mechanical homogenization (2). In the bottom part, protein A chromatography columns after three purification cycles from the corresponding extracts are shown.

4.1.9 Gel-filtration analysis of purified mAb H10

The assembly and the molecular weight of the purified mAb H10 from total or lyophilized leaves extracts and from IFs, after the preliminary analysis performed by Western blot, was further characterized by size-exclusion chromatography with a Superdex 200 5/150 GL Column (GE Healthcare) (**Figure R15**). Results of the gel filtration analysis obtained in the case of the antibody purified from the total extract showed the presence of a major peak at a value of 0.34 Kav, perfectly matching with that obtained for a full-size human purified IgG1 used as a control (**Figure R15** panel **a**, broken line). After the evaluation of the peak area, it was demonstrated that 75 % was represented by intact IgG (**Figure R15** panel **a** and **Table R1**). On the other hand, in the case of mAb H10 purified from IFs, only 40 % of the antibody consisted of full size IgG (elution peak at 0.35 Kav) (**Figure R15** panel **b** and **Table R1**). In this case, a major degradation product (elution peak at about 0.5 Kav) with a calculated molecular weight of ~50 kDa was observed. This result was also confirmed by the non-reducing SDS-PAGE, in which a band of the same molecular weight was observed (**Figure R13**).

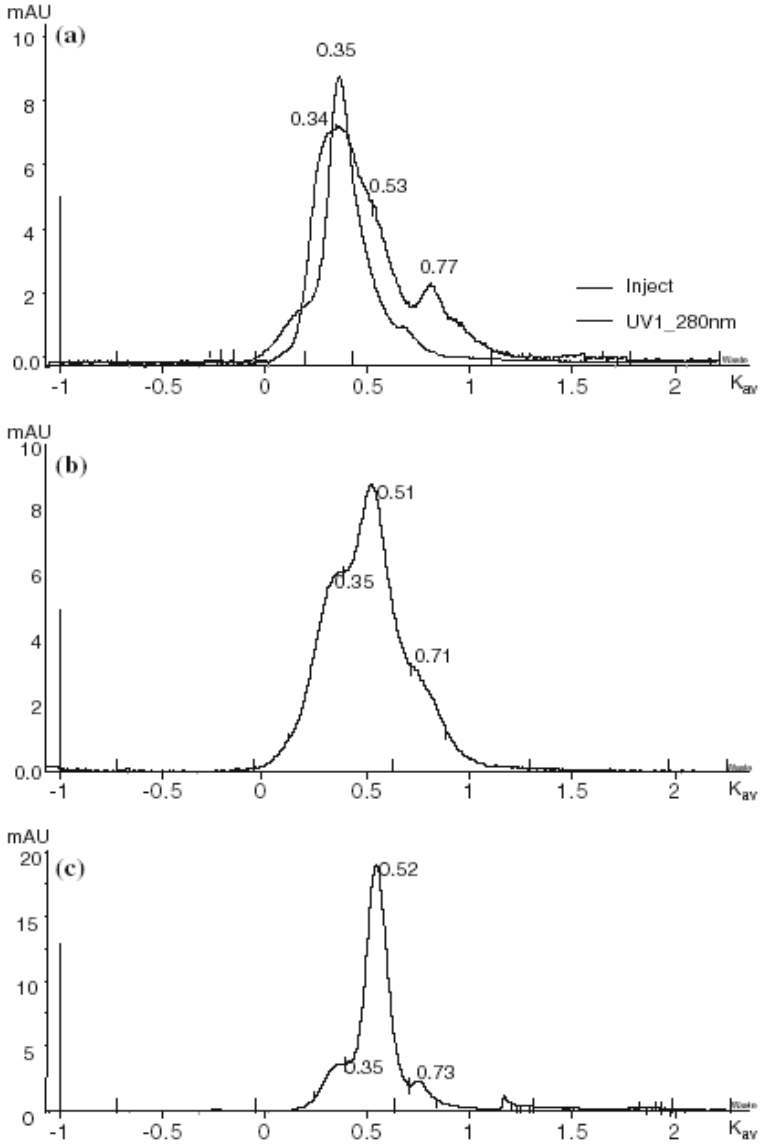


Figure R15: Characterization of H10 purified from different agroinfiltrated *N. benthamiana* leaf extracts by size-exclusion chromatography. H10 antibody was purified by protein A chromatography from batches of 40 g of agroinfiltrated leaves and was analyzed on a SuperdexTM 200 5/150GL column as described in the ‘‘Materials and Methods’’. For a better interpretation of results, the chromatograms are presented over Kav = (elution volume - void volume)/(column volume - void volume) instead of elution volume or retention time. Kav values of the major peaks are reported. A typical chromatogram obtained for mAb H10 purified from total extracts of fresh agroinfiltrated leaves. The broken line in this panel represents the chromatogram resulting from a commercially available purified human IgG1 used as reference (Kav value of about 0.3). Typical chromatograms of mAb H10 purified from intercellular fluids (IFs) (b) and from lyophilized leaf extracts (c).

The degradation reported for the H10 purified from IFs was even more evident in the lyophilized leaf extract, in which only 10 % of the purified antibody resulted fully assembled (**Figure R15** panel **c**), confirming what previously observed in the non-reducing SDS-PAGE (**Figure R13**).

4.1.10 Pilot-scale purification and characterization of H10 from fresh agroinfiltrated leaves

Pilot-scale purifications were carried out starting from batches of 250 g of agroinfiltrated *N. benthamiana* leaves. Extraction was achieved by mechanical homogenization to a final volume of 750 ml. The solution was clarified by centrifugation and the pH was adjusted to a value of 7.5. A second centrifugation and a filtration through 0.22 μm filters was performed in order to further clarify the extracts. The solution obtained was stable up to 24 hours at 4 °C without the appearence of any precipitate. In order to increase the recovery of clear eluted fractions and reduce the antibody loss, the column was further washed with 1 X PBS 5 % isopropyl alcohol, 1 M NaCl and Glycine pH 4.0 and then antibody elution with Glycine pH 3.0 was performed. From this first chromatographic step, we obtained an average yield of 80-100 mg/Kg FW of purified H10. The efficiency of every purification step was monitored by functional ELISA on mouse tenascin-C, with the aim to verify purification efficiency at every step (**Figure R16**).

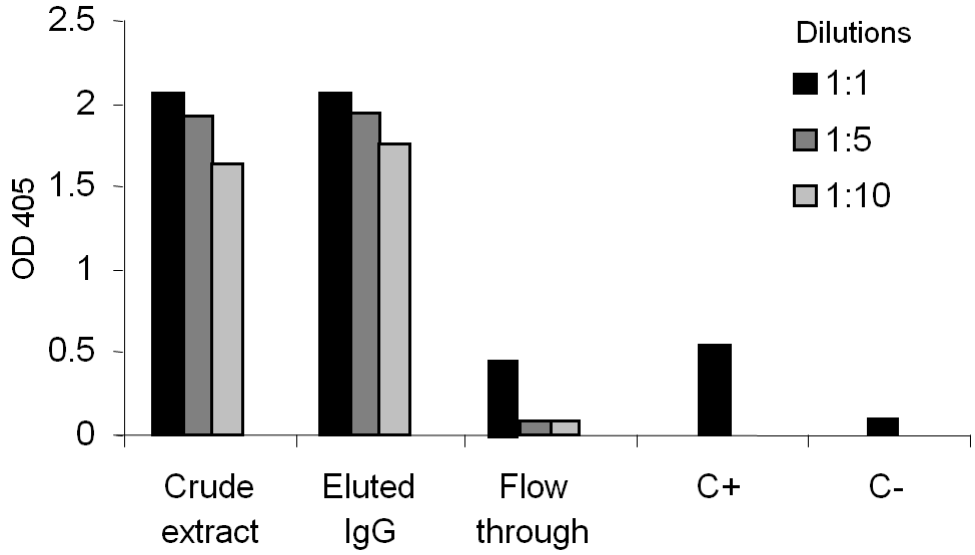


Figure R16: ELISA assay performed on each step of the mAb H10 pilot-scale purification. Batches (250 g) of fresh vacuum-agroinfiltrated *N. benthamiana* leaf extracted by mechanical homogenization were used.

The analysis of the quality of the H10 fractions eluted from protein-A affinity chromatography was achieved both by gel filtration using a Superdex 200 10/300 GL column and reducing SDS-PAGE analysis. Gel filtration assay showed the presence of a major elution peak at a Kav value of 0.33, corresponding to the intact immunoglobulin, flanked by a small fraction at about 0.51 Kav (degradation product) corresponding to a molecular weight of about 50 kDa (**Figure R17** panel **a**).

Reducing SDS-PAGE analysis revealed the presence of some barely visible degradation bands corresponding to minor degradation products (**Figure R17** panel **d**, lane 1).

Elution fractions obtained from protein-A affinity chromatography were further purified by cation-exchange chromatography (CEX). This analysis was performed using a Source 30S resin and elution was performed with a 0-50 % linear gradient. The typical chromatogram obtained from this analysis showed the presence of two partially overlapping peaks (A and B) (**Figure R17** panel **b**).

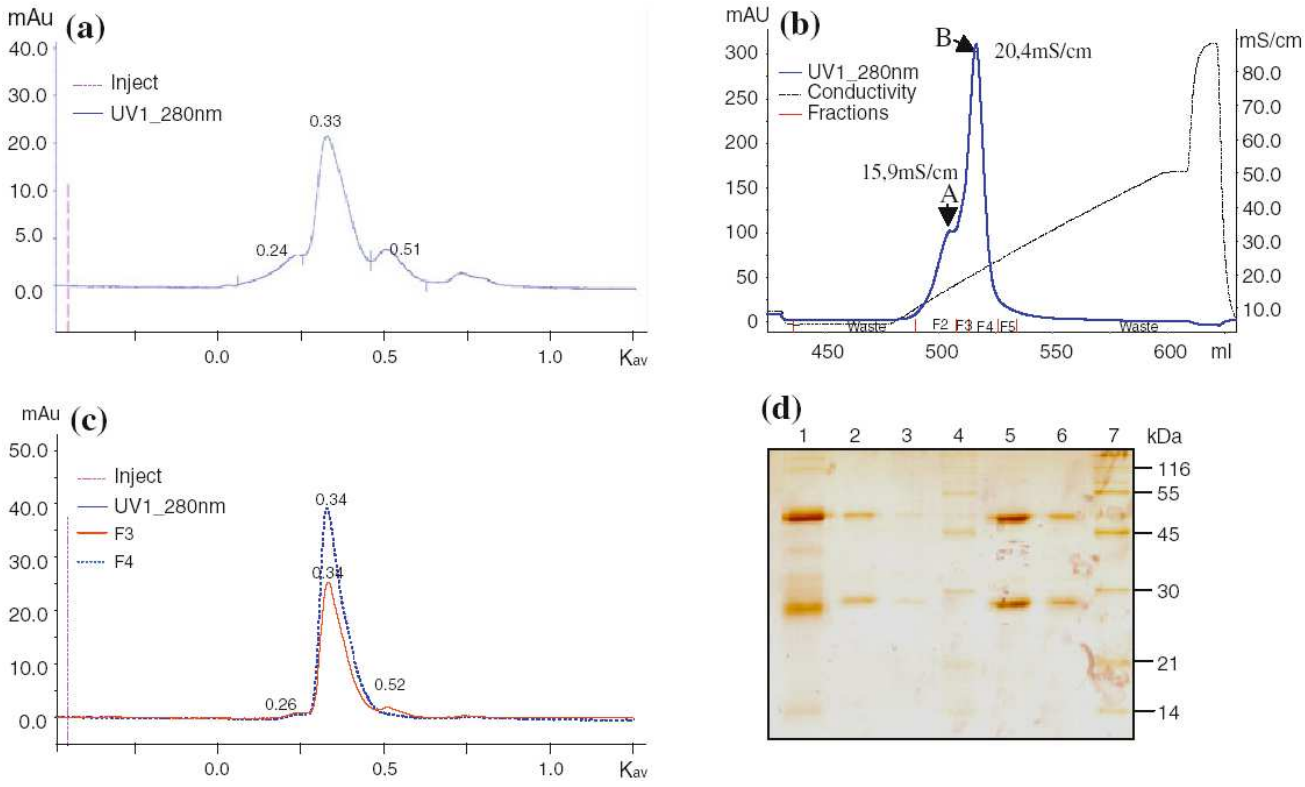


Figura R17: H10 pilot-scale purifications from batches (250 g) of fresh vacuum-agroinfiltrated *N. benthamiana* leaves extracted by mechanical homogenization. **a)** Typical size exclusion chromatogram obtained from the pooled fractions eluted from preparative protein A affinity chromatography. Analysis was performed using a calibrated SuperdexTM 200 10/ 300 GL. Kav values of the major peaks are indicated. **b)** Fractions eluted from the preparative protein A affinity column were subjected to cation-exchange chromatography (CEX) using Source 30S resin. Elution was performed with a 0–50 % linear gradient using a high ionic strength buffer (50 mM acetic acid, 1 M NaCl, 5.0 mM EDTA, pH 6.0). Two protein peaks are distinguishable and are indicated as A and B; eluted fractions collected from the column are indicated as F2, F3, F4 and F5. **c)** Typical size-exclusion chromatogram obtained from the F3 and F4 fractions eluted from preparative CEX. Analysis was performed using a calibrated SuperdexTM 200 10/300 GL column. Kav values of the major peaks are indicated, with the 0.3 Kav peak corresponding to full-size IgG. **d)** Analysis of eluted fractions from pilot-scale H10 purification by reducing SDS-10 % (w/v) PAGE stained with silver nitrate. Lane 1: fraction eluted from preparative protein A (1 µg); 2 and 3: purified human IgG1 used as a control (500 and 200 ng respectively); 4 and 7: protein molecular weight marker; 5 and 6: pooled F3 and F4 CEX elution fractions (1 µg and 500 ng respectively)

Peak B, corresponding to fraction 3 and 4 was additionally analyzed by gel filtration, functional ELISA and reducing SDS-PAGE. Gel filtration analysis using a Superdex 200 10/300 GL column showed that both fractions had a unique elution peak at about 0.3 Kav, corresponding to the fully

assembled antibody with a high purity grade of 99.4 % (**Figure R17** panel **c**). The quality of the CEX purified antibody was verified by reducing SDS-PAGE confirming a very high level of integrity of the IgG (**Figure R17** panel **d**, lanes 5 and 6). Furthermore, the analysis of phenolic and alkaloid compounds demonstrated that H10 was highly pure and free from plant contaminants. Antibody purification yields after this second chromatographic step were in the range of 40 mg/Kg FW, corresponding to a 45 % recovery of total antibody loaded on the CEX column.

4.1.11 Endotoxin Removal

The final eluted product was further analyzed with the Lymulus Amebocyte Lysate Test (LAL) to verify the presence of endotoxins. The plant purified antibody after CEX (mAb H10 concentration of 0.5 mg/ml) showed an endotoxin level between 125 and 250 EU/ml, and in order to decrease this value, an endotoxin removal was performed using an Endo Trap Red affinity chromatography column as described in ‘‘Material and Methods’’. After two cycles of affinity chromatography, the remaining endotoxin levels in the sample were 1 EU/ml and a 20 % loss of antibody was observed with a final mAb H10 concentration of 0.4 mg/ml. Moreover, the specificity of plant purified H10 to tenascin-C was assayed by Surface Plasmon Resonance analysis, revealing a KD value of 10 nM, perfectly matching the previously reported mAb H10 antigen-binding kinetics data (Villani et al., 2009)

4.2 DISCUSSION

In the last years significant progresses have been made in the development of novel transient expression platforms to increase the levels of recombinant immunoglobulin production. These systems initially used for rapid validation of expression constructs, are now being employed for the production of high amounts of recombinant proteins within a few weeks (Vézina et al., 2009). In particular, in the case of the H10 antibody, the use of a transient expression system based on vacuum-agroinfiltration boosted by the use of the Artichoke mottled crinckle virus (AMCV) p19 gene silencing suppressor protein, it was possible to obtain high levels of antibody in *N. benthamiana* plants in a reduced period of time. Furthermore, using a protein-A affinity chromatography, it was possible to purify up to 100 mg of antibody per Kg FW (Villani et al., 2009).

However, upstream technological achievements observed in the last years have not been matched by downstream processing advancements since information on the main factors influencing this step are still limited. In fact, even if some progresses have been achieved in terms of extraction/purification strategies, the methodologies used have been optimized for several proteins just on a case-by-case basis, leading to the development of platform-dependent approaches.

Based on this concern, the first part of the thesis has been focused on different aspects of protein extraction from agroinfiltrated *N. benthamiana* leaves with the aim to optimize the purification process of the H10 antibody and to investigate alternative purification strategies able to provide additional information for the large-scale production.

Extraction is an essential step to be addressed during the purification of plant derived proteins because it could affect the quality, the purity and the final yield of the protein product (De Muynck et al., 2010). In particular, in the case of purification of plant-derived antibodies, the choice of the optimal extraction methodology could dramatically influence the yield of the purified IgG modulating also the amount of plant contaminants in the purified protein. In fact, as shown by Hassan et al., (2008), the efficiency of the extraction technique could be influenced by the target location of IgG in the plant cell, and by the physical extraction methodology used. Moreover, it was shown that the less vigorous extraction methodologies such as freeze thaw, led to the accumulation of antibody levels perfectly comparable to the more severe mechanical extraction procedures. Furthermore, the use of mild extraction techniques provided a reduction in the level of unwanted plant contaminants in the extract increasing the purity of the final product (Hassan et al., 2008).

In this study, the evaluation of different extraction strategies from agroinfiltrated *N. benthamiana* leaves emphasized that mechanical homogenization from fresh leaves provided the highest antibody yields (640 mg/Kg FW), with an accumulation level reaching 16 % TSP. However, this extract showed also high levels of total polyphenol contaminants (420 μg/g FW). This data, in accordance with the results obtained in a previous work reported in *Nicotiana tabacum* (Platis and Labrou, 2006), confirmed that extraction obtained with mechanical homogenization of plant leaves increased the level of plant contaminants reducing the purity of the final product.

The same extraction procedure was applied also to lyophilized agroinfiltrated leaves stored at room temperature. This strategy was assayed since lyophilization could represent a significant methodology for long-term storage of agroinfiltrated material for the possibility to reduce the cold-chain costs needed for the preservation of recombinant proteins. The reported antibody yield was sensibly lower (160 mg/Kg FW), with a high grade of antibody degradation observed both in size exclusion chromatography and in SDS-PAGE analysis. This observation represents a limitation for this strategy leading to a dramatic reduction of intact IgG. A second extraction procedure based on the recovery of intercellular fluids (IFs) from agroinfiltrated leaves was assayed. In this case, the levels of purified antibody were comparable with those obtained using mechanical homogenization of lyophilized leaves (160 mg/Kg), but considerably lower in comparison to the previously reported yield obtained from the mechanical homogenization of fresh agroinfiltrated leaves (25 % reduction). However, it must be noted that antibody concentration in the IFs was considerably higher, reaching 26.6 % TSP, which corresponded to about a two fold increase compared to the concentration in the total extract (16 % TSP). The most interesting data obtained from this extraction methodology was represented by the analysis of polyphenol contaminants contained in the plant IFs before antibody purification with protein-A. In fact, a 10 fold decrease compared to the total leaf extract obtained by mechanical homogenization was observed, with levels (21 μg/g FW) comparable to the results obtained using an aqueous two-phase partitioning system (ATPS) previously reported by Platis et al., (2008). This reduction in contaminants (phenolics, alkaloids and pigments) represented a crucial point in the protein-A purification steps. In fact, a chromatography step performed using a clarified extract could favor the preservation of Protein-A affinity column performances even after repeated use, having an impact on downstream processing costs reduction. Another important consideration is related to the quality of the plant derived antibodies obtained from different extraction procedures based on the observed degradation profiles in both gel filtration analysis and non-reducing SDS-PAGE. In the case of the extraction of H10 from total fresh leaves, almost 75 % of the purified antibody was in its intact tetrameric form. In particular, two degradation bands at about 35 and 20 kDa were observed in the Western blot analysis of the

HC indicating specific antibody degradation. No detectable degradation was observed in the case of the light chain analysis. A fine analysis of H10 degradation in plant will be treated in **Paragraph 4.3**. Size exclusion analysis performed on H10 purified from IFs showed that only about 40 % of H10 was recovered as full-size mAb while major degradation fragments with a molecular weight lower than 50 kDa were observed. In particular, SDS-PAGE analysis, showed three major HC degradation fragments at 35 and 20 kDa as well as an abundant lower molecular weight band at about 12 kDa. The 35 and 20 kDa bands confirmed the results obtained in the previously described Western blot analysis of total fresh leaves crude extract, indicating that these fragments have been specifically bound by protein-A purification chromatography and represent degradation products derived from the plant cell processing. It must be noted that both the 35 and 12 kDa bands were already evidenced in the IFs of tobacco leaves expressing the chimeric rat/human LO-BM2 mAb (De Muynck et al., 2009). The presence of a more severe antibody degradation observed in the IFs could be ascribed to the significantly higher level of plant peptidases in the apoplast, as previously demonstrated both in *N. benthamiana* and *N. tabacum* (Delannoy et al., 2008; De Muynck et al., 2009; Drake et al., 2009). Surprisingly, also in the case of IFs, no degradation of the light chain was observed, in contrast with what already documented in Intercellular Fluids of transgenic tobacco, where a strong degradation of the light chain of the Lo-BM2 antibody was observed (De Muynck et al., 2009).

Antibody degradation was even more evident in the lyophilized leaves where the size exclusion analysis revealed a 10 % of full size IgG. SDS-PAGE analysis highlighted the presence of additional degradation bands at around 100 and 75 kDa. Based on these results, it is possible to suppose that the lyophilization process and the long-term storage could further affect the integrity of the whole IgG. Nevertheless, in spite of the antibody degradation, the H10-derived fragments resulted functional in ELISA.

In general, it must be noted that all purification processes yielded a low antibody recovery. This result could be explained hypothesizing that an unwanted overestimation of antibody expression yields had occurred in the quantitative ELISA, due to the specific degradation of the plant expressed antibody, leading to the formation of functional Fab fragments. The extraction/purification results obtained indicated that the optimal strategy to ensure the highest amount of IgG recovery was represented by mechanical homogenization. For this reason, using this strategy, a pilot-scale purification from 250 g of fresh agroinfiltrated leaves was performed, in order to obtain a robust and reproducible purification protocol on a larger scale. After clarification of the extract, obtained by pH adjustment and two sequential centrifugations, it was verified that no precipitate formation occurred within 24 hours. Due to the high extract volume to be passed through the affinity column,

the purification step was necessarily conducted over-night, therefore, to ensure the best performance of this process, an high grade of stability of the extract was required. Additionally, it was shown that to increase final product purity and column binding an additional washing step with Glycine pH 4.0 was required before antibody elution. This procedure allowed the recovery of a clear eluate exhibiting low levels of polyphenols, without any substantial antibody loss in the flow through. According to this optimized extraction/purification protocol, after protein A affinity chromatography, it was possible to obtain an antibody yield in the range of 20-25 mg starting from an amount of 250 g of fresh leaves. These values, corresponding to an amount of 80-100 mg/Kg FW, were in the range of those obtained from small-scale purifications achieved starting from 40 g FW. The second purification step was conducted in order to increase the antibody purity and further clarify the eluate from plant typical contaminants. In fact, purity of the final product is essential for antibodies to be used in biopharmaceutical applications. Based on this concern, we decided to use a purification procedure normally applied to animal-cell culture supernatants, based on protein A affinity chromatography followed by a cation-exchange chromatography step aiming to further purify the antibody eluate from contaminants (Birch and Racher, 2006; Shukla et al., 2007). This cation-exchange chromatography column (CEX) allowed to separate the protein fraction corresponding to the intact fully-assembled human IgG from the antibody degradation products, obtaining a 99.4 % pure mAb H10 free of phenolics and alkaloids. Other examples in literature reported the use of cation-exchange chromatography as a first column step for the removal of unwanted compounds from the extract, giving substantial advantages in the next purification steps (Platis et al., 2008). In this work authors used a first CEX step followed by immobilized metal ion affinity chromatography (IMAC). Although, the use of protein A column was shown to be still required to obtain a highly purified final product free from other protein contaminants.

In conclusion, this work shows the possibility to use the vacuum-agroinfiltration system, boosted by a plant virus gene silencing suppressor protein, as a successful strategy for antibody production. This new methodology has been recently validated also by other research groups that demonstrated the possibility of producing antibodies endowed with a humanized glycosylation profile with a similar approach (Vézina et al., 2009; Strasser et al., 2009). Furthermore, it was demonstrated that this platform could ensure antibody levels perfectly comparable to those obtained using viral vectors. Moreover, we described the use of different extraction processes from agroinfiltrated leaves and showed that each extraction methodology differentially affected the integrity and yield of a purified tumour-targeting antibody, offering additional information about the purification/extraction processes. Best results, in terms of antibody recovery, were typically obtained using mechanical homogenization of fresh leaves, but the strategy of purification from IFs appeared to be a very

promising methodology in terms of ease of extraction, low contaminants content and maintenance of protein A column performances. Finally, we also described a reproducible pilot-scale purification protocol from fresh vacuum-agroinfiltrated *N. benthamiana* leaves extracted by mechanical homogenization, that allowed the purification of mg quantities of highly pure mAb H10, providing useful indications for large-scale antibody production.

4.3 MODULATION OF THE GLYCOSYLATION PROFILE OF mAb H10: INFLUENCE ON EXPRESSION YIELD AND ANTIBODY DEGRADATION.

The aim of this part of the thesis was to investigate different strategies for the production of H10 optimized glycoforms in order to study the effect of the different glycosylation profiles on antibody quality and plant expression yield. The glycosylation pattern of plant produced H10 antibody was modulated using three different strategies: a) mutation of the glycosylation site of H10 heavy chain in order to obtain an aglycosylated antibody (H10 Mut); b) retention of H10 in the Endoplasmic Reticulum (ER) of plant cells by fusing the SEKDEL retention sequence to the C-terminal of both HC and LC (H10 SEKDEL); c) expression of the H10 in *N. benthamiana* plants in which *fucosil transferase* and *xylosil transferase* gene expression is downregulated by RNAi (Δxyl, Δfuc plants).

4.3.1 Gene constructs

In addition to the previously described H10 heavy chain (p35:HC) and light chain (p35:LC) and P19 (p35:p19) plant expression constructs that allowed the expression of the secretory mAb H10, we engineered additional expression cassettes to produce H10 variants. In particular, for the ER retained H10 SEKDEL, we obtained the p35:HC-SEKDEL and p35:LC-SEKDEL constructs by fusing the DNA sequence coding for the SEKDEL ER retention signal, to the 3' of both HC and LC coding genes. Moreover, in order to produce an aglycosylated H10-Mut variant we generated a mutated H10 HC construct (p35:HC-Mut) in which the glycosylation site (Asn297) was mutated to Ala by site-directed mutagenesis. All genes were cloned into the pBI-Ω plant expression vector under the control of the CaMV 35S promoter, the TMV Ω translational enhancer and the NOS terminator sequences (Marusic et al., 2007). All plasmids were electroporated into *A. tumefaciens* strain LBA4404 and used in co-agroinfiltration experiments. A schematic representation of the constructs is reported in **Figure R18**.

In parallel, we performed vacuum co-agroinfiltration of XylT/FucT RNAi-silenced *N. benthamiana* plants, in which endogenous β 1, 2-*xylosyltransferase* and α 1, 3-*fucosyltransferase* gene expression was down-regulated (Strasser et al., 2008), with a mixture of two *A. tumefaciens* clones harbouring p35:HC and p35:LC obtaining the $H10^{XylT/FucT}$ variant. In this case, the p19 silencing suppressor was not used to avoid any interference with down regulation of the glycosylation enzymes. Leaves

were collected 3 d.p.i. since maximum expression levels in the absence of p19 was typically observed at this time point.

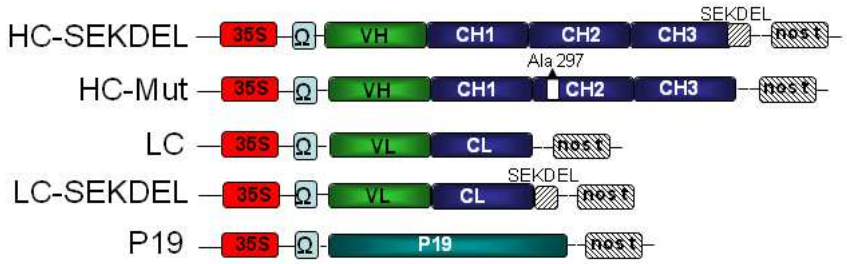


Figure R18: Schematic representation of the constructs used in the agroinfiltration experiments. A description of the constructs is presented in the Material and methods section. All genes were cloned in the pBI-Ω plant expression vector under the control of the CaMV 35S promoter and containing the TMV Ω translational enhancer and the NOS terminator sequence.

4.3.2 Transient expression of H10 variants in tobacco protoplasts

The different H10 formats (H10, H10-Mut and H10-SEKDEL), were transiently expressed in mesophyll protoplasts of *N. tabacum* SR1 in order to assess their accumulation and localization in plant cells. This evaluation was achieved co-transforming separately tobacco protoplasts with pGEM plasmids harbouring HC and LC (H10); HC-Mut, and LC (H10-Mut); HC-SEKDEL and LC-SEKDEL (H10-SEKDEL) plant expression constructs (**Table M2**).

Pulse labeling of the protoplasts was carried out with ^{35}S-methionine and ^{35}S-cysteine for 1 hour and chasing was performed at 2.5 and 5 hours. Cellular (C) and secreted (S, culture media) protein fractions were mixed with a protein-A/protein-G Sepharose resin mix and then subjected to immunoprecipitation and autoradiography (**Figure R19** panel **a**). Analysis of the expression performed by Western blot revealed that in C protein fractions H10 level was uniform for all the time points demonstrating a comparable expression profile for all variants. In the case of the S fractions, an high secretion of the full size H10 in the media was observed, for H10 and H10-Mut, 5 hours after pulse. In fact, at this time point, a very strong band at 150 kDa, corresponding to the complete immunoglobulin, was detected. On the contrary, in the case of H10-SEKDEL, the same band at 150 kDa was barely visible, demonstrating low secretion of the antibody in the culture media. In addition, H10-Mut profile showed several weak bands at lower molecular weight, indicating possible degradation products. Negative control panel obtained from the analysis of untransformed protoplasts (**Figure 19** panel **a**, right picture) did not show cross-reaction of protein-A and protein-G with endogenous plant proteins.

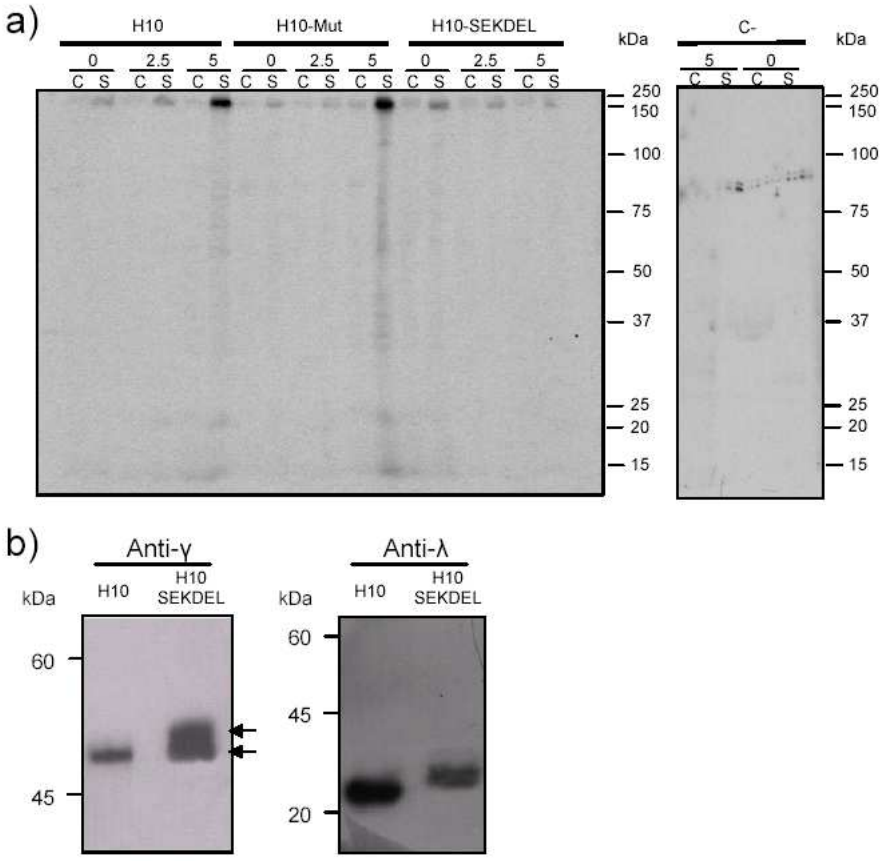


Figure R19: a) Pulse chase analysis performed in SR1 *N. tabacum* mesophyll protoplasts transfected with plasmids, separately encoding the heavy and light chain of each antibody glycoform (H10, H10 SEKDEL, H10-Mut), or with empty vector (negative control, C-). Transfected cells were labelled for 1h and chased at three time points: 0 (pulse), 2,5 hours, 5 hours; the analysis involved cellular (C) and secreted (S) fractions. A non-reducing sodium dodecylsulphate-polyacrylamide 15 % gel electrophoresis SDS-15 % (w/v) PAGE and fluorography was performed. Numbers at right indicate molecular weight markers in kDa.
b) Analysis by sodium dodecylsulphate-polyacrylamide 9 % gel electrophoresis (SDS-9 % (w/v) PAGE, 25 mA for 1 h 40 minutes) and Western blot of the plant extract obtained from vacuum agroinfiltrated *N. benthamiana* plants with H10 and H10 SEKDEL constructs. Immunoblotting was performed with a peroxidase-conjugated anti-human γ-chain (left) or anti-human λ chain (right). Plant extracts were normalized for TSP content and a total of 1 µg TSP were loaded on each well in both gels. Arrows indicate the band shift produced by presence/absence of SEKEL peptide.

4.3.3 Transient expression of H10 antibody variants in *N. benthamiana* plants

The expression of H10 antibody was obtained by vacuum-agroinfiltration of *N. benthamiana* plants as previously described. In order to evaluate the expression levels and the degradation profile of antibody glyco-variants Western blot analysis, using an anti-γ antibody, was performed on fresh plant extracts. The analysis emphasized that all antibody variants showed a similar degradation pattern. In fact, a band at 50 kDa, corresponding to the HC, and a major degradation band around 20 kDa were clearly visible. In the case of the secretory H10, the degradation bands were essentially less intense compared to other variants, while H10-Mut showed a more severe degradation pattern with a very strong band at 20 kDa and an additional fragment around 12 kDa (**Figure R20** panel **a**).

On the contrary, the analysis of plant extracts using an anti-λ antibody showed only the expected band at 25 kDa indicating that no degradation bands were detectable (**Figure R20** panel **b**).

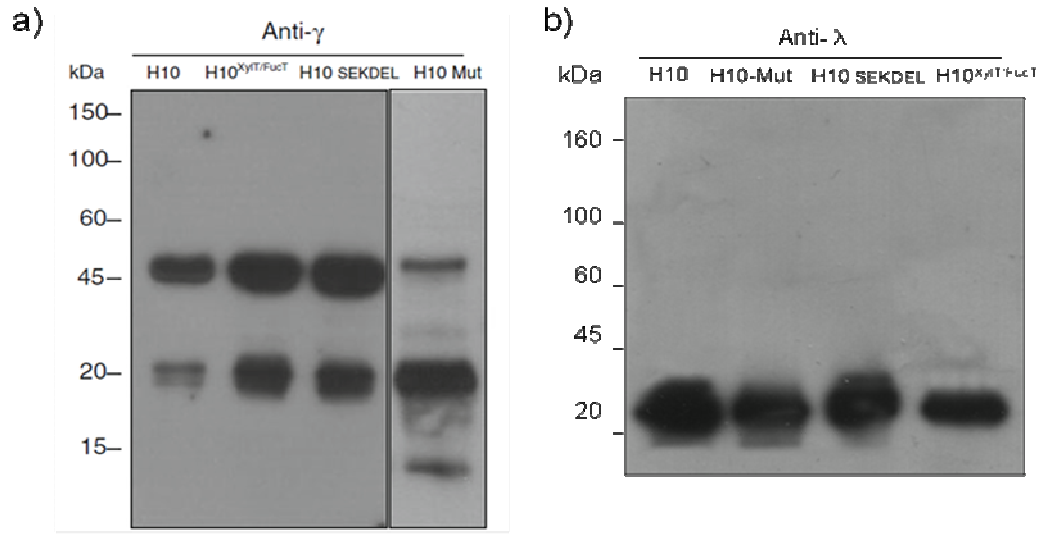


Figure R20: a) Western Blot analysis of H10 glycosylation variants performed on crude extracts using peroxidase-conjugated anti-human γ-chain (reducing SDS-12.5 % (w/v) PAGE analysis). b) Western Blot analysis of H10 glycosylation variants performed on crude extracts using peroxidase-conjugated anti-human γ-chain (reducing SDS-10 % (w/v) PAGE analysis). H10, H10-Mut: 1 µg TSP; $H10^{XylT/FucT}$: 10 µg TSP; H10-SEKDEL: 2 µg TSP.

A comparison of H10 variants expression levels in plant extracts was obtained by quantitative antigen-binding ELISA. This assay revealed an average expression level ranging from 46 mg/Kg FW for $H10^{XylT/FucT}$ produced in XylT/FucT *N. bentamiana* plants to 620 mg/Kg in the case of H10-Mut (**Table R2**). Surprisingly, expression levels of the ER retained antibody H10-SEKDEL were lower (480 mg/Kg FW) if compared to its secretory counterpart H10, used as a reference (640 mg/Kg FW). Antibody levels reported as TSP percentage, showed values of 15.5 and 12 % TSP for H10-Mut and H10 SEKDEL, respectively, while H10 values (16 %) matched closely those previously reported (Lombardi et al., 2010). The lower values observed for the $H10^{XylT/FucT}$ (1.15 % TSP) were caused by the different agro-infiltration protocol used in this case, since the transient expression was performed in the absence of the AMCV P19 gene silencing suppressor protein. It is important to point out that the expression levels obtained in ELISA, estimated all functional antibody products in the extracts and not necessarily intact fully assembled H_2L_2 immunoglobulins.

A detailed analysis of the H10-SEKDEL expression in agro-infiltrated *N. benthamiana* plant extracts was obtained by Western blot using both anti-γ and anti-λ detection antibodies (**Figure R19** panel **b**). Western blot analysis performed with the anti-γ antibody (**Figure R19 b**, left panel) revealed the presence of two close bands around 50 kDa, with the lower band corresponding to the molecular weight of the H10 HC used as a reference. The second band exhibited a higher molecular

weight than 50 kDa, probably indicating a gel shift due to the presence of an HC population still harbouring SEKDEL C-terminal sequence. Western blot analysis performed with an anti λ antibody (**Figure R19 b**, right panel), differently to what observed for the HC, showed a unique higher band at about 25 kDa compared to the H10 LC used as reference. This shift is probably due to the presence of a unique population of LC maintaining the SEKDEL peptide.

Table R2: Results of plant production of H10-Mut, H10-SEKDEL and H10XylT/FucT in terms of antibody expression yield, purification yield and percentage of intact IgG.

IgG Form	[a] Yield (mg/Kg)	[a] IgG levels (% TSP)	[b] IgG purification yield (mg/Kg FW)	[c] Percentage of intact IgG
H10	640± 20	16	75	75
H10-Mut	620± 18	15.5	73	3
H10-SEKDEL	480± 20	12	60	24
$H10^{XylT/FucT}$	46± 12*	1.15*	10*	32*

[a] IgG concentration in the extracts was calculated by quantitative ELISA as described in Materials and Methods and values are the mean ± standard error of the mean (SEM) of triplicate samples.
[b] The purification yield is the mean value obtained from three purifications of different batches (40 g) of agroinfiltrated leaves using protein A affinity chromatography.
c Percentage of intact H10 was calculated from size-exclusion chromatography analysis.
* Expression yields of $H10^{XylT/FucT}$ are lower because agroinfiltration of *N. benthamiana* plants was performed without the AMCV P19 gene silencing suppressor protein.

4.3.4 Purification and characterization of H10 antibody variants from agroinfiltrated leaves

Purification of H10 variants was carried out, as previously described, starting from batches of 40 g of agroinfiltrated *N. benthamiana* leaves by protein-A affinity chromatography. Based on the previous results, protein extraction was carried out from fresh vacuum-agroinfiltrated leaves using mechanical homogenization. For the secretory H10 and H10-Mut similar purification yields were achieved (75 and 73 mg/Kg FW, respectively). In the case of the ER retained H10-SEKDEL, lower but comparable values were obtained (60 mg/Kg FW). As expected, in the case of $H10^{XylT/FucT}$, as agro-infiltration experiments were performed in the absence of the P19 silencing suppressor, a significantly lower purification yield of 10 mg/Kg FW was obtained (**Table R2**). The binding activity of all purified antibody formats was confirmed through an ELISA assay against the recombinant C domain of human TNC-C (**Figure R21**). Results revealed a similar binding activity for H10, H10-SEKDEL and $H10^{XylT/FucT}$, while a lower binding activity was observeed for H10-Mut probably due to the high degradation of the plant purified product.

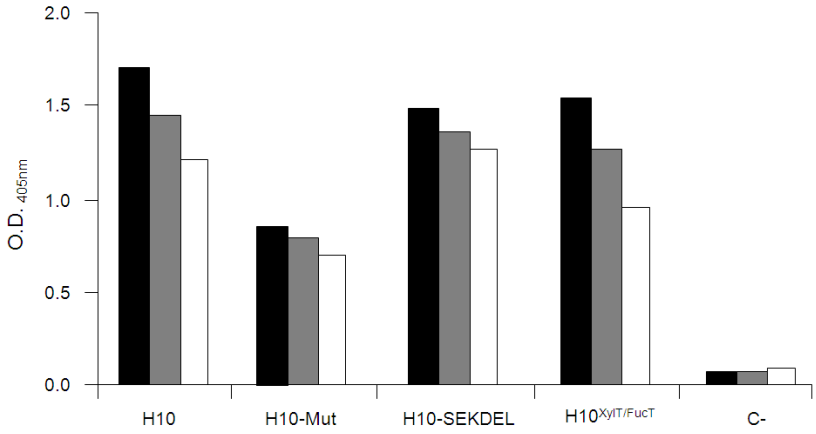


Figure R21: Functional ELISA assay of all H10 glycosylation variants. The antigen binding activity of plant produced purified H10 variants was assayed by ELISA. Plates were coated with recombinant human tenascin-C (C-domain) and detection was achieved using a peroxidase conjugated anti-human λ chain antibody.

Analysis of the quality of the purified immunoglobulins was performed through SDS-PAGE, in order to compare the degradation profile of each H10 variant. The reducing gel analysis revealed the presence of two expected bands at about 50 kDa and 25 kDa, corresponding to the intact IgG, even if an additional strong degradation product was visible around 23 kDa (**Figure R22** panel **a**). In the case of H10-Mut, a very faint band at 50 kDa (corresponding to the whole HC) was visible, while a strong band at 25 kDa as well as at about 23 kDa were present. Most notably, the H10-SEKDEL variant showed an additional degradation product at about 28 kDa (as indicated by the arrow). As expected, HC-SEKDEL band showed a slightly higher molecular weight if compared to H10, due to the presence of the SEKDEL peptide. This result was also confirmed by reducing anti-γ chain Western blot analysis on purified H10-SEKDEL, in which HC showed a band shift compared to H10 (**Figure R22** panel **b**).

In parallel, non-reducing SDS-PAGE analysis performed on H10, H10-SEKDEL and $H10^{XylT/FucT}$ **(Figure R22** panel **c**) showed, for all antibody formats, an expected band of about 150 kDa corresponding to the full size immunoglobulin. On the contrary, in the case of H10-Mut, a barely visible band at 150 kDa was detectable. Furthermore, in all antibody glyco-variants, additional degradation fragments with different intensities were visible at about 100, 70, 50 and 40 kDa (indicated by arrows). In contrast to what observed in the reducing SDS-PAGE analysis, in which only strong bands at about 25 kDa were present in H10-Mut (**Figure R 22** panel **a**), under non-reducing conditions, bands at 50 and 40 kDa were evident indicating the presence of assembled degradation fragments (**Figure R22** panel **c**).

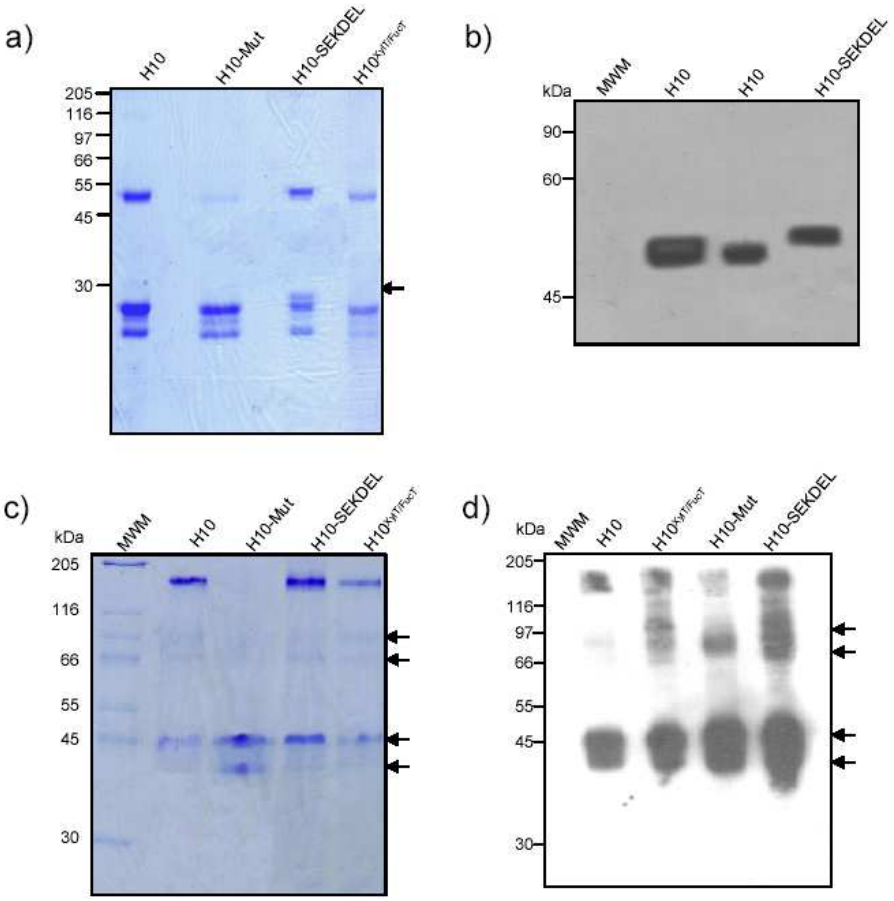


Figure R22: Characterization of purified H10 variants. (a) Reducing SDS-12.5 % (w/v) PAGE analysis of purified H10, H10-Mut, H10-SEKDEL and $H10^{XylT/FucT}$ stained with Coomassie blue. Two micrograms of H10 and 1 μg of H10-Mut, H10-SEKDEL, $H10^{XylT/FucT}$ were loaded on gel. The arrow indicates the additional degradation band at about 28 kDa observed in H10-SEKDEL. (b) Western blot analysis of purified H10 and H10-SEKDEL on reducing SDS-12.5 % (w/v) PAGE. Immunoblotting was developed with an anti-human γ-chain. Lanes 1 and 2: 50 and 25 ng of purified H10 respectively; lane 3: purified H10-SEKDEL (25 ng). (c) Non-reducing SDS-9 % (w/v) PAGE analysis of purified H10 (2 μg), H10-Mut (2 μg), H10-SEKDEL (2 μg) and $H10^{XylT/FucT}$ (1 μg). Arrows indicate degradation bands at about 100, 70, 50 and 40 kDa present with different intensities in all samples. (d) Western blot analysis of purified H10 variants on non-reducing SDS-9 % (w/v) PAGE. Immunoblotting was developed with an anti-human λ-chain. Fifty ng of each antibody were loaded. Arrows indicate degradation bands at about 100, 70, 50 and 40 kDa present in all samples.

Moreover, protein profile of all antibody variants was also assayed by a reducing anti-λ Western blot assay. This analysis showed the presence of the full-size antibody in all samples as a band at 150 kDa, even if at a significantly lower intensity, in the case of H10-Mut (**Figure R 22** panel **d**). Two major bands at about 40 and 50 kDa (indicated by arrows) and two minor bands at about 70 and 100 kDa at different intensities between the samples were also clearly visible, perfectly matching those observed on non reducing SDS-PAGE (**Figure R22** panel **c**), indicating the presence of LC in all these antibody degradation products.

4.3.5 Gel-filtration analysis of purified IgGs

To assess the assembly and integrity of all IgGs purified from *N. benthamiana* agro-infiltrated leaves, size exclusion chromatography was performed using a Superdex S-200 column (GE Healthcare) (**Figure R23**). Analysis of H10-SEKDEL showed that only 24 % of the purified IgG was a full-size antibody (peak at a K_{av} value of about 0.3) with a major degradation product (elution peak at about 0.5 Kav) corresponding to a calculated molecular weight of about 45-50 kDa (**Figure R23** panel **a**, **Table R2**). This degradation product was also clearly visible in the non-reducing SDS-PAGE analysis, lane of the H10 SEKDEL (**Figure R22** panel **c**). The same degradation profile was observed for the purified $H10^{XylT/FucT}$ with only 32 % of intact antibody (peak at a K_{av} value of about 0.3) (**Figure R23** panel **c**).

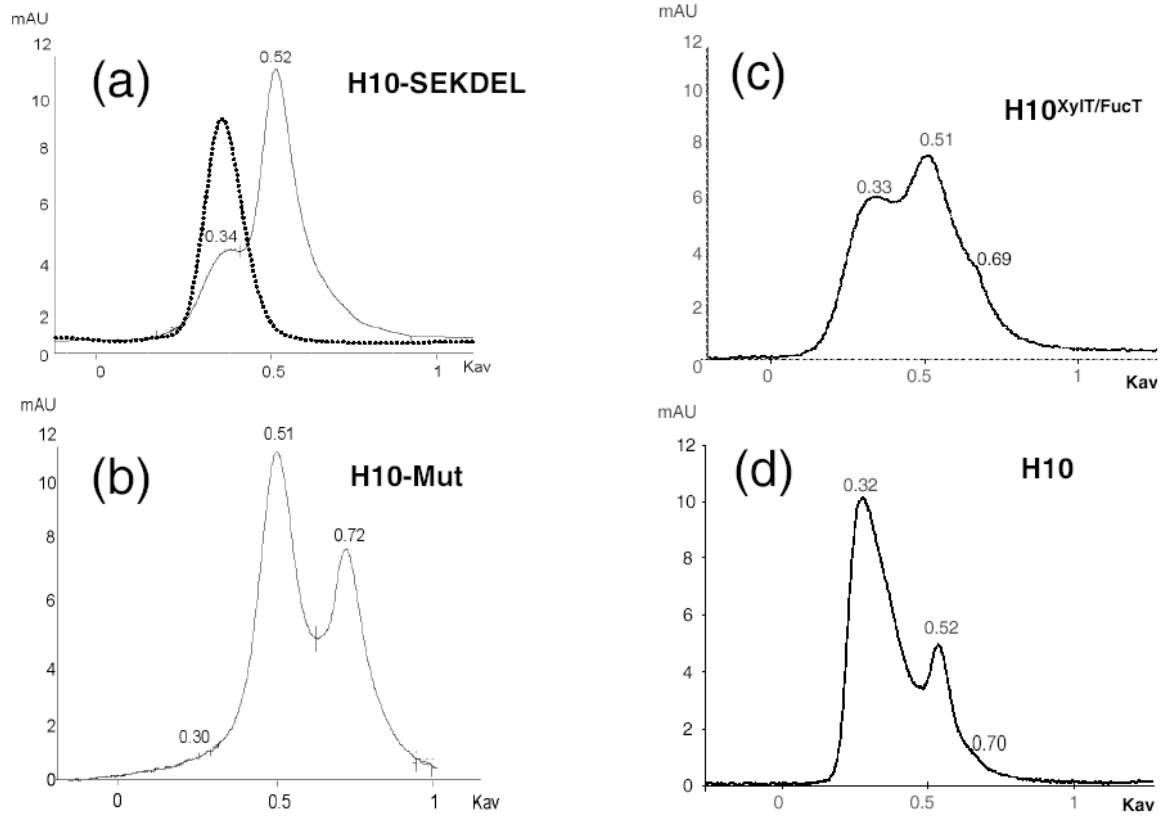


Figure R23: Characterization of purified H10 variants by size-exclusion chromatography. Protein A purified H10 variants were analyzed on a Superdex^{TM}200 5/150 GL column as described in Material and Methods. For a better interpretation of results, the chromatograms are presented over K_{av}= (elution volume- void volume)/ (column volume- void volume) instead of elution volume or retention time. K_{av} values of major peaks are reported. (**a**) Chromatogram obtained for H10-SEKDEL. The broken line represents the chromatogram obtained from a commercially available purified human IgG used as a reference (K_{av} value of about 0.3). Typical chromatograms obtained from purified H10-Mut (**b**), $H10^{XylT/FucT}$ (**c**) and H10 (**d**).

Even stronger degradation was observed in the gel filtration analysis of the purified H10-Mut. In this case, only 3 % of the purified IgG appeared as full-size antibody (the peak at about 0.3 K_{av}) with two major specific degradation products, at about 0.5 and 0.7 K_{av} (**Figure R23** panel **b**).
This result confirmed the data observed in the non-reducing SDS-PAGE in which the fully assembled H10-Mut was barely detectable and a strong band at about 50 kDa was present (**Figure R22** panel **c**). As a reference, we report the chromatogram obtained for the purified H10 in which a major peak at about 0.3 K_{av} (75 % of intact antibody) and a minor degradation peak at about 0.5 K_{av} were observed confirming the results previously reported.

4.3.6 N-glycan analysis

The absence of HC N-glycosylation in the H10-Mut variant was first confirmed by gel-shift analysis in a reducing SDS-7.5 % (w/v) PAGE analysis (**Figure R24** panel **a**). A clear lower molecular weight of the purified H10-Mut HC compared to H10 HC was visible. Glycan analysis of H10, H10-Mut and H10-SEKDEL variants was also performed by concanavalin A (Con A) Western blot. Normalized amounts of the intact HC of each purified antibody (since H10-Mut HC was heavily degraded, a five times higher amount of total purified antibody was loaded on gel with respect to H10 and H10 SEKDEL) were subjected to Western blot analysis using Con A, a lectin able to preferentially bind high mannose sugars of glycoproteins (**Figure R24** panel **b**). As expected, in the case of H10 and H10-SEKDEL a clear band at 50 kDa, corresponding to the HC was visible while no bands were detected in H10-Mut, confirming the lack of sugar chains.

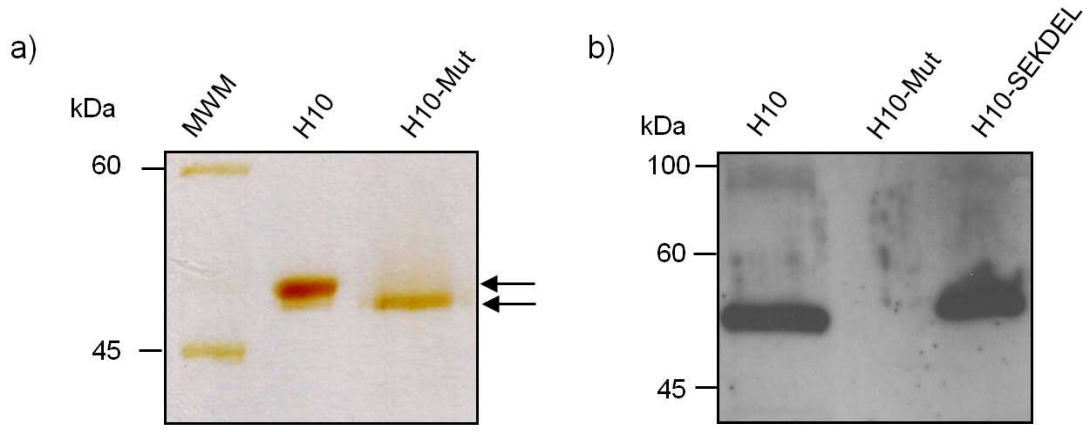


Figure R 24: Analysis of H10-Mut variant. (**a**) Silver Stained reducing SDS-7.5 % (w/v) PAGE of purified H10 and H10-Mut. One µg of total protein for each sample was loaded on gel. Arrows indicate band shift between H10 and H10-Mut. (**b**) Glycosylation analysis by Western blot on non-reducing SDS-10 % (w/v) PAGE of purified H10, H10-Mut and H10-SEKDEL. In order to normalize the amount of intact HC (since H10-Mut HC was heavily degraded) a five times higher amount of total purified H10-Mut (1 µg) was loaded on gel compared to H10 and H10 SEKDEL (200 ng). Proteins were blotted on a nitrocellulose membrane and detection was performed probing with biotinylated Concanavalin-A.

The N-glycan structures of H10, H10-SEKDEL, and H10$^{XylT/FucT}$ were determined and compared. N-glycans were released by hydrazynolysis and the resultant free glycans were 2-aminopyridine (PA)-labeled, analyzed by RP-HPLC (**Figure R25** and **R26**), followed by LC-MS/MS (**Figure R27**).

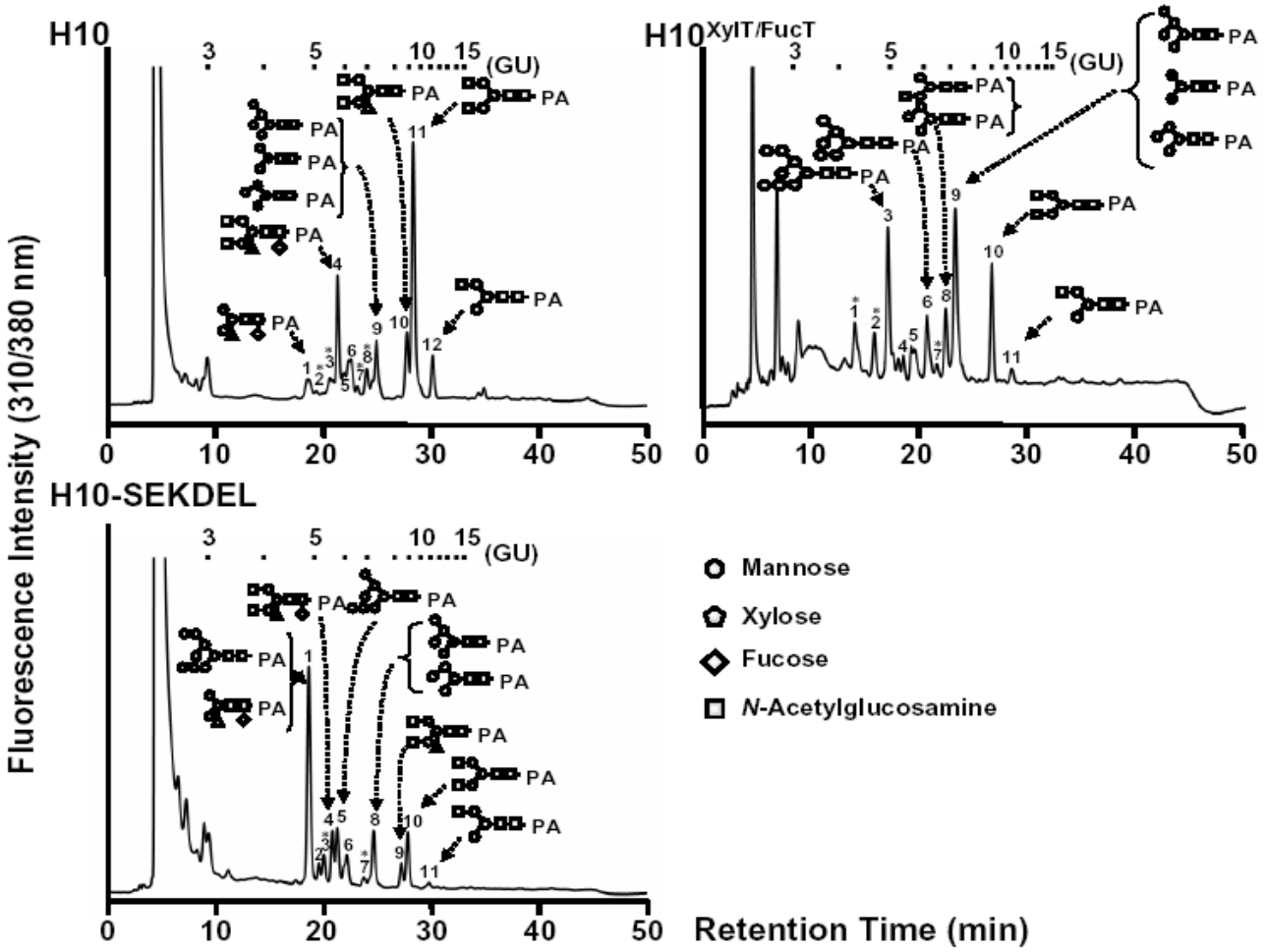
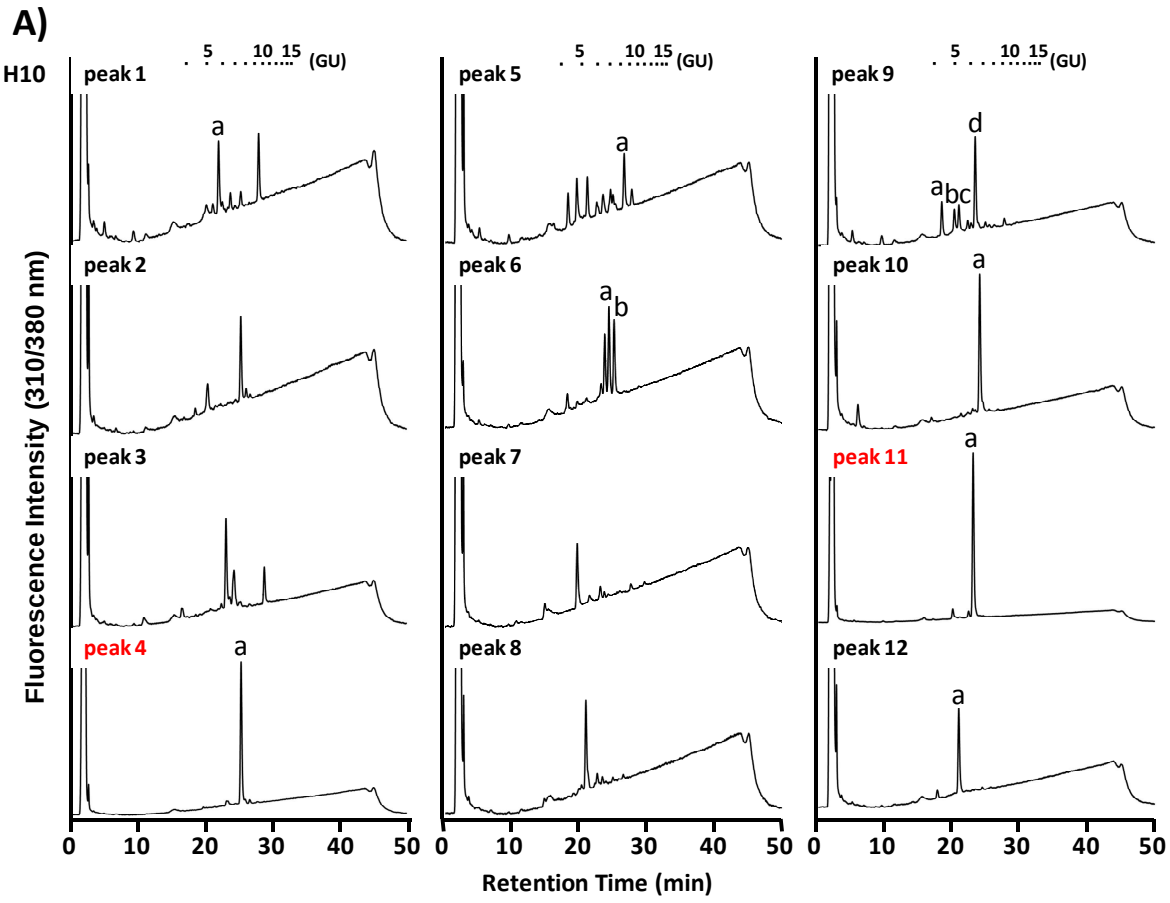
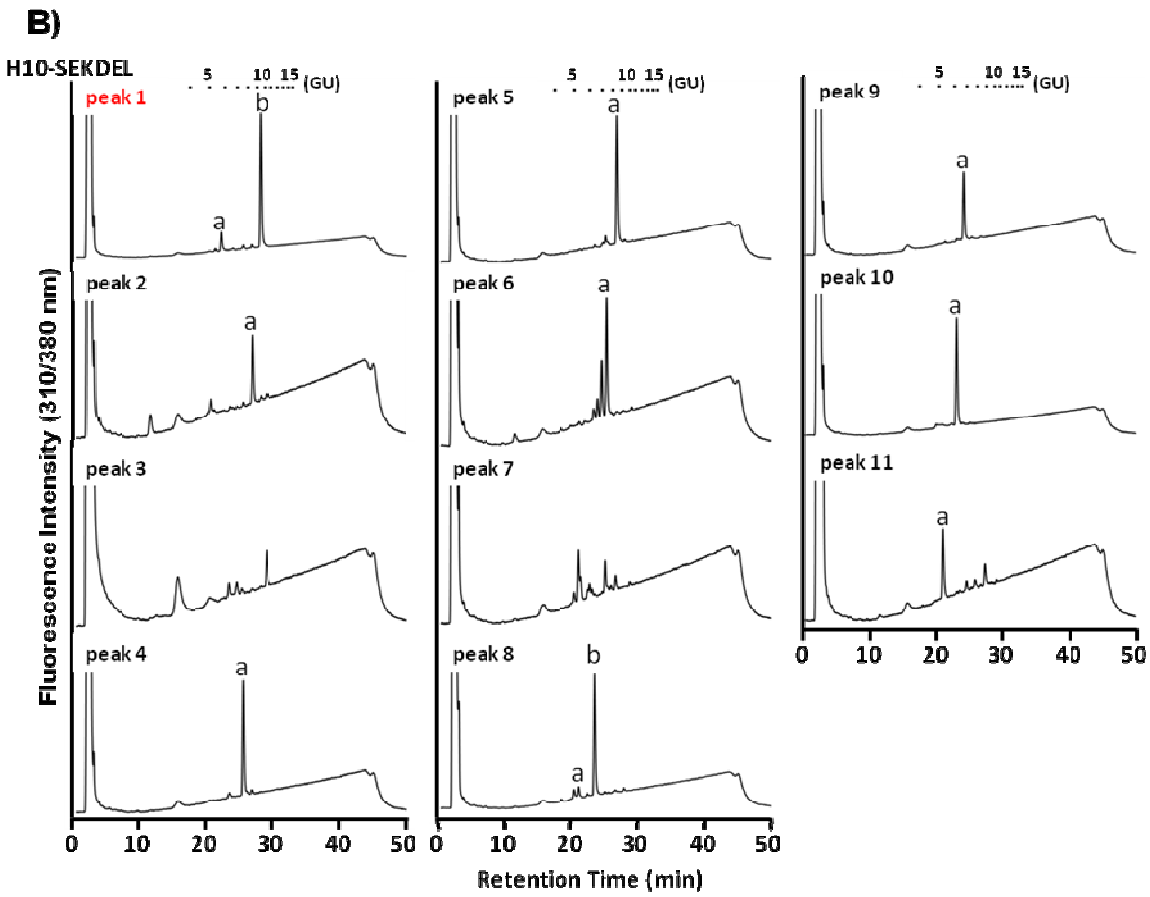


Figure R25: *N*-glycan structures analysis of H10, H10-SEKDEL, and H10$^{XylT/FucT}$. All peaks (indicated by a number) were subjected to LC-MS/MS. Furthermore, *N*-glycan composition was determined by ESI-MS/(MS) analysis by comparing spectra with in-house PA (2-aminopyridine) -sugar chain libraries (Kaulfürst-Soboll, 2011). For simplicity, only predominant structures are indicated in chromatograms. All structures associated to each peak are shown in detail in **Table R3**. Analyzed peaks belonging to non PA-*N*-glycan derivatives or contaminants are indicated by an asterisk. Glucose Oligomer Unit (3-15mer) (GU) is indicated on the upper part of chromatograms to better compare results between chromatograms.


A)
H10
peak 1
peak 2
peak 3
peak 4
peak 5
peak 6
peak 7
peak 8
peak 9
peak 10
peak 11
peak 12
5 10 15 (GU)
Fluorescence Intensity (310/380 nm)
0 10 20 30 40 50
Retention Time (min)


B)
H10-SEKDEL
peak 1
peak 2
peak 3
peak 4
peak 5
peak 6
peak 7
peak 8
peak 9
peak 10
peak 11
5 10 15 (GU)
Fluorescence Intensity (310/380 nm)
0 10 20 30 40 50
Retention Time (min)

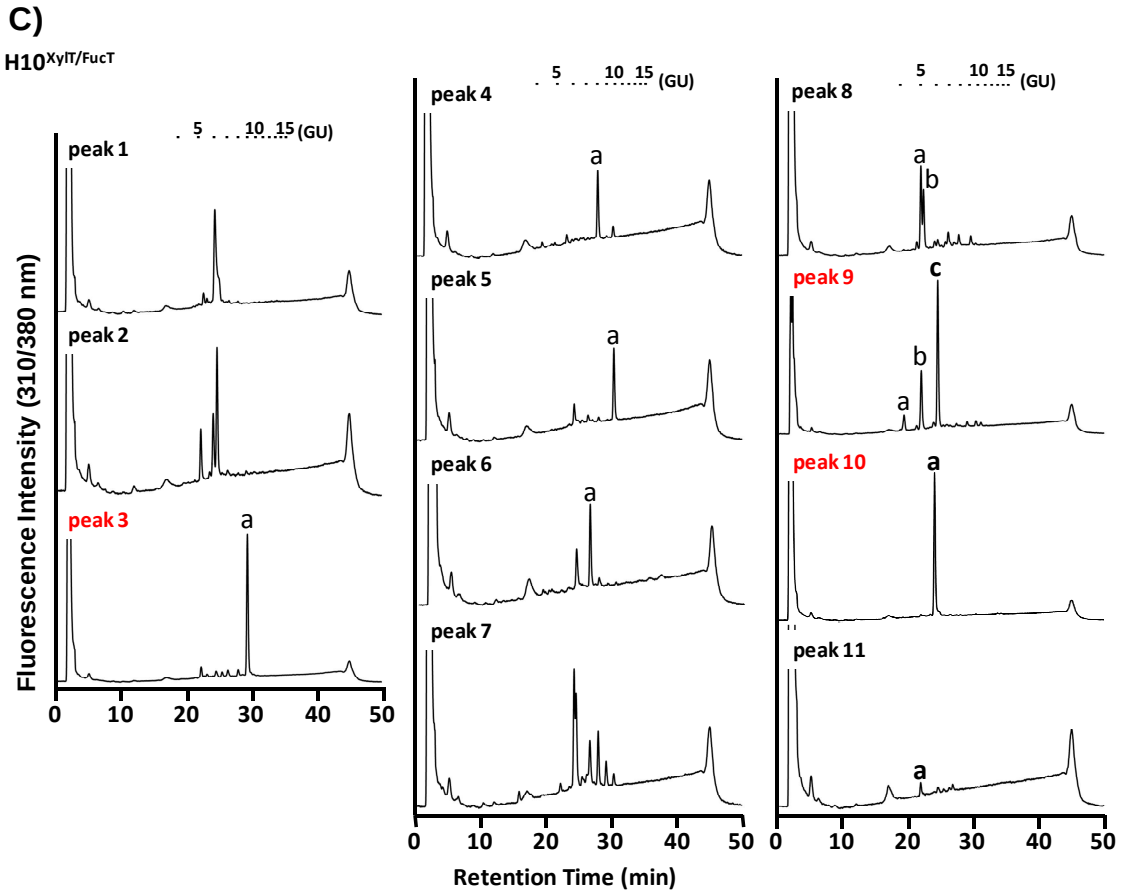
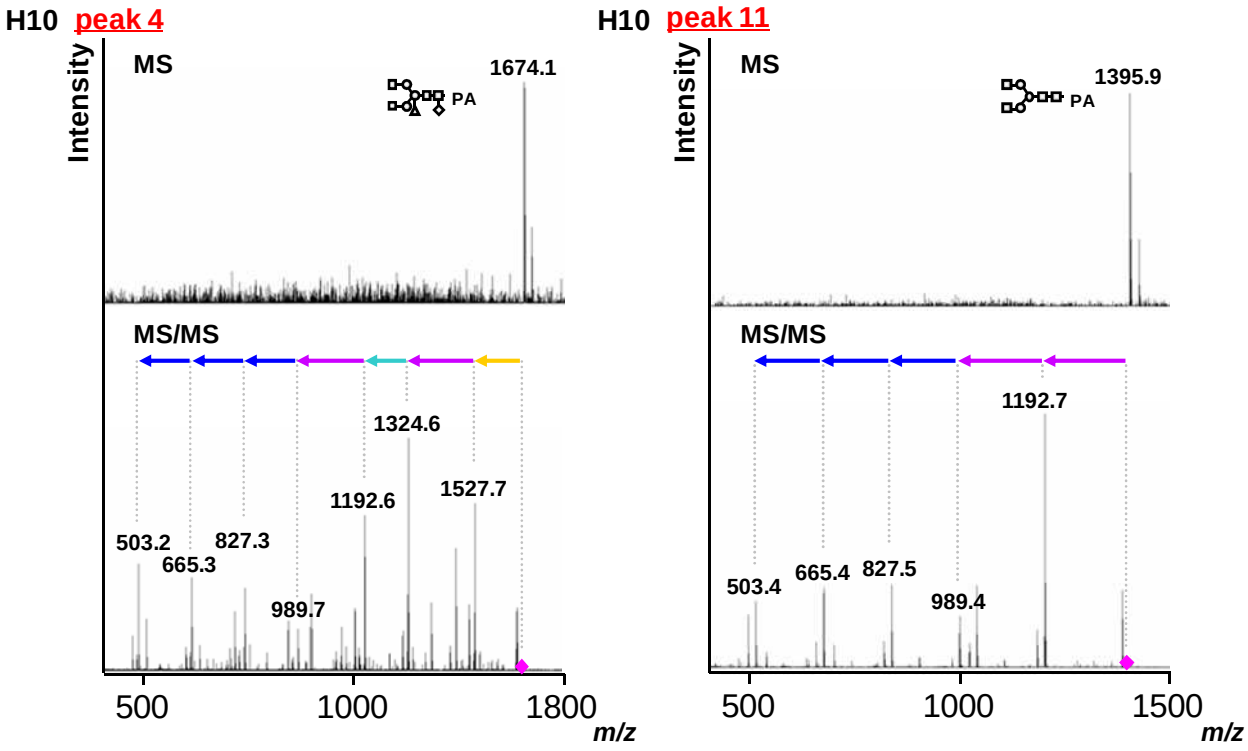


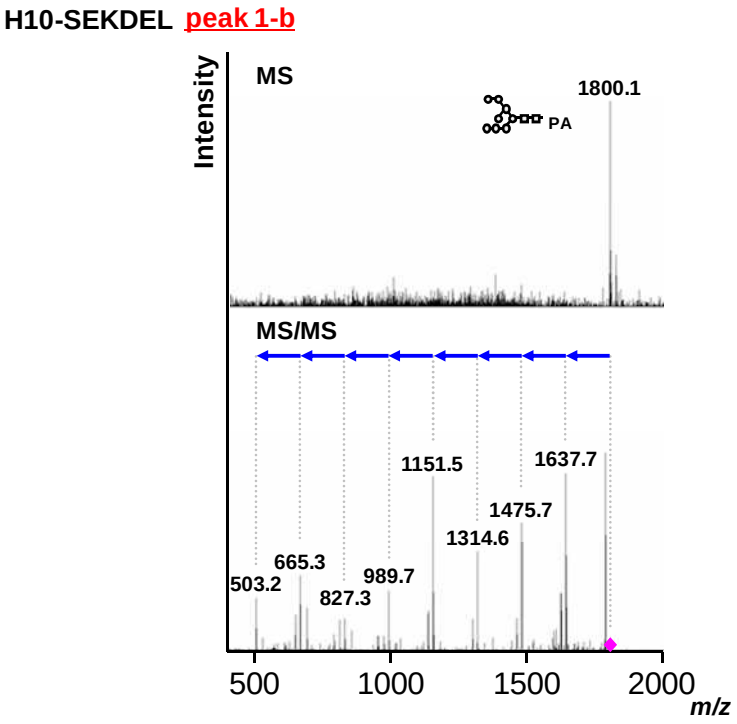
Figure R26: Peaks eluted from the RP-HPLC analysis of H10 (A), H10-SEKDEL (B) and H10$^{XylT/FucT}$ (C) (**Figure R25**) were subjected to LC-MS/MS. The LC part was performed by size-fractionation HPLC analysis using Shodex Asahipak NH2P-50 4E column allowing to separate similar *N*-glycan structures and their ratio was calculated from the peak area of resulting chromatograms. *N*-glycan structures (corresponding to the peaks indicated by letters in the chromatograms) are shown in **Table R4**. Peaks that are not identified by letters belong to non PA-*N*-glycan derivatives or contaminants. To better compare results between chromatograms Glucose Oligomer Unit (3-15mer) (GU) is indicated.

The relative amounts of N-glycans detected in H10 variants were calculated on the basis of their HPLC peak areas detected in LC-MS (**Figure R27**). RP-HPLC demonstrated that H10, H10-SEKDEL, and H10$^{XylT/FucT}$ contained predominant peaks composed of PA-labeled glycans. The relative amounts of N-glycans detected in H10, H10-SEKDEL, and H10$^{XylT/FucT}$ are summarized in **Table R3**.

A)


H10 peak 4
Intensity
MS
1674.1
PA
MS/MS
1324.6
1192.6
1527.7
503.2
827.3
665.3
989.7
500
1000
1800
m/z
H10 peak 11
Intensity
MS
1395.9
PA
MS/MS
1192.7
503.4
665.4
827.5
989.4
500
1000
1500
m/z

B)


H10-SEKDEL peak 1-b
Intensity
MS
1800.1
PA
MS/MS
1151.5
1637.7
1475.7
1314.6
665.3
503.2
989.7
827.3
500
1000
1500
2000
m/z

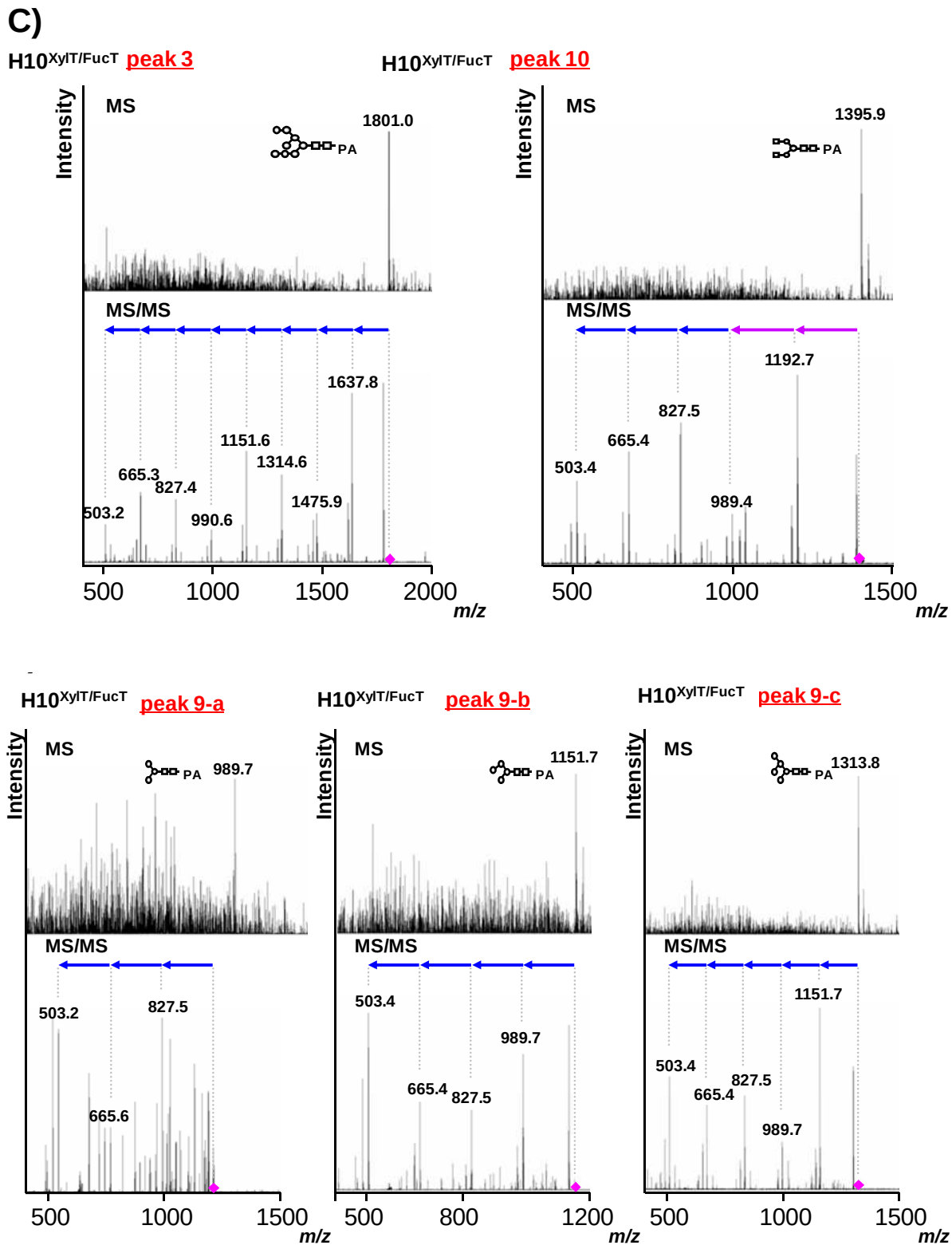


Figure R27: Typical MS spectra for predominant structures are shown. *N*-glycan structures were determined by comparing with Glucose oligomer unit and spectra with in-house PA-sugar chain libraries (Kaulfürst-Soboll, 2011). Results for H10, H10 SEKDEL and H10-Mut are reported in panels A, B, C respectively.

Table R3 Relative amounts of N-glycan structures detected in H10, H10-SEKDEL, and H10XylT/FucT. The relative ratio (%) of structures was calculated on the basis of peak area of SF-HPLC analysis performed in LC-MS/MS.

N-Glycan structure		Ratio (%)		
		H10	H10-SEKDEL	$H10^{XylT/FucT}$
Mannose type	M3	2.4	-	4.8
	M4	2.4	1.3	7.0
	M5	6.9	12.6	20.7
	M6	2.8	6.7	3.4
	M7A	-	3.4	2.9
	M7B	0.7	12.9	-
	M8A	-	26.3	22.0
	M9	-	-	3.4
GlcNAc linked	^{GN}M3	5.6	1.2	0.5
	$_{GN}$M3	-	-	6.4
	GN2M3	46.5	14.6	28.9
Complex type	M3X	2.3	-	-
	GN2M3X	10.2	6.8	-
	M3FX	2.7	3.7	-
	^{GN}M3FX	2.3	-	-
	GN2M3FX	15.2	10.5	-
Total mannose type		15.2	63.2	64.2
Total GlcNAc-linked		79.8	33.1	35.8
Total complex type		32.7	21.0	0

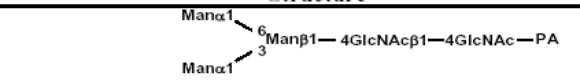
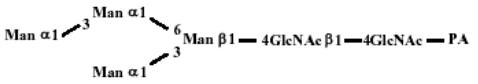
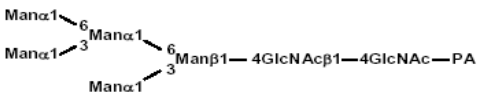
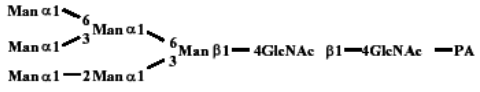
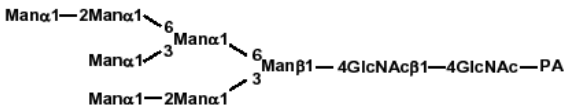
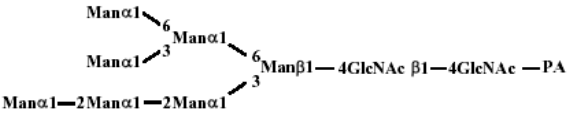
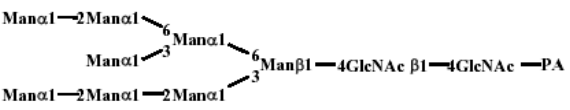
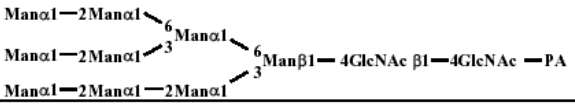
H10 antibody variants contained high-mannose type N-glycans, such as M3, M4, M5, M6, M7A, M7B, M8A, and M9 (abbreviations of sugar structures are summarized in **Table R5**), however, the ratios were substantially different. Particularly, in H10-SEKDEL and $H10^{XylT/FucT}$, the ratios accounted for approximately 64 % of total N-glycan, while the ratio of H10 was ~15 %. On the other hand, H10 contained GN2M3, which is a common precursor of mammalian-type N-glycan, as the most abundant N-glycan, which accounts for 46.5 %. These results suggest that ER retention signal in H10-SEKDEL successfully worked to retrieve glycoproteins from Golgi to the ER, resulting in accumulation of M8A.

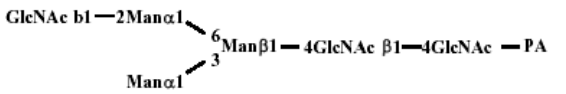
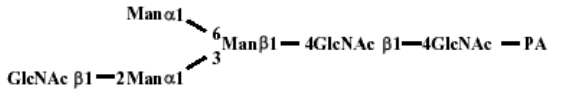
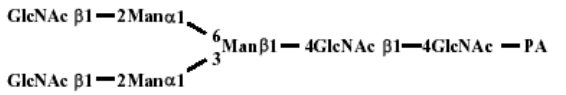
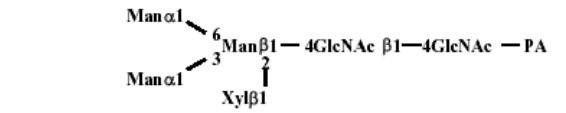
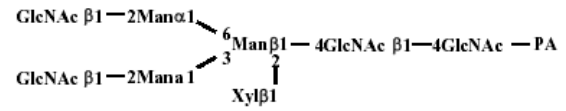
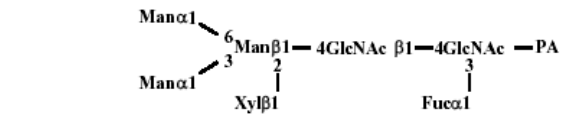
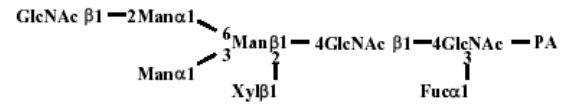
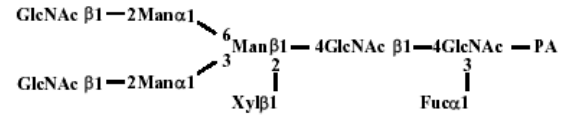
However, results also showed that about 33 % of H10-SEKDEL was transported to medial and late Golgi and modified with Golgi-N-glycosylation machineries, which resulted in residues carrying β 1,2-xylose and α 1,3-fucose, indicating a sort of escape from ER retrieval system. Moreover, while $H10^{XylT/FucT}$ did not contain potentially immunogenic complex plant sugars, 33 % of H10 N-glycans harboured β 1,2-xylose and α 1,3-fucose residues. As for $H10^{XylT/FucT}$ N-glycans, high-mannose type N-glycans, especially M8A and M5, were added.

Table R4: N-glycan structures determined by LC-MS/MS analysis.

H10		H10-SEKDEL		$H10^{XylT/FucT}$	
Peak n.	Sugar type	Peak n.	Sugar type	Peak n.	Sugar type
1-a	M3FX	1-a	M3FX	3-a	M8A
4-a	GN2M3FX	1-b	M8A	4-a	M7A
5-a	M7B	2-a	M7A	5-a	M9
6-a	^{GN}M3FX	4-a	GN2M3FX	6-a	M6
6-b	M6	5-a	M7B	8-a	M4
9-a	M3	6-a	M6	8-b	$_{GN}$M3
9-b	M3X	8-a	M4	9-a	M3
9-c	M4	8-b	M5	9-b	M4
9-d	M5	9-a	GN2M3X	9-c	M5
10-a	GN2M3X	10-a	GN2M3	10-a	GN2M3
11-a	GN2M3	11-a	^{GN}M3	11-a	^{GN}M3
12-a	^{GN}M3				

Table R5: Table indicating abbreviations of glycan structures. In parenthesis are shown numbers of the corresponding SF-HPLC peaks**.**

	Structure	Abbreviations	H10	H10-SEKDEL	$H10^{XylT/FucT}$
Mannose type		M3	2.4 (9-a)	-	4.8 (9-a)
		M4	2.4 (9-c)	1.3 (8-a)	7.0 (8-a, 9-b)
		M5	6.9 (9-d)	12.6 (8-b)	20.7 (9-c)
		M6	2.8 (6-b)	6.7 (6-a)	3.4 (6-a)
		M7A	-	3.4 (2-a)	2.9 (4-a)
		M7B	0.7 (5-a)	12.9 (5-a)	-
		M8A	-	26.3 (1-b)	22.0 (3-a)
		M9	-	-	3.4 (5-a)

GlcNAc linked		^{GN}M3	5.6 (12-a)	1.2 (11-a)	0.5 (11-a)
		$_{GN}$M3	-	-	6.4 (8-b)
		GN2M3	46.5 (11-a)	14.6 (10-a)	28.9 (10-a)
		M3X	2.3 (9-b)	-	-
		GN2M3X	10.2 (10-a)	6.8 (9-a)	-
		M3FX	2.7 (1-a)	3.7 (1-a)	-
		^{GN}M3FX	2.3 (6-a)	-	-
		GN2M3FX	15.2 (4-a)	10.5 (4-a)	-

4.4 DISCUSSION

In the previous section we showed the possibility to express high levels of mAb H10 in *N. benthamiana* leaves using a vacuum agro-infiltration system based on the use of the P19 gene silencing suppressor protein. Moreover we demonstrated that H10 could be efficiently purified obtaining an extremely pure product (99.4 %), free from plant contaminants and endotoxins. However, analysis of the final product showed that the antibody exhibited a typical plant glycosylation profile with the presence of complex-type oligosaccharides exposing β 1,2-xylose and α 1,3-fucose moieties. The presence of these sugars represents a limitation to its use in immunotherapy, as it has been suggested that xylose and fucose moieties may cause immunogenic reactions in mammals (Gomord et al., 2010). Moreover, it has been demonstrated that plant complex glycan structures may also interfere with the binding to Fcγ receptors (Forthal et al., 2010). In this section, we present data on three different H10 antibody glyco-variants in which the glycosylation profile was modulated through three different procedures: site-directed mutagenesis to abolish the glycosylation site, Endoplasmic Reticulum retention (C-terminal SEKDEL fusion) to ensure predominantly high-mannose type glycans, and expression in a *N. benthamiana* RNAi down-regulated line in which β 1,2- xylosyltransferase and α 1,3- fucosyltransferase gene expression was silenced.

Prior to transient over-expression in leaves, we preliminarily characterized H10, H10-Mut and H10-SEKDEL formats by transient expression in tobacco protoplasts, in order to identify the sub-cellular localization of each variant. This study, carried out through a pulse-chase experiment, showed that H10 and H10-Mut were efficiently secreted by tobacco cells while H10-SEKDEL was barely detectable in the culture medium, indicating the substantial retention of this antibody in the ER. However, a partial escape of the antibody from the Endoplasmic Reticulum could not be excluded. In fact, in a previous study presented by Fujiyama and colleagues (2009), the ER retained form of a mouse antibody expressed in suspension-cultured tobacco BY2 cells, bearing the SEKDEL signal on both heavy and light chain, exhibited a partial escape of the immunoglobulin from the ER, in spite of the retention signal. As evidence, this IgG showed the presence of complex-type oligosaccharides with β 1,2-xylose and α 1,3-fucose moieties typical of the Golgi compartment, indicating a partial escape from ER retention/cis-Golgi retrieval. To further investigate the H10-SEKDEL behavior during the expression process in agro-infiltrated *N. benthamiana* leaves we verified the preservation of the SEKDEL peptide fused to both H and L chains. Surprisingly, Western blot analysis of crude extracts of the agro-infiltrated H10-SEKDEL carried out using an heavy chain detection antibody, revealed the presence of two consecutive bands, one matching with

the HC of the secretory H10 used as control, the second, with higher molecular weight. This result indicated the coexistence of two populations of heavy chains deriving either from a partial cleavage of the SEKDEL or to a different glycosylation pattern. On the contrary, Western blot analysis of the H10-SEKDEL light chain from the crude extract, revealed the presence of a unique band with an higher molecular weight compared to secretory H10 LC used as control. This reduced electrophoretic mobility, indicated the presencence of the SEKDEL peptide fusion at the C-terminus. However, analysis of the purified H10-SEKDEL carried out both by Coomassie stained SDS-PAGE and Western blot detected with an anti-γ antibody, did not confirm the presence of a double band in the case of the HC analysis. In fact, in this case a unique heavy chain (~50 kDa) and a unique light chain band (~25 kDa) both showing a lower electrophoretic mobility, compared to H10 were detected, indicating that the purified antibody maintained the SEKDEL retention peptide. This result was confirmed by the LC–MS glycosylation analysis performed on the purified H10-SEKDEL due to the high percentage of typical high-mannose ER sugar moieties compared to the secretory H10. In fact, in this case, in comparison to the secretory H10, an high percentage (63 % compared to 15 % of H10) of the sugar residues, were typical high-mannose ER glycans (M5 - M6 - M7 - M8 forms), indicating the retention of the H10 SEKDEL in the Endoplasmic Reticulum. In fact, as reported in literature, Man7 (Tekoah et al., 2004; Triguero et al., 2005) or Man7/8 (Sriraman et al., 2004) were the most abundant *N*-glycans in mAbs expressed in plants with KDEL ER-retention signal. So, the finding of M8 as the major *N*-glycan in H10-SEKDEL, strongly supports the full functionality of the ER-retention signal. Nevertheless, glycan analysis showed also the presence of a fraction (21 %) of complex sugars (β 1,2-xylose and α 1,3-fucose residues) as result of a specific modification of H10-SEKDEL by the Golgi-*N*-glycosylation machineries. This effect revealed an escape of the antibody from the ER retrieval system in spite of the presence of the SEKDEL retention peptide. However, a similar evidence was previously described by other groups that reported the same result for antibodies harbouring the KDEL fusion peptide (Tekoah et al., 2004; Triguero et al., 2005; Rademacher et al., 2008; Floss et al., 2008). In these studies, it was shown that antibody forms bearing an ER retention signal did not always guarantee a complete retention of the immunoglobulin in the cells since in all these cases a low percentage of β 1,2-xylose and α 1,3-fucose residues was reported. On the contrary, other studies on the expression of immunoglobulins harbouring the KDEL tag fusion, described the complete retention of the antibody in the ER of both transgenic tobacco plants and agro-infiltrated *N. benthamiana* (Sriraman et al., 2004; Petruccelli et al., 2006; Floss et al., 2009; Sainsbury et al., 2010; Loos et al., 2011). Many factors can explain the variability of the glycosylation pattern such as the sensitivity of the methodology used for *N*-glycans identification or, most likely, the plant expression system used or

the antibody sequence itself which may influence accessibility to the KDEL sequence. In the case of the H10 produced in a XylT/FucT *N. benthamiana* mutant, analysis of the glycosylation profile showed a high percentage of high-mannose type *N*-glycans as well as a high amount of GN2M3, a common precursor of mammalian-type *N*-glycans. Most interestingly, as expected, the glycan profile showed the absence of the typical plant complex residues β 1,2-xylose and α 1,3-fucose confirming the efficiency of these plants to block the activity of the fucosiltransferase and xilosiltransferase glyco-enzymes. These findings substantially matched those obtained in the transient expression of the 2G12 antibody in *N. benthamiana* XylT/FucT mutants by agro-infiltration (Forthal et al., 2010; Strasser et al., 2008). On the contrary, in the case of the analysis performed on the secretory H10 expressed in wild type *N. benthamiana* plants, the presence of higher percentages of complex *N*-glycans (32 %) represented by M3X, GN2M3X, M3FX, GN2M3FX, GNM3X was observed. Surprisingly, the most represented sugar structure identified for H10 was the GN2M3, with a percentage of 46.5 %. Several studies presented in literature emphasized that this sugar was normally present at low percentages in antibodies targeted to the secretory pathway. Nevertheless, it must be noted that in a recent work reported by Vezina et al., (2009), high percentages of GN2M3 were described for the secretory murine mAb C5-1 produced in *N. benthamiana* plants using a vacuum agroinfiltration system (Vezina et al., 2009).

The analysis of expression levels of the H10-SEKDEL glyco-variant, surprisingly emphasized that this value was lower compared to the secretory H10 counterpart (480 mg/kg FW instead of 640 mg/kg FW). This result is somehow unexpected as the ER retention strategy is normally used to increase antibody accumulation in plant cells (Schillberg et al., 2003; Petruccelli et al., 2006). Nevertheless, as reported by a recent work, ER targeting of a mouse IgG produced in agro-infiltrated *N. benthamiana* plants, using a viral suppressor of gene silencing (HCPro), did not show any significant higher accumulation of the antibody in leaves if compared to its secretory counterpart (Vezina et al., 2009), confirming that in some expression systems, the ER retention could even result detrimental for antibody accumulation.

Characterization of the integrity of the purified antibodies was conducted by SDS-PAGE analysis and gel filtration chromatography. Electrophoresis performed on the purified antibodies revealed that all H10 variants had very similar specific degradation profiles with a major band at 50 kDa in the non reducing analysis, likely corresponding to Fab fragments, and minor products at about 100, 70 and 40 kDa. We previously demonstrated that protein-A affinity chromatography specifically captured not only the Fc portion of H10 but also the Fab fragment due to the presence of an HC V_H3 variable domain able to bind D domain of protein A. This double binding valency explained the specific enrichment for H10 Fab fragments in the purified product, rendering this molecule an

interesting model system to study antibody degradation in plant (Villani et al., 2009). Major striking differences were found for the aglycosylated H10-Mut. In fact, the reducing gel evidenced a very faint band corresponding to the intact heavy chain (~50 kDa) and a strong band at ~25 kDa that indicated a specific degradation of the heavy chain around the hinge region. This hypothesis was confirmed by the non-reducing analysis, in which an almost unique band at ~50 kDa was present. These data were supported also by the Western blot analysis performed on the crude extracts.

This analysis highlighted that H10 SEKDEL and H10 produced in the XylT/FucT *N. benthamiana* mutant did not show significantly different profiles in comparison to the H10 secretory form, making difficult to find a direct correlation between glycosylation pattern and antibody stability. In contrast, a direct correlation between stability and glycosylation status was evidenced by the comparison between H10 and H10-Mut. In this case, the Western blot analysis revealed a more severe degradation profile, in which, compared to the secretory H10, the band corresponding to the intact HC was significantly reduced and the presence of additional degradation bands was observed. These antibodies differ only by a point mutation in the Asn 297 glycosylation site and were expressed in plants using exactly the same conditions. Besides, pulse-chase analysis revealed a similar secretory performance in plant cells indicating that glycosylation has no influence in antibody localization within the cell.

Moreover, gel filtration analysis revealed that almost 97 % of the antibody was degraded with a major elution peak at a K_{av} value of 0.51, corresponding to a molecular weight of 50 kDa. This evidence, together with the antigen binding activity of H10-Mut suggests that this antibody variant is specifically degraded in plants, leading to the formation of functional Fab fragments. A possible explanation of this tendency is the susceptibility to a specific proteolytic cleavage of the H10 heavy chain hinge region. Furthermore, we give evidence that the aglycosylated H10-Mut produced in agro-infiltrated *N. benthamiana* is significantly more prone to proteolytic degradation if compared to its glycosylated counterpart (H10). There is a large body of work demonstrating that N-glycosylation of the heavy chain is fundamental not only for Ab effector function but also for protein stability and prevention of aggregation phenomena (Kayser et al., 2011). It should also be pointed out that H10-SEKDEL showed higher degradation if compared to its secretory counterpart (24 and 75 % of the antibody being an intact H_2L_2 molecule, respectively). In this case, reducing analysis revealed the presence of a band with a molecular weight of about 28 kDa, which was not present in all other samples, indicating a peculiar degradation product of HC-SEKDEL. Apparently, this result is in contrast with the general assumption that ER retention protects antibodies from degradation.

Recent results reported no significant differences in the degradation profile between secretory and ER retained form of the transiently expressed 2G12 in *N. benthamiana* plants (Sainsbury et al., 2010). On the contrary, the expression of 2G12 in *Arabidopsis thaliana* seeds, showed that retention in the ER was detrimental for both expression yields and antibody integrity, although, in this case, antibody accumulation was specifically directed to the protein storage vacuoles (Loos et al., 2011). Most surprisingly, also the secretory H10 produced in the XylT/FucT *N. benthamiana* mutant showed higher degradation (32 % of intact antibody) if compared to H10. In this case, it is important to point out that transient expression was performed without the use of the p19 silencing suppressor protein, resulting in lower accumulation levels in leaves.

Data herein provided a thorough characterisation of different variants of the tumour targeting H10 antibody produced at high yields in agro-infiltrated *N. benthamiana* plants. Protein stability and degradation is known to be influenced by different factors (peptide sequence, protein targeting compartment, genetic constructions and purification procedures), overall data obtained for H10 variants suggest that also glycosylation might play an important role. It is evident that both ER retention and secretory expression of H10 in the XylT/FucT *N. benthamiana* double mutant are promising strategies for the modulation of antibody glycosylation. Future work will be directed towards the identification of possible ‘fragile’ sites in the H10 antibody hinge region particularly prone to be cleaved by the plant protease machinery in order to explore new strategies to reduce antibody degradation and increase the yield of intact IgG.

4.5 CHARACTERIZATION OF THE DEGRADATION PROFILE OF mAb H10 PRODUCED IN PLANT

Proteolytic processes occurring in the plant cell, are essential to ensure metabolic functions but could represent, in some cases, a drawback for the production of biologically active heterologous proteins in plants. In particular, in the case of plant-derived antibodies unintended proteolysis driven by this complex proteolytic machinery can dramatically affect the final yield of intact IgG causing the degradation of more than 90 % of the final purified immunoglobulin (Villani et al., 2009). Many studies reported a strong degradation for a number of plant-derived antibodies detectable in SDS-PAGE analysis as additional smaller fragments between 20 and 150 kDa under non-reducing conditions (De Muynck et al., 2010). In this part of the work, I characterized the proteolysis of the plant-derived mAb H10 in order to study *in planta* antibody degradation profile and to identify specific cleavage sites of the immunoglobulin. Furthermore, an *in silico* analysis permitted the identification of proteases responsible of the cleavage. In order to protect the antibody from cleavage, a site directed mutagenesis of specific aminoacid sequences recognized by these proteolytic enzymes was performed.

4.5.1 Purification of H10 from plant extracts

Purification of mAb H10 was performed using three different affinity chromatography columns: anti γFc, protein A and protein G column. The use of different affinity columns enabled the comparison of the purification efficiency of each methodology, in terms of final recovery yield and quality of the purified immunoglobulin. Plant extracts were prepared from 40 g of vacuum-agroinfiltrated *N. benthamiana* leaves without the addition of any protease inhibitor and, after clarification, were loaded on the affinity column. The non reducing SDS-PAGE analysis of H10 purified using three different strategies, is shown in **Figure R28**. In all cases, the protein profile presented a major band at 150 kDa, corresponding to the intact form of the mAb H10. Nevertheless, several bands at lower molecular weight, corresponding to partially assembled or degradation products of the antibody, were visible.

In the case of H10 purified with the Anti γFc-affinity column, nine bands were detected between 140 and 10 kDa. This profile was comparable to that obtained after purification with protein G column. In fact, except for the fragment around 10 kDa, absent in the protein G purifications, all bands seem to match with Western blot, ELISA and N-terminal sequencing.

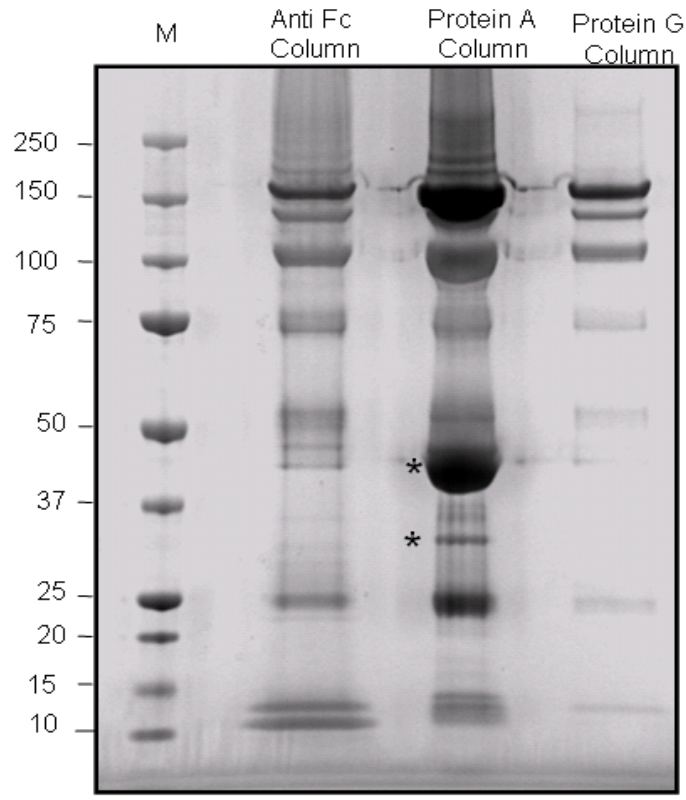


Figure R28: Analysis of H10 purified from extracts of vacuum-agroinfiltrated *N. benthamiana* leaves using different affinity chromatography strategies (Anti γFc, protein A and protein G affinity chromatography column). Analysis was performed loading 20 µl of antibody purified from batches of 40 g of vacuum-agroinfiltrated leaves onto non-reducing SDS 4-20 % (w/v) PAGE stained with Coomassie blue. Each purified sample was concentrated 10 times by lyophilization before gel loading. Asterisks indicate the two additional major bands present in the purification of mAb H10 performed with protein A column.

Even if the protein profile obtained with the three techniques is generally comparable, two major differences are clearly evident in the case of protein A column in which the presence of two additional bands between 50 and 25 kDa is clearly observable (indicated by an asterisk). The higher band (around 45 kDa) is present at barely detectable levels also in the purification profile obtained with the γFc column while the lower band (around 30 kDa) is not present in any protein profile analyzed. The band at 45 kDa shows a strong intensity compared to the one at 30 kDa, confirming the high affinity of protein A for this specific fragment. It must be noted that all purifications were conducted using the same batch of agroinfiltrated material starting from 40 g of leaf material and in the case of protein A, a higher purification efficiency (75 mg of total purified proteins from Kg FW compared to 15 mg/Kg FW in the case of Anti γFc, and protein G columns) is clearly evident as well as the difference in the relative abundance of some protein products compared to the Anti γFc, and protein G columns. All degradation bands (nine in total, **Figure R30**) were excised from the gel and analyzed using different techniques (**Figure R29**).

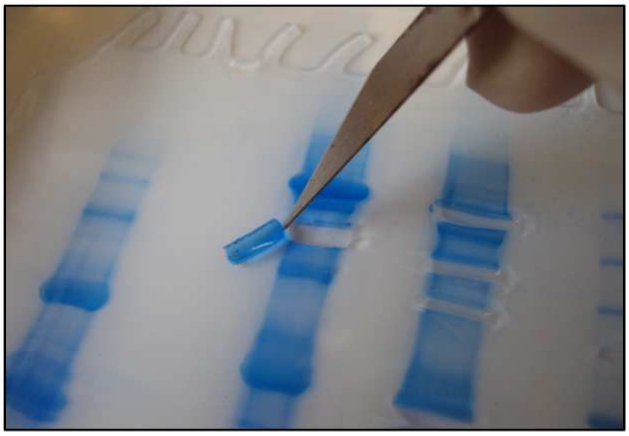


Figure R29: Example of cut band from SDS-PAGE Coomassie blue. Each excised band containing a single degradation fragment was analysed with different techniques (Western Blot, ELISA, N-terminal sequence).

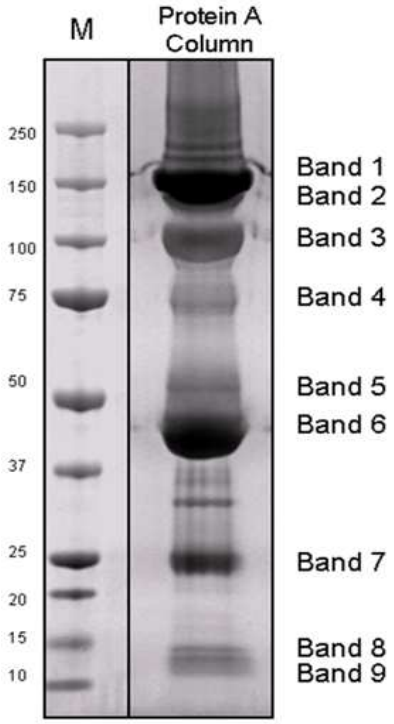


Figure R30: Coomassie staining of the SDS-PAGE analysis of the purified mAb H10. To each band was assigned an identification number.

4.5.2 Western blot analysis of purified H10 bands excised from SDS-PAGE gel

Affinity purified mAb H10 from vacuum-agroinfiltrated *N. benthamiana* plants was analyzed in order to characterize the observed protein degradation products. Protein A affinity-chromatography revealed to be the best technique to isolate the most abundant H10 degradation fragments. Therefore, the 9 most abundant Coomassie stained SDS-PAGE protein bands were excised from the gel (**Figure R29**), proteins were eluted and again individually analyzed by SDS-PAGE followed by

Western blot analysis using an anti-human γ IgG1 (**Figure R31** panel **A**) or anti human-λ antiserum (**Figure R31** panel **B**). This method allowed to reveal the presence of HC and/or LC derived fragments in each excised protein band.

Western blot analysis performed using the anti-human γ antibody showed the presence of HC in all degradation bands, except for band 8 (15 kDa) and 9 (13 kDa). On the other hand, the anti-λ Western blot analysis of excised bands proved the presence of the LC in all bands except for band 5 (50 kDa), 8, and 9 (15 and 13 kDa, respectively) indicating an absence of LC.

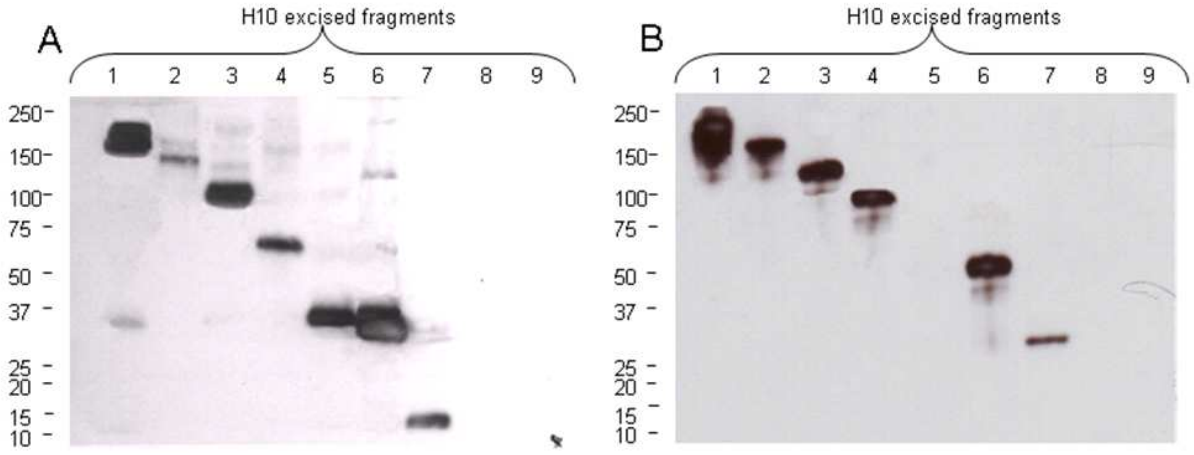


Figure R31: Western blot analysis performed on proteins eluted from the bands excised from the SDS-PAGE analysis (analyzed bands are shown in **Figure R30** and identified by a number). Bands were excised from gel, homogenized in PBS 1 X and separated again by 4-20 % SDS-PAGE under non-reducing conditions. Proteins were blotted to a PVDF membrane and probed with either anti-human γ IgG1 antiserum (A) or anti human-λ antiserum (B).

Degradation bands where further studied by Western blot analysis using Concanavalin A, a lectin with an high saccharide binding specificity. As previously described, mAb H10 contains N-glycans attached to the asparagine-297 (Asn297) residue of the C_H2 domain of the immunoglobulin HC. Western blot analysis in reducing conditions of the single protein fragments with Concanavalin A, allowed the identification of bands harbouring the glycan chain on the C_H2 domain (**Figure R32**).

The presence of the glycan chain was detected in all protein fragments, except for band 3 and 6, indicating that these bands do not contain the C_H2 domain of the antibody. Moreover, band 1, 2 and 4 showed a molecular weight of 50 kDa corresponding to the size of the intact HC. Interestingly, band 5 in this reducing Western blot analysis showed a molecular weight reduced by half (25 kDa), indicating the presence of a truncated HC fragment.

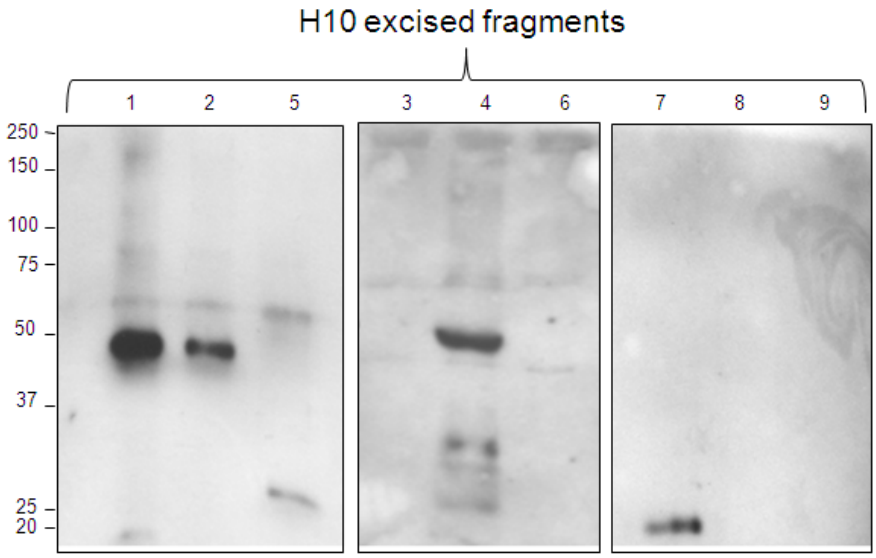


Figure R32: Western blot analysis of mAb H10 degradation bands excised from the SDS-PAGE Coomassie blue (**Figure R30**) of the mAb H10 purified from *N. benthamiana* leaves by protein A. Bands were excised from gel, homogenised in PBS 1X and separated again by 4-20 % SDS-PAGE under reducing conditions. Proteins were blotted to a nitrocellulose membrane and probed with biotinylated Concanavalin A, a lectin with an high saccharide binding specificity, and detected with peroxidase conjugated Streptavidin. Numbers indicates the analysed bands as reported in the Coomassie blu gel.

4.5.3 ELISA of purified H10 bands excised from SDS-PAGE

The study of the degradation fragments of the mAb H10 was improved by ELISA, in order to verify which of the H10 fragments maintained the capability to bind to the antigen. To this aim, each band of interest was ground in presence of PBS 1 X and centrifuged. The recovered supernatant, containing the eluted fragment, was loaded on an ELISA plate coated with Human Lung Cancer cells (A 549). These cells expose on their surface the large isoform of tenascin including the C domain specifically recognized by the mAb H10 (Fitch et al., 2011). Bands 1, 3, 4, 5 and 6, that showed a molecular weight above 40 kDa, were selected for the analysis. As positive control 150 ng of a plant purified mAb H10 were used. As the intensity between the bands in SDS-PAGE was markedly different and some bands were very faint (bands 4 and 5 in particular), it was not possible to normalize for the TSP content loaded on each well. Results obtained in the ELISA experiment (**Figure R33**) revealed that band 1, representing the intact IgG as expected is able to bind the antigen. Moreover we show that also band 3 and 6 have antigen binding specificity. In the case of the weaker bands 4 and 5, only band 4 showed a significant binding activity to tenascin-C. It must be noted that Western blot analysis performed on bands 3, 4 , and 6 evidenced the presence of both HC and LC while in the case of band 5 only HC was detected (**Figure 31** panel **A**, **B**). This data support the hypothesis of the presence of the binding site in bands 3, 4 and 6.

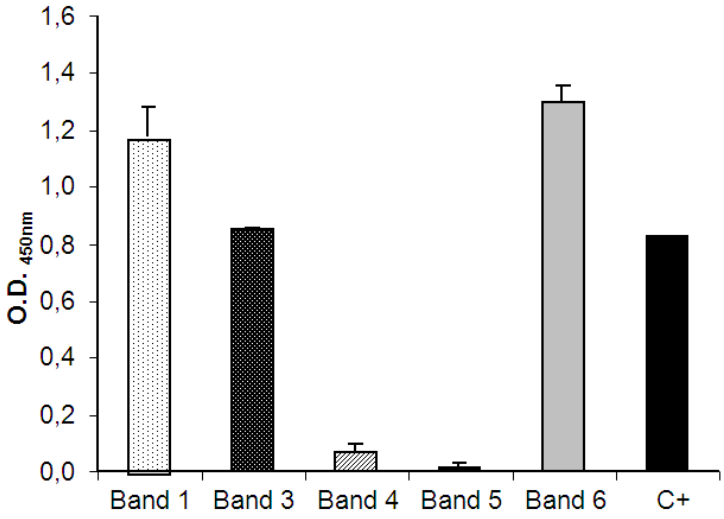


Figure R33: Whole cell ELISA performed using Human Lung Cancer cells (A 549) performed loading on the wells the band coming from the SDS-PAGE analysis of the H10 (**Figure R30**). The experiment was carried out loading on the wells the supernatant coming from each ground band in 50 µl of 1 X PBS. The detection was performed with a peroxidase anti human-λ chain antibody. C+ H10 (positive control): 150 ng of plant purified mAb H10. The absorbance is detected at a wave length of 450 nm.

4.5.4 Two-dimensional gel electrophoresis of protein A purified mAb H10

The study of the protein profile of mAb H10 purified with protein A was also conducted using a reducing two-dimensional gel electrophoresis (2-DE) analysis. As shown in **Figure R34** all degradation spots were localized around 15-25 kDa with an experimental isoelectric point (pI) range of 3-7. Furthermore, spots 1-6 are all restricted around a molecular weight of 25 kDa and distributed in a pI range of 4-7. In the pI range of 3-4, four major spots are identified: spot 7 at a molecular weight of ~23 kDa; spots 9 and 10 at a molecular weight of ~20 kDa and spot 8 at about~18 kDa. In order to identify each degradation spot, a MALDI-TOF analysis was performed. Spots 1- 6 were identified as LC, while spots 7-9 as HC derived fragments; surprisingly spot 10 showed to contain both HC and LC peptides. Molecular weight of spots 1- 6 (25 kDa) confirmed the presence of the intact form of the LC, while the different pI values observed are probably related to different post-translational modifications. Based on these results, spots 7 and 10 were selected for the N-terminal sequencing analysis.

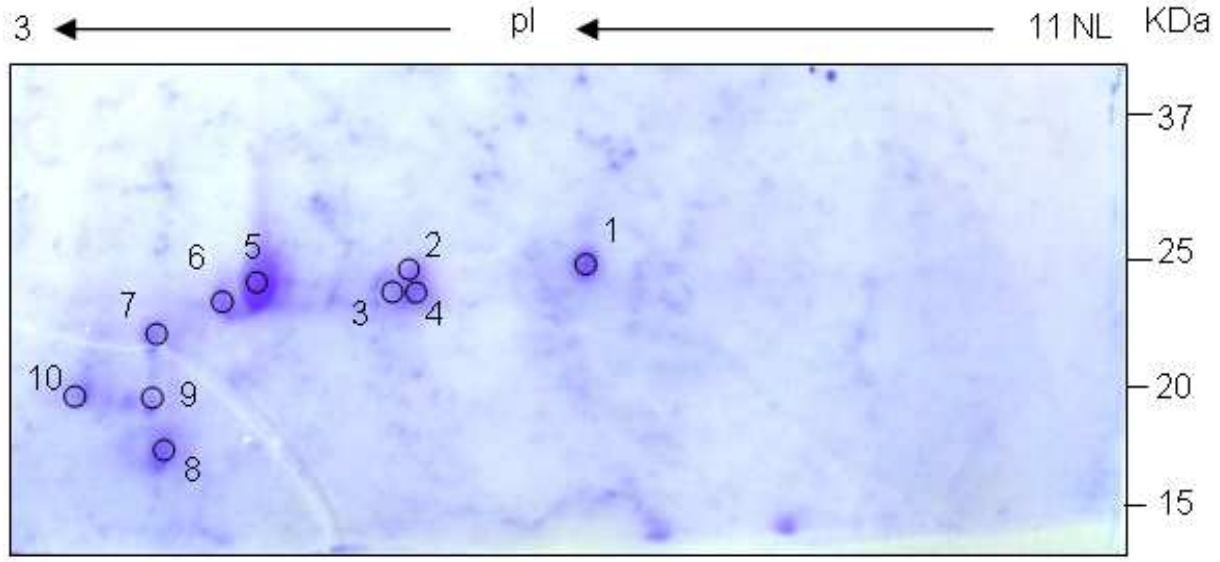


Figure R34: Coomassie stained two-dimensional gel electrophoresis of the the mAb H10 purified with protein A from vacuum-agroinfiltrated *N. benthamiana* plants. 800 µl of purified mAb H10 were separated by isoelectrofocusing (IEF) at pH 3-11 Non Linear (NL) strips, followed by SDS-12.5 % (w/v) PAGE. The major visible spots are indicated by numbers.

4.5.5 N-terminal sequencing analysis

N-terminal sequencing was performed with the aim to identify mAb H10 amminoacid sequences prone to proteolytic cleavage in plants. This analysis involves a series of chemical reactions that derivatize and remove one amino acid at a time from the N-terminus of purified peptides or protein, enabling the identification of the residues belonging to the N-terminus. Analysis was performed on all 9 protein bands obtained in the mono-dimensional gel electrophoresis (1-DE) of the protein A purified mAb H10 (**Figure R30**) as well as on spots 7 and 10 obtained after 2-DE gel electrophoresis. In both cases proteins were blotted on a PVDF membrane and subsequently processed. N-terminal analysis of proteins was limited to five residues, representing the minimum amount of aminoacids required to univocally identify a proteolytic cleavage site. The N-terminal analysis of the bands/spots obtained with 1-DE or 2-DE separation are summarized in **Table R8** panel **A** and **Table R8** panel **B**, respectively.

N-terminal sequencing performed on band 1 (1-DE analysis) showed, as expected, the presence of amminoacid sequences corresponding to the most N-terminus sequence of the intact LC (positions 1-5) and HC. In the case of band 3 and 4, N-terminal analysis produced a similar result identifying sequences corresponding to HC and LC (positions 1-5). In particular, the amminoacid sequences found for band 3 exhibited a strong detection signal (> 2 pmol) for all the amminoacids identified, indicating the reliability of the result. On the other hand, in the case of the analysis of band 4, the

first two amminoacids (t and s in lower case letters), both in heavy and light chain, showed a weaker detection (< 2 pmol), but the strong signal for the next amminoacids in position 3-5 validated the identification of the sequence. No other sequence belonging to HC or LC was identified. Band 5 N-terminal analysis did not reveal any sequence corresponding to the LC matching perfectly with the results obtained by Western blot analysis (**Figure 31**, panel **A**, **B**). However, the presence of an internal sequence of the HC was identified at the position 232-236 (KKVEP), corresponding to the end of the C_H1 (KKV) and the starting point of the hinge region (EP) of mAb H10. This position, contained in the hinge region, produced a protein fragment corresponding to the Fc part of the mAb H10. This hypothesis was confirmed by the size of the analyzed band (55 kDa) corresponding to the theoretical mass of two assembled Fc fragment of the mAb H10. Furthermore, we also showed that Concanavalin A Western blot analysis under reducing conditions of band 5 highlighted the presence of a single band with a molecular weight of about 25 kDa.

Band 6 and 7 showed a similar result for band 3 and 4, obtaining sequences corresponding to the N-terminus of intact HC and LC. In the case of band 6, the positions 1-2 of the light chain presented a missing amminoacid in position 1 and a weak signal for the amminoacid in position 2. In spite of the first two positions, all the other amminoacids in positions 3-5 clearly matched (signal > 2 pmol) the N-terminal part of the of LC. In the case of HC, the signal was weak for positions 1-2, but clear for the positions 3-5 confirming the presence of an intact HC N-terminus. Band 6 most likely corresponded to a Fab fragment (molecular weight of 45 kDa), with the proteolytic cleavage occurring in position 232-236, as observed in band 5. This conclusion is strengthened by the results previously shown in Western blot analysis (**Figure R31** panel **A**, **B**) and ELISA (**Figure R33**). The analysis of protein fragments contained in band 7 indicated the presence of two sequences corresponding to the position 1-5 of both heavy and light chain. In the case of LC the signal was weak for amminoacids in position 1-4 (< 2 pmol), but stronger for the amminoacid in position 5. For the HC, analysis identified the N-terminus of the intact chain (position 1-5) with a strong signal (>2 pmol for all the amminoacids). From these results and from the size of the band in SDS-PAGE (25 kDa) it can be hypothesized that it corresponds to different protein species co-banding at the same molecular weight (unassembled intact LC and degradation fragment of HC). This result is confirmed by the western blot analysis in which a band at 25 kDa is recognized by both anti-γ and anti-λ antibodies. Furthermore, we also showed that Concanavalin A Western blot analysis under reducing conditions highlighted the presence of a single band with a molecular weight of about 25 kDa (**Figure R32**).

A single N-terminal analysis was performed for both bands 8 and 9 because of the difficulty to isolate the single bands on the PVDF membrane. This analysis enabled the identification of three amminoacid sequences corresponding to positions 353-357 (KTISK), 355-359 (ISKAK) and 357-361 (KAKGQ) of the HC.

The first two sequences (KTISK and ISKAK) corresponded to the final part of C_H2, while the third sequence (KAKGQ) was localized between the end of C_H2 (KAK) and the early C_H3 (GQ). The sequences overlapped in three positions possibly identifying three possible overlapping cleavage sites on the C_H3 interdomain region. A strong signal for all the amminoacids was obtained (> 2 pmol), enabling the unambiguous recognition of the HC sites. No detection for LC sequences was observed. These bands both corresponded to a short fragment of HC (15-13 kDa) which was not recognized on Western blot analysis by anti-γ antibody.

N-terminal sequence of spot 7 (pI 4, 23 kDa) and spot 10 (pI 3, 20 kDa) from the 2-DE gel was also performed (**Table R8**, panel **B**). Analysis of spot 7 indicated the presence of the sequence STKGP (> 2 pmol) corresponding to position 138-142 of the HC. The sequence was localized at the level of the interdomain region, at the beginning of the C_H1 domain. The molecular weight of 23kDa, most likely, indicated that the most C-terminal sequence of this protein fragment was localized in the interdomain region C_H2-C_H3 in which a cleavage sequence was identified in the 1-DE bands 8 and 9. The analysis of spot 10 (20 kDa) revealed the unexpected presence of an amminoacid sequence corresponding to the N-terminus of the intact LC.

4.5.6 Structural assignment of 1DE bands and 2DE spots.

From all experimental results obtained from Western blot analysis (**Figure R31**), antigen binding by ELISA (**Figure R33**), Fc sugar-chain analysis (using Concanavalin-A on Western blot, **Figure R32**), which are summarized in **Table R7,** and from N-terminal sequencing results, we were able to assign a structural footprint to each 1-DE band or 2-DE spot (**Table R8,** panel **A** and **Table R8,** panel **B**).

In the case of band 1, as summarized in **Table R7**, both HC and LC were present, and both binding site and sugar chain were detected (**Figures R31**, **R33** and **R32** respectively). In addition, N-terminal analysis showed the presence of the intact LC (**Table R8** panel **A**) while an experimental molecular weight of 150 kDa was observed. These data indicated that this band corresponded to the full size H_2L_2 H10 form. Band 3 showed all the features of the band 1 except for the absence of the sugar chain (**Figure R32**). This evidence indicated that this antibody fragment lacked of the Fc region (40-50 kDa), as confirmed also by the molecular weight of about 110 kDa. Based on these

results, the fragment can be described as a Fab2 like fragment (**Table R8** panel **A**). In the case of band 4, the presence of both HC and LC (**Figure R31**), binding site (**Figure R33**) and sugar chain (**Figure R32**) was confirmed. Furthermore, the N-terminal sequencing revealed the presence of the intact starting point of both HC and LC (**Table R8** panel **A**). However, a molecular weight corresponding to about half of the whole immunoglobulin size was found (about 80 kDa) indicating that this antibody fragment represents a partially assembled IgG represented by an H_1L_1 molecule. An interesting result was obtained with the analysis of band 5 that allowed the identification of the first internal cleavage site of the immunoglobulin. This fragment, as confirmed by Western blot analysis (**Figure R31**), contained only the HC and the sugar chain (**Figure R32**) and the signal of the antigen binding site (**Figure R33**) was absent, while LC was not detected. The most N-terminal sequence of this of this fragment corresponded to the end of C_H1 domain close to the hinge region (**Table R8** panel **A**). This was confirmed by the size of the analyzed band (55 kDa) matching the theoretical molecular weight of the Fc fragment. Furthermore, the Concanavalin A Western blot analysis of band 5 under reducing conditions highlighted the presence of a single band with a molecular weight of about 25 kDa indicating half of an intact Fc (**Figure R32**). Band 6 (about 50 kDa) showed different features, the antigen binding site was present (**Figure R33**), the HC and LC were both recognised in Western blot analysis (**Figure R31**) while the sugar chain was not (**Figure R32**). In addition, N-terminal sequencing confirmed the presence of the first aminoacid residue of both HC and LC indicating that this fragment matched the Fab portion of the immunoglobulin (**Table R8** panel **A**). In the case of band 7, HC and LC were identified, but also the sugar chain was observed. From these results and from the size of the band verified on SDS-PAGE (25 kDa) it can be hypothesized that it corresponded to different protein species co-banding at the same molecular weight (unassembled intact LC and degradation fragments of HC). Moreover, N-terminal sequencing of bands 8 and 9 indicated that these two fragments were the result of a cleavage occurring in the C_H2-C_H3 region (**Table R8** panel **A**).

In conclusion, the most informative results were obtained from the analysis of bands 5, 8 and 9 in 1-DE and from spot 7 in 2-DE (**Table R8** panel **A, B** red square). The N-terminal sequencing analysis of these peptides allowed the identification of the internal cleavage site KKVEP (C_H1-C_H2 region) in the case of band 5, of the cleavage sites KTISK, ISKAK and KAKGQ (C_H2-C_H3 region) for bands 8 and 9, and the cleavage site STKGP (V_H-C_H1 region) in the case of spot 7 (**Table R8** panel **A, B**).

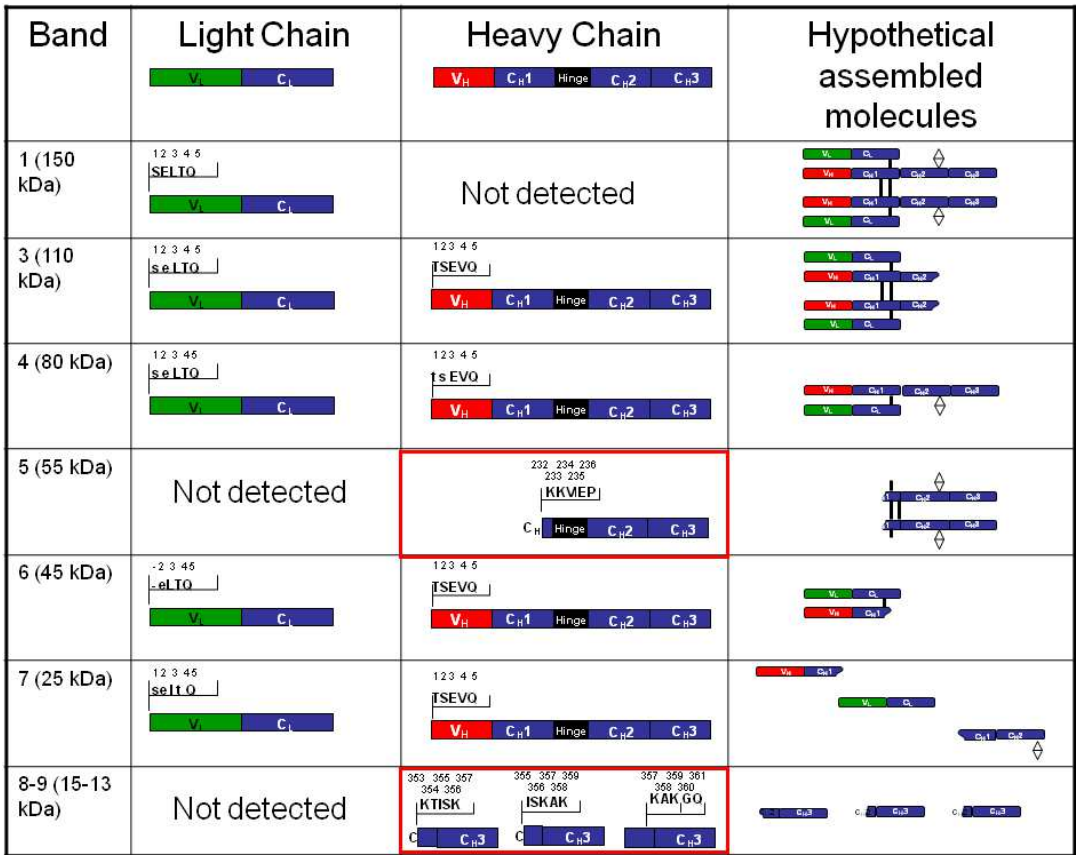
Table R7: Summary of the experimental results obtained from the mAb H10 degradation bands analyzed with the different strategies. (+)present; (-)absent.

Band	WB Hc fragment recognition	WB Lc fragment recognition	Sugar chain	Binding site	MW (kDa)
1	+	+	+	+	150-145
2	+	+	+	na	140
3	+	+	-	+	110
4	+	+	+	+	80
5	+	-	+	-	55
6	+	+	-	+	40
7	-	-	+	na	28
8	-	-	-	na	15
9	-	-	-	na	13

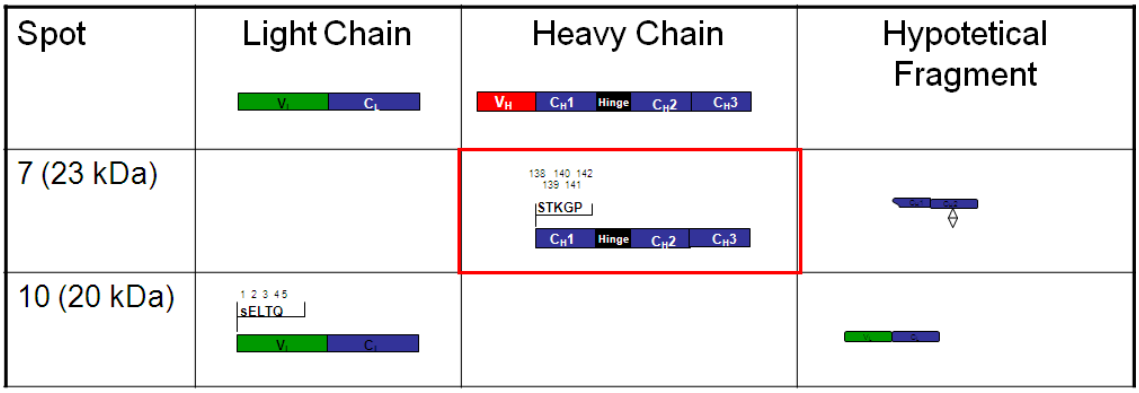
n.a.**:** not analyzed**.**

Table R8 A/B: Summary of the N-terminal sequencing analysis. For every band is reported the position of the N-terminal sequence detected on HC and LC and the deducted molecular species corresponding to each band. na: bands not analyzed.

A)



B)



Red square: fragments containing internal cleavage sites.

4.5.7 Analysis of potential cleavage sites on H10 susceptible to plant proteases

A list of proteolytic enzymes encoded by the genomes of plants belonging to the *Nicotiana* genus has been obtained from the MEROPS database (Rawlings et al., 2010). The five potential cleavage sites identified in the HC of the H10 Ab purified from *N. benthamiana* are shown in **Table R9**.

Table R9: Potential cleavage sites identified in the H10 aminoacid sequence. (|) cleavage position (P) aminoacid position before the cleavage site; (P') aminoacid position after the cleavage. In red are indicated the sequences recognized by N-terminal sequencing. In green are highlighted the residues specifically recognized by plant proteases.

Cleavage site	**Positions of the cleavage** P5 P4 P3 P2 P1|P1'P2'P3'P4'P5'	**Position in the aminoacid sequence**
1st	T V S R A | S T K G P	138-142
2nd	N T K V D | K K V E P	232-236
3rd	P A P I E | K T I S K	253-257
4th	P I E K T | I S K A K	255-259
5th	E K T I S | K A K G Q	257-261

Analysis of these cleavage sites allowed the identification of several residues potentially responsible for the recognition and cleavage by *Nicotiana* peptidase. In particular, from MERPOS database analysis it was found that in the 1st cleavage site (region V_H-C_H1), Arginine in P2 was recognized with high specificity by cathepsin B (Peptidase family C1, Cystein protease) and frequently also by Phytocalpain (Peptidase family C2, Cystein protease), both of which are encoded by the *N.*

benthamiana genome, as well as other *Nicotiana* species. In the 2nd cleavage site, close to the hinge region, two residues are specifically recognized by plant proteases. In particular Valine in position P2, recognized by phytocalpain, and the Aspartic acid in position P1 recognized by a Legumain (Peptidase family C13, Cystein proteases). In the case of the 3rd and 5th cleavage sites (region C_H2-C_H3) Phytocalpain with a specificity for bulky hydrophobic residues, might recognize Isoleucine in position P2.

Interestingly, it was observed that identified cleavage sites occurred both within antibody non structured loop regions (V_H-C_H1), or within secondary structural elements such as beta-strands (C_H1-C_H2 and C_H2-C_H3). Cleavage sites are all located in antibody regions accessible to the solvent (**Figure R35**).

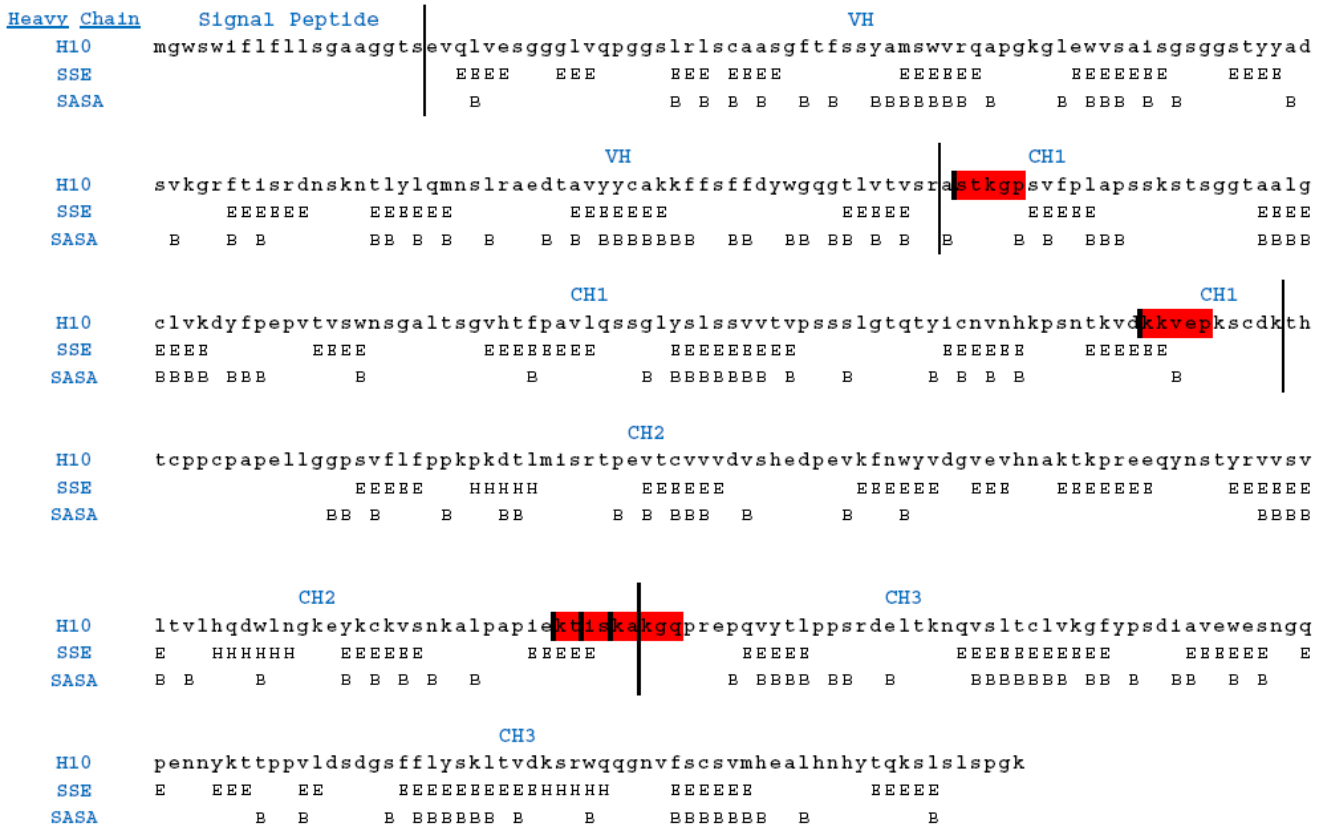


Figure R35: H10 Heavy Chain aminoacid sequence. The cleavage sites identified are reported in red and the cleavage positions are indicated by black vertical bars. H10 Antibody structural domains (i.e. V_H, C_H1, C_H2 and C_H3 of the HC) and signal sequences are labelled and separated by black vertical bars. Details about secondary structure and solvent accessible surface area were obtained with antibodies of known 3D structure found in literature. Secondary structure elements (SSE) are indicated by 'E' (extended, i.e., beta-strand), 'H' (alpha-helix). Residues whose solvent accessible surface area (SASA) is ≤ 20 Å^2 are indicated by 'B' (buried).

4.5.8 Site directed mutagenesis of identified H10 internal cleavage sites

We engineered three H10 HC mutants (-$H10_{VH\text{-}CH1}$, $H10_{CH1\text{-}CH2}$, $H10_{VH\text{-}CH1\text{-}CH2}$-) in which key aminoacid residues in the identified consensus sequences recognized by specific plant proteases were mutated. All genes were cloned into the pBI-Ω plant expression vector under the control of the CaMV 35S promoter, the TMV Ω translational enhancer and the NOS terminator sequences (Marusic et al., 2007). All plasmids were electroporated into *A. tumefaciens* strain LBA4404 and used in co-agroinfiltration experiments. A schematic representation of the constructs is reported in **Figure R36**.

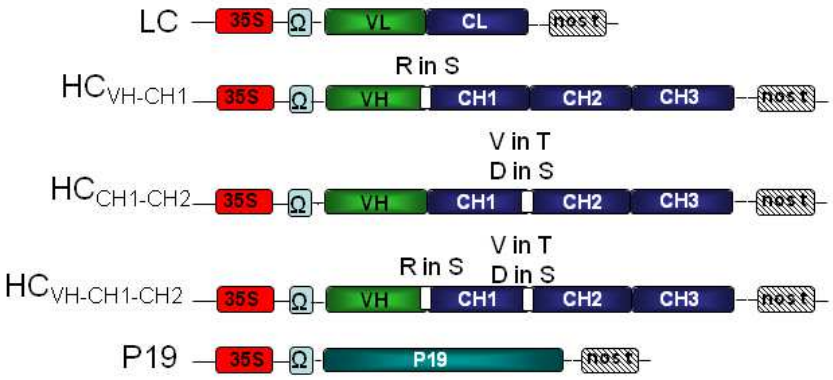


Figure R36: Schematic representation of constructs used for the expression of H10 mutants. Specific aminoacid mutations are indicated on every construct. A description of the constructs is presented in Material and methods section. All genes were cloned in the pBI-Ω plant expression vector under the control of the CaMV 35S promoter and containing the TMV Ω translational enhancer and the NOS terminator sequence.

4.5.9 Analysis of mAb H10 mutants

In order to protect H10 from plant proteolysis, three mutant mAb H10 constructs were generated: $HC_{VH\text{-}CH1}$, $HC_{CH1\text{-}CH2}$, $HC_{VH\text{-}CH1\text{-}CH2}$. Key residues were mutated in two of the identified cleavage sites in order to avoid specific recognition by Cathepsin B, Phytocalpain and Legumain. All mutations were performed based on the homology with other IgG1 aminoacid sequences found in literature in order to preserve the same physic-chemical characteristics (i.e. pI, pH, hydrophobicity) of replaced residues without altering antibody structure and assembly. We decided to mutate only aminoacids of the cleavage sites of V_H-C_H1 and C_H1-C_H2 since these cleavages generated the most visible degradation bands in the 1-DE of purified H10. The $HC_{VH\text{-}CH1}$ construct was obtained replacing the Arginine in position P2 with a Serine, since this residue is usually found in most IgG1 sequences and likely not recognized by Cathepsin B. In the case of $HC_{CH1\text{-}CH2}$ both Valine in P2 and

Aspartic acid in P1 were modified. In particular, Valine was replaced by a Threonine since it maintained the same Valine's hydrophobic interaction, while in the case of Aspartic acid it was replaced by a Serine since it preserved the orientation towards the solvent and did not seem to make specific interactions with other plant proteases. The $H10_{VH1\text{-}CH1\text{-}CH2}$, instead, was obtained inserting both $HC_{VH\text{-}CH1}$ and $HC_{CH1\text{-}CH2}$ together on the same construct.

The three constructs ($HC_{VH\text{-}CH1}$, $HC_{CH1\text{-}CH2}$, $HC_{VH\text{-}CH1\text{-}CH2}$) were separately expressed in *N. benthamiana* plants by co-agroinfiltration with the LC. The crude extracts obtained from agroinfiltrated plants, were subsequently analyzed by reducing and non reducing SDS-PAGE Western blot assay with an anti-human-γ antiserum (**Figure R37**). The mutated constructs were compared to the mAb H10 in order to verify if the mutation could have produced any difference in the degradation profile. A preliminary Western Blot analysis showed that all H10 mutants were expressed at high levels and showed a degradation profile comparable to the original H10 molecule. This preliminary result represents only an early indication of the behaviour of the mutated antibodies in the plant cell. Further analysis to evaluate the effectiveness of these mutations needs to be performed in order to evaluate their potential to confer protection to the plant-derived immunoglobulin.

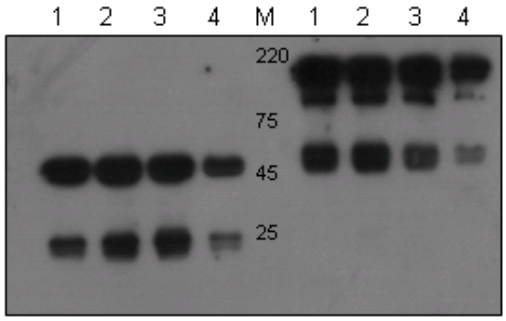


Figure R37: Western blot analysis of the different plant extracts on reducing SDS 4-20 % (w/v) PAGE. The immunoblotting was performed with a peroxidase-conjugated anti-human γ chain in non reducing (right side) and reducing (left side) conditions. Plant extracts were normalised for TSP content and a total of 500 ng TSP were loaded on each well in the gel. 1: H10, 2: $H10_{VH\text{-}CH1}$, 3: $H10_{CH1\text{-}CH2}$, 4: $H10_{VH\text{-}CH1\text{-}CH2}$. M: molecular marker (kDa).

4.6 DISCUSSION

Many studies showed that degradation phenomena in a range of antibodies expressed in plants, represented a major issue to be addressed for the optimization of plant production (**Table 11,** Introduction) (Villani et al., 2009; de Neve et al., 1993; Ma et al., 1994; Stevens et al., 2000; Sharp and Doran 2001). Several strategies have been used in the past few years to overcome protein degradation by plant proteases, either by stabilizing the extraction medium with protease inhibitors or by targeting the antibody to sub-cellular compartments considered to have a lower proteolyitc activity (Nuttall et al., 2002; Faye et al., 2005). However, only few of the above mentioned approaches demonstrated to be efficient in preventing antibody proteolysis.

In this study, we characterized the degradation profile of the tumour targeting H10 antibody purified from agroinfiltrated *N. benthaminana* plants, in the attempt to identify specific internal cleavage sites of the plant derived immunoglobulin. To this aim, we performed a detailed analysis of all degradation fragments and spots obtained from 1-DE and 2-DE separation of the protein A purified mAb H10. In the case of 1-DE analysis, we used three different affinity columns (Protein A, Protein G and anti-γFc column) in order to compare the purification efficiency of each methodology in terms of recovery of antibody fragments and quality of the purified immunoglobulin. Even if the protein profiles obtained with the three techniques were generally comparable, two major differences were evident in the case of protein A column in which the presence of two stronger protein bands at 50 and 25 kDa was clearly observable. This different pattern was related to the fact that H10 HC variable domain belongs to the subgroup III (V_H3) of human IgGs which binds to the D domain of Protein A (Bouvet, 1994). Thus, protein A affinity chromatography specifically captured not only the Fc but also the Fab portion of H10. This double binding valency explained the specific enrichment of H10 Fab fragments in the protein A purified product. This evidence renders H10 an interesting model to study antibody proteolysis in plant. H10 degradation profile was also reported in a previous work (Villani et al., 2009) in which the antibody purified from transgenic *N. tabacum* leaves revealed a high degradation (more than 90 % of the final product), leading to the accumulation of antigen-binding fragments (Fab) observable in the gel as a main degradation band around 50 kDa.

In the second part of the work all H10 protein bands/spots obtained from both 1-DE and 2-DE analysis were isolated and studied in detail using different techniques (Western Blot, ELISA) and finally characterised by N-terminal sequencing. The main strategy used to assess degradation of antibodies has usually been Western Blot analysis, but this strategy presents some limitations. In fact, the detection could be affected by the affinity of the secondary antibody used for the analysis,

or by the fact that smaller fragments may not be recognized at all, leading to an underestimation of degradation phenomena. In other studies, degradation bands of plant purified immunoglobulins separated by SDS-PAGE were also analyzed using mass spectrometry (MS) (Ramessar et al., 2008; Villani et al., 2009) although, an accurate determination of internal cleavage sites could not be made. The only example of the specific identification of an antibody plant protease cleavage site was reported by De Muynck and colleagues (2009) that determined, by Edman degradation, the sequence of the N-terminal part of a heavy chain degradation product of a human/mouse chimeric antibody expressed in tobacco. In our methodology we used a combination of different detection strategies allowing to specifically identify the N-terminal part of antibody degradation fragments.

Using this approach, we were able to identify five specific internal cleavage sites, one in the interdomain region between V_H and C_H1, another in the C_H1 close to the hinge region and three very close sites that were in the interdomain C_H2-C_H3 region. It must be noted that all cleavage sites occurred in inter-domain regions, within a solvent surface accessible area, indicating that these exposed antibody regions are more accessible by plant peptidases. Most interestingly, we identified three very close sites in the C_H2-C_H3 region, one is KTISK while the other two sites are generated by the sequential removal of two residues from the N-terminus. This could be interpreted as the result of the sequential trimming of the exposed N-terminus by specific plant peptidases. These identified aminoacid sequences were analyzed using the MEROPS database in order to identify possible specific plant proteolytic enzymes responsible of the recognition. This analysis allowed the identification of three Cystein proteases (Cathepsin B, Legumain and Phytocalpain) able to recognize specific residues in position P2 and/or P1 of the five identified aminoacid sequences. Interestingly, a recent work presented by Goulet and colleagues, identified intracellular Cysteine proteases and some Sereine proteases of *N. benthamiana* as responsible for the cleavage of a human IgG1, confirming the susceptibility of antibodies to Cystein protease family (Goulet et al., 2011). In our case, this indicates that proteolysis of the secretory H10 could take place in the cell along the cell secretory pathway. This could be confirmed by the observation that the H10-SEKDEL variant retained in the Endoplasmic Reticulum has a higher degradation compared to the secretory antibody. We also demonstrated that this variant is not fully retained in the ER but shows Golgi typical glycosylation patterns. This data was supported by many other research groups that extensively studied the degradation patterns of several antibodies in different plant expression systems. In these works, it was suggested that antibody degradation in plants could probably occur in the apoplast as well as during secretion, (Sharp and Doran 2001, De Muynck et al., 2010). In fact, it has been shown that ER retained antibodies harbouring a SEKDEL retention signal were

subjected to degradation as the native counterpart and, in some cases, degradation profile was more severe than the native IgG (Loos et al., 2011, Vezina et al., 2009).

Accordingly, in order to protect the antibody from proteolysis, we mutated the aminoacids of the cleavage sites specifically recognized by these cysteine proteolytic enzymes. The effects of mutations on H10 stability were verified by Western Blot on crude extract. This preliminary analysis showed that the antibody, in spite of the mutations, exhibited a proteolytic pattern almost comparable to the one observed for the cognate antibody indicating that, possibly, the mutations inserted seemed to not influence the specific protease recognition. However, further investigation are needed to collect additional data, since proteolysis is a mechanism likely occurring on different close sites equally accessible to the solvent and operated by different proteases. Besides, it is important to point out that the information about proteases collected so far in the MEROPS database are still limited and could lead to ignore additional data about proteolysis.

In conclusion, it is possible to assert that this work represents the first example of the specific characterisation of the degradation of a human IgG1 antibody in plants and provides new important data for the development of a successful strategy for the study of the proteolysis of plant-derived immunoglobulins. Additionally, in our study we found that the three proteases responsible of the cleavage of the mAb H10 were all belonging to the Cystein proteases family. Based on these results, and supported also by the findings obtained by Goulet et al., (2011), next step will be to perform new experiments in which specific inhibitors of the Cystein proteases family of *N. benthamiana* will be co-expressed with H10, in order to induce a specific protection from the plant proteolytic attack and increase the final yield of intact antibody.

5. THESIS CONCLUSIONS

Plant-based expression systems have emerged as a competitive platform for the production of recombinant biopharmaceuticals and, among the molecules effectively produced in plants, antibodies are undoubtedly those most successful and near to marketableness as herbal recombinant molecule. Compared to other expression platforms, plants present several advantages for the expression of recombinant molecules such as the ability to assemble complex multimeric proteins, the possibility to achieve post-translational modifications similar to those found in mammalian cells, low management costs and ease of scalability. Despite these advantages, some drawbacks need to be addressed. An important limit, in the plant system, is represented by downstream purification steps, in which many factors, such as low antibody concentration, extraction methodologies and purification strategy, could influence the effectiveness of the purification. In addition, the presence of several unwanted plant compounds, such as alkaloids, phenolics, polysaccharides, and proteases able to interact with the recombinant IgGs, could affect their final quality and yield, reducing in some way the efficacy of protein A chromatography step.

Another important issue to be addressed in the use of plants for antibody expression, is represented by plant glycosylation. Differences between sugar moieties harbored by proteins expressed in plants and mammals, represents a major drawback in the application of plant-derived immunoglobulins in human therapy. In fact, the presence of typical plant sugars has been demonstrated capable of inducing immunogenic responses in humans, leading also to a remarkable reduction of pharmacokinetic properties and biological activity of plant derived proteins (Sethuraman & Stadheim, 2006).

Besides, an additional drawback of recombinant antibodies obtained from plants is represented by the final recovery yield, that can be, in some cases, unsatisfactory. One of the factors playing a significant role in the reduction of the final yield and quality of heterologous immunoglobulins, is represented by the unintended proteolytic processes taking place in the plant cell. Particularly, in the case of antibody expression, degradation could produce a protein reduction of about 90 % of the final purified product as reported in many studies.

In this work, we described the expression of the human recombinant tumour targeting mAb H10 in *Nicotiana benthamiana* using a transient expression system based on vacuum agroinfiltration, focusing our research efforts on expression and purification strategies, glycosylation profile and proteolysis.

As a first approach, we optimized the antibody plant production system by analyzing different extraction procedures (mechanical homogenization of fresh agroinfiltrated leaves and recovery of intercellular fluids -IFs-) in terms antibody expression levels, percentage of intact IgG, purification yields and presence of alkaloids and polyphenols in the final product. The results showed that mechanical grinding from fresh leaves allowed to achieve the highest purification yield (75 mg/Kg Fresh Weight -75 % intact tetrameric IgG-) while extraction from IFs guaranteed the lowest presence of polyphenols (21 μg/g FW) in the extract. Based on these results, we optimized a pilot-scale purification protocol for the extraction of mAb H10, obtaining an average yield of **40 mg per Kg fresh weight** of intact IgG with **99.4% purity**, with **endotoxin levels <1 EU/ml**, free of phenolic, alkaloid compounds.

The second part of the work was focused on the analysis and modulation of the glycosylation pattern of plant produced H10 using different strategies: a) mutation of the H10 antibody glycosylation site of H10 heavy chain in order to obtain an aglycosylated antibody (H10 Mut); b) retention of H10 in the Endoplasmic Reticulum (ER) of plant cells by fusing the SEKDEL retention sequence to the C-terminal of both HC and LC (H10 SEKDEL); c) expression of the H10 in *Nicotiana benthamiana* plants in which *fucosyl transferase* and *xylosil transferase* genes were downregulated (Δxyl, Δfuc plants) preventing the addition of complex plant sugars to the glycan chain of the antibody ($H10^{XylT/FucT}$). As a result, all antibody formats showed similar expression levels compared to H10, except for $H10^{XylT/FucT}$ that exhibited a lower expression yield because the over-expression was carried on without the boost of the P19 gene silencing suppressor protein in these downregulated mutants. Analysis of the plant-purified aglycosylated H10 Mut confirmed the lack of the sugar chain showing its higher susceptibility to the unintended proteolysis. In addition, retention of the H10 SEKDEL in endoplasmic reticulum was proved, and also the lack of complex plant sugars in the case of the $H10^{XylT/FucT}$ glycoform was observed.

In the last part of the work, the study was based on the analysis of proteolysis of the plant-derived mAb H10, in order to evaluate the degradation profile and to identify the cleavage sites of the immunoglobulin. We found three specific cleavage sites localized in the three inter-domain regions of the H10 heavy chain. Moreover, based on bioinformatics analysis performed using the MEROPS proteases database, three proteolytic enzymes were individuated, belonging to the cystein proteases family. In spite of that, no clear evidences of antibody protection were observed from a preliminary experiment, in which the aminoacids within the cleavage sites responsible of the proteases recognition were modified. Based on these preliminary results, a future perspective to avoid the cleavage of the H10 in plants could reside in the use of specific cysteine protease inhibitors to be co-

expressed in plants together with the antibody,in order to induce a specific protection from plant resident proteases thus increasing the final yield of intact product.

6. BIBLIOGRAPHY

Ahmad A, Pereira EO, Conley AJ, Richman AS, Menassa R. Green biofactories: recombinant protein production in plants. Recent Pat Biotechnol. 2010; 4:242-259.

Aldridge S. Downstream processing needs a boost. Gen Eng News. 2006; 26:1-51.

Altmann F. The role of protein glycosylation in allergy. Int Arch Allergy Immunol. 2007; 142:99-115.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997; 25: 3389-3402.

Andersen DC, Krummen L. Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol. 2002; 13:117–123.

Andreeva A, Howorth D, Chandonia JM, Brenner SE, Hubbard TJ, Chothia C, Murzin AG. Data growth and its impact on the SCOP database: new developments. Nucleic Acids Res. 2008; 36: D419-D425.

Arlen PA, Singleton M, Adamovicz JJ, Ding Y, Davoodi-Semiromi A, Daniell H. Effective plague vaccination via oral delivery of plant cells expressing F1-V antigens in chloroplasts. Infect Immun. 2008; 76:3640–3650.

Artsaenko O, Kettig B, Fiedler U, Conrad U and During K. Potato tubers as a biofactory for recombinant antibodies. Mol. Breed. 1998; 4: 313–319.

Aviezer D, Almon-Brill E, Shaaltiel Y, Galili G, Chertkoff R, Hashmueli S, et al. Novel enzyme replacement therapy for Gaucher disease: ongoing phase III clinical trial with recombinant human glucocerebrosidase expressed in plant cells. Mol Genet Metab. 2009a; 96:S13-14.

Aviezer D, Brill-Almon E, Shaaltiel Y, Hashmueli S, Bartfeld D, Mizrachi S. A plant derived recombinant human glucocerebrosidase enzyme - a preclinical and phase I investigation. PLoS ONE. 2009b; 4:e4792.

Awram P, Gardner RC, Forster RL, Bellamy AR. The potential of plant viral vectors and transgenic plants for subunit vaccine production. Adv Virus Res. 2002; 58:81-124.

Azhakanandam K, Weissinger SM, Nicholson JS, Qu R, Weissinger AK. Amplicon-plus targeting technology (APTT) for rapid production of a highly unstable vaccine protein in tobacco plants. Plant Mol Biol. 2007; 63:393-404.

Azzoni AR, Farinas CS, Miranda EA. Transgenic corn seed for recombinant protein production: relevant aspects on the aqueous extraction of native components. J Sci Food Agric. 2005; 85:609–14.

Bardor M, Faveeuw C, Fitchette AC, Gilbert D, Galas L, Trottein F, Faye L and Lerouge P. Immunoreactivity in mammals of two typical plant glyco-epitopes, core α 1,3-fucose and core xylose. Glycobiology. 2003; 13: 427–434.

Basaran P, Rodriguez-Cerezo E. Plant molecular farming: opportunities and challenges. Crit Rev Biotechnol. 2008; 28:153–172.

Beers EP, Jones AM, and Dickerman AW. The S8 serine, C1A cysteine and A1 aspartic protease families in Arabidopsis. Phytochemitry. 2004; 65: 43–58.

Benchabane M, Goulet C, Rivard D, Faye L, Gomord V and Michaud D. Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol J. 2008; 6, 633–648.

Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, Herz S, Fröde R, Inogés S, Lòpez-Dìaz de Cerio A, Soria E, Villanueva H, Vancanneyt G, McCormick A, Tusé D, Lenz J, Butler-Ransohoff JE, Klimyuk V, Gleba Y. Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. Ann Oncol. 2010; 21:2420-7.

Birch JR, Racher AJ. Antibody production. Adv Drug Deliv Rev. 2006; 58:671–685.

Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, Murray E, Morck D, Moloney MM. Seed-based expression systems for plant molecular farming. Plant Biotechnol J. 2010; 8:588-606.

Borsi L, Balza E Bestagno M, Castellani P, Carnemolla B, Biro A, Leporini A, Sepulveda J, Burrone O, Neri D and Zardi L. Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. Int. J. Cancer. 2002; 1102, 75–85.

Bouvet JP. Immunoglobulin Fab fragment-binding proteins. Int J Immunopharmacol. 1994; 16:419-24.

Boyhan D, Daniell H. Low-cost production of proinsulin in tobacco and lettuce chloroplasts for injectable or oral delivery of functional insulin and C-peptide. Plant Biotechnol J. 2010.

Brisson N, Hohn T. Nucleotide sequence of the dihydrofolate-reductase gene borne by the plasmid R67 and conferring methotrexate resistance. Gene. 1984; 28:271-4.

Brodzik R, Glogowska M, Bandurska K, Okulicz M, Deka D, Ko K, van der Linden J, Leusen, JHW, Pogrebnyak N, Golovkin M, Steplewski Z and Koprowski H. Plant derived anti-Lewis Y mAb exhibits biological activities for efficient immunotherapy against human cancer cells. Proc. Natl. Acad. Sci. U S A. 2006; 103, 8804–8809.

Büttner-Mainik A, Parsons J, Jérôme H, Hartmann A, Lamer S, Schaaf A, Schlosser A, Zipfel PF, Reski R, Decker EL. Production of biologically active recombinant human factor H in Physcomitrella. Plant Biotechnol J. 2011; 9:373-83.

Cao QH, Qu WJ, Li JG, Deng YX. Study on purification of total flavonoids from seed residue of Hippophae rhamnoides with macroporous resin. Zhongguo Zhong Yao Za Zhi. 2004; 29:225-8.

Capodicasa C, Chiani P, Bromuro C, De Bernardis F, Catellani M, Palma AS, Liu Y, Feizi T, Cassone A, Benvenuto E, Torosantucci A. Plant production of anti-β-glucan antibodies for immunotherapy of fungal infections in humans. Plant Biotechnol J. 2011; 9:776-87.

Cardi T, Lenzi P, Maliga P. Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev Vaccines. 2010; 9:893–911.

Carnemolla B, Castellani P, Ponassi M, Borsi L, Urbini S, Nicolo G, Dorcaratto A, Viale G, Winter G, Neri D and Zardi L. Identification of a glioblastoma-associated tenascin-C isoform by a high affinity recombinant antibody. Am. J. Pathol. 1999; 154: 1345–1352.

Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R, Altmann F, Steinkellner H. *In planta* protein sialylation through overexpression of the respective mammalian pathway. J Biol Chem. 2010; 285:15923–15930.

Chen M, Liu M, Wang Z, Song J, Qi Q, Wang PG. Modification of plant N-glycans processing: the future of producing therapeutic proteins in transgenic plants. Med Res Rev. 2005; 25:343–60.

Circelli P, Donini M, Villani ME, Benvenuto E, Marusic C. Efficient Agrobacterium-based transient expression system for the production of biopharmaceuticals in plants. Bioeng Bugs. 2010; 1:221-4.

Conley AJ, Joensuu JJ, Jevnikar AM, Menassa R, Brandle JE. Optimization of elastin-like polypeptide fusions for expression and purification of recombinant proteins in plants. Biotechnol Bioeng. 2009; 103:562–73.

Conrad U, Fiedler U. Expression of engineered antibodies in plant cells. Plant Mol Biol. 1994; 26:1023-30.

Cox KM, Sterling JD, Regan JT, Gasdaska JR, Frantz KK, Peele CG, et al. Glycan optimization of a human monoclonal antibody in the aquatic plant Lemna minor. Nat Biotechnol. 2006; 24:1591–7.

Craig W, Lenzi P, Scotti N, De Palma M, Saggese P, Carbone V. Transplastomic tobacco plants expressing a fatty acid desaturase gene exhibit altered fatty acid pro.les and improved cold tolerance. Transgenic Res. 2008; 17:769–82.

Daniell H, Ruiz G, Denes B, Sandberg L, Langridge W. Optimization of codon composition and regulatory elements for expression of human insulin like growth factor-1 in transgenic chloroplasts and evaluation of structural identity and function. BMC Biotechnol. 2009a; 9:33.

Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci. 2009b; 14:669–79.

D'Aoust MA, Couture MMJ, Charland N, Trepanier S, Landry N, Ors F, et al. The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. Plant Biotechnol. 2010; 8:607–19.

Davoodi-Semiromi A, Schreiber M, Nalapalli S, Verma D, Singh ND, Banks RK. Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. Plant Biotechnol J. 2010; 8:223-42.

De Cosa B, Moar W, Lee SB, Miller M, Daniell H. Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat Biotechnol. 2001; 19:71-4.

De Jaeger G, Scheffer S, Jacobs A, Zambre M, Zobell O, Goossens A, Depicker A, Angenon G. Boosting heterologous protein production in transgenic dicotyledonous seeds using Phaseolus vulgaris regulatory sequences. Nat Biotechnol. 2002; 20:1265-8.

De Jesus M, Wurm FM. Manufacturing recombinant proteins in kg-ton quantities using animal cells in bioreactors. Eur J Pharm Biopharm. 2011; 78:184-8.

De Muynck B, Navarre C, Nizet Y, Stadlmann J, Boutry M. Different subcellular localization and glycosylation for a functional antibody expressed in *Nicotiana tabacum* plants and suspension cells. Transgenic Res. 2009; 18:467-82.

De Neve M, De Loose M, Jacobs A, Van Houdt H, Kaluza B, Weidle U, Van Montagu M and Depicker A. Assembly of an antibody and its derived antibody fragment Nicotiana and Arabidopsis. Transgenic Res. 1993; 2:227–237.

De Pourcq K, De Schutter K, Callewaert N. Engineering of glycosylation in yeast and other fungi: current state and perspectives. Appl Microbiol Biotechnol. 2010; 87:1617-31.

De Wilde C, De Neve M, De Rycke R, Bruyns AM, De Jaeger G, Van Montagu M, Depicker A and Engler G. Intact antigen-binding MAK33 antibody and Fab fragment accumulate in intercellular spaces of *Arabidopsis thaliana*. Plant Sci. 1996; 114:233–241.

De Wilde C, Peeters K, Jacobs A, Peck I and Depicker A. Expression of antibodies and Fab fragments in transgenic potato plants: a case study for bulk production in crop plants. Mol. Breed. 2002; 9:271–282.

Delannoy M, Alves G, Vertommen D, Ma J, Boutry M, Navarre C. Identification of peptidases in *Nicotiana tabacum* leaf intercellular fluid. Proteomics. 2008; 8:2285-2298.

Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv. 2009; 27:297-306.

Drake PM, Barbi T, Sexton A, McGowan E, Stadlmann J, Navarre C, Paul MJ, Ma JK. Development of rhizosecretion as a production system for recombinant proteins from hydroponic cultivated tobacco. FASEB J. 2009; 23:3581-3589.

Drake PM, Chargelegue DM, Vine ND, van Dolleweerd CJ, Obregon P and Ma JKC. Rhizosecretion of a monoclonal antibody protein complex from transgenic tobacco roots. Plant Mol. Biol. 2003; 52:233–241.

Drake PM, Chargelegue D, Vine ND, Van Dolleweerd CJ, Obregon P, Ma JK. Transgenic plants expressing antibodies: a model for phytoremediation. FASEB J. 2002; 16:1855-1860.

Engler C, Gruetzner R, Kandzia R, Marillonnet S. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One. 2009; 4:e5553.

Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. PLoS One. 2008; 3:e3647.

Escobar MA, Dandekar AM. *Agrobacterium tumefaciens* as an agent of disease. Trends Plant Sci. 2003; 8:380-6.

Farran I, Rio-Manterola F, Iniguez M, Garate S, Prieto J, Mingo-Castel AM. High-density seedling expression system for the production of bioactive human cardiotrophin-1, a potential therapeutic cytokine, in transgenic tobacco chloroplasts. Plant Biotechnol J. 2008; 6:516–27.

Faye L, Boulaflous A, Benchabane M, Gomord V and Michaud D. Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. Vaccine. 2005; 23:1770–1778.

Fischer R, Liao YC, Drossard J. Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. J Immunol Methods. 1999; 226:1–10.

Fischer R, Schillberg S, Twyman RM. Molecular farming of antibodies in plants. In: Kirakosyan A, Kaufman PB, editors. Recent advances in plant biotechnology. New York: Springer. 2009; 35-63.

Fischer R, Stoger E, Schillberg S, Christou P and Twyman RM. Plant-based production of biopharmaceuticals. Curr. Opin. Plant Biol. 2004. 7:152–158.

Fitch PM, Howie SE, Wallace WA. Oxidative damage and TGF-β differentially induce lung epithelial cell sonic hedgehog and tenascin-C expression: implications for the regulation of lung remodelling in idiopathic interstitial lung disease. Int J Exp Pathol. 2011; 92:8-17.

Floss DM, Sack M, Arcalis E, Stadlmann J, Quendler H, Rademacher T, Stoger E, Scheller J, Fischer R and Conrad U. Influence of elastin-like peptide fusions on the quantity and quality of a tobacco-derived human immunodeficiency virus-neutralizing antibody. Plant Biotechnol. J. 2009; 7:899–913.

Floss DM, Sack M, Stadlmann J, Rademacher T, Scheller J, Stoger E, Fischer R and Conrad U. Biochemical and functional characterization of anti-HIV antibody-ELP fusion proteins from transgenic plants. Plant Biotechnol. J. 2008; 6:79–391.

Forthal DN, Gach JS, Landucci G, Jez J, Strasser R, Kunert R, Steinkellner H. Fc-glycosylation influences Fcγ receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. J Immunol. 2010; 185:6876-82.

Fötisch K, Vieths S. N- and O-linked oligosaccharides of allergenic glycoproteins. Glycoconj J. 2001; 18:373-90.

Fouad WM, Altpeter F. Transplastomic expression of bacterial L-aspartate-alpha- decarboxylase enhances photosynthesis and biomass production in response to high temperature stress. Transgenic Res. 2009; 18:707–18.

Franconi R, Demurtas OC, Massa S. Plant-derived vaccines and other therapeutics produced in contained systems. Expert Rev Vaccines. 2010; 9:877–92.

Franconi R, Demurtas OC, Massa S. Plant-derived vaccines and other therapeutics produced in contained systems. Expert Rev Vaccin. 2010; 9:877–92.

Fujiyama K, Furukawa A, Katsura A, Misaki R, Omasa T, Seki T. Production of mouse monoclonal antibody with galactose-extended sugar chain by suspension cultured tobacco BY2 cells expressing human beta(1,4)-galacto- syltransferase. Biochem Biophys Res Commun. 2007; 58:85–91.

Gallie DR and Kado CI. A translational enhancer derived from Tobacco mosaic virus is functionally equivalent to a Shine- Dalgarno sequence. Proc. Natl. Acad. Sci. USA. 1989; 86**:**129–132.

Gallie DR, Sleat DE, Watts JW, Turner PC and Wilson TMA. The 5'-leader sequence of Tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. Nucleic Acids Res. 1987; 15:3257–3273.

Gasser B, Maurer M, Gach J, Kunert R. Engineering of Pichia pastoris for improved production of antibody fragments. Biotechnol. Bioeng. 2006; 94:353–361.

Girard LS, Fabis MJ, Bastin M, Courtois D, Petiard V and Koprowski H. Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture. Biochem. Biophys. Res. Commun. 2006; 345:602–607.

Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci. 2006; 103:14701-14706.

Gleba Y, Klimyuk V, Marillonnet S. Viral vectors for the expression of proteins in plants. Curr. Opin. Biotechnol. 2007; 18:134–141.

Gleba Y, Marillonnet S and Klimyuk V. Plant virus vectors (gene expression systems). Enc. Virology. 2008; 4:471–479.

Gomord V, Chamberlain P, Jefferis R, Faye L. Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol. 2005; 23:559–565.

Gomord V, Faye L. Post-translational modification of therapeutic proteins in plants. Curr Opin Plant Biol. 2004; 2:171-81.

Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L. Plant-specific glycosylation patterns in the context of therapeutic protein production. Plant Biotechnol J. 2010; 8:564-87.

Gomord V, Chamberlain P, Jefferis R and Faye L. Biopharmaceutical production in plants: problems, solutions and opportunities. Trends Biotechnol. 2005; 23**:**559–565.

Gottschalk U. Bioseparation in antibody manufacturing: the good, the bad and the ugly. Biotechnol Progr. 2008; 24:496–503.

Goulet C, Benchabane M, Anguenot R, Brunelle F, Khalf M and Michaud D. A companion protease inhibitor for the protection of cytosol-targeted recombinant proteins in plants. Plant Biotechnol. J. 2010; 8:142–154.

Goulet C, Khalf M, Sainsbury F, D'Aoust MA and Michaud D. A protease activity–depleted environment for heterologous proteins migrating towards the leaf cell Apoplast Plant Biotechnology Journal. 2011; 1:1–12.

Goulet C, Dallaire C, Vaillancourt LP, Khalf M, Badri MA, Preradov A, Duceppe MO, Cloutier C and Michaud D. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. Plant Physiol. 2008; 146:1010–1019.

Gray BN, Ahner BA, Hanson MR. High-level bacterial cellulase accumulation in chloroplast-transformed tobacco mediated by downstream box fusions. Biotechnol Bioeng. 2009; 102:1045–54.

Gray BN, Yang H, Ahner BA, Hanson MR. An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol Biol. 2011; 76:345-55.

Griffin TJ, Seth G, Xie H, Bandhakavi S, Hu WS. Advancing mammalian cell culture engineering using genome-scale technologies. Trends Biotechnol. 2007; 25:401–8.

Guan Y, Ramalingam S, Nagegowda D, Taylor PW, Chye ML. Brassica juncea chitinase BjCHI1 inhibits growth of fungal phytopathogens and agglutinates gram-negative bacteria. J Exp Bot. 2008; 59:3475–84.

Hadlington J, Santoro A, Nuttal J, Denecke J, Ma JK-C, Vitale, A and Frigerio, L. The C-terminal extension of a hybrid immunoglobulin A/G heavy chain is responsible for its Golgi-mediated sorting to the vacuole. Mol Biol Cell. 2003; 14:2592-2602.

Hassan S, van Dolleweerd CJ, Ioakeimidis F, Keshavarz- Moore E, Ma JK. Considerations for extraction of monoclonal antibodies targeted to different subcellular compartments in transgenic tobacco plants. Plant Biotechnol J. 2008; 6:733–748.

Hellwig S, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. Nat Biotechnol. 2004; 22:1415–22.

Hiatt A, Cafferkey R. and Bowdish K. Production of antibodies in transgenic plants. Nature. 1989; 342:76–78.

Hitchman RB, Possee RD, King LA. Baculovirus expression systems for recombinant protein production in insect cells. Recent Pat Biotechnol. 2009; 3:46-54.

Hober S, K. Nord, M. Linhult, J. Chromatogr. B. 2007; 848:198-206.

Holler C, Zhang C. Purification of an acidic recombinant protein from transgenic tobacco. Biotechnol Bioeng. 2008; 99:902–9.

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breeding. 1997; 3:291–306.

Hooft RW, Sander C, Scharf M, Vriend G. The PDBFINDER database: a summary of PDB, DSSP and HSSP information with added value. Comput Appl Biosci. 1996; 12:525-529.

Horn ME, Woodard SL, Howard JA. Plant molecular farming: systems and products. Plant Cell Rep. 2004; 22:711–20.

Horsch RB, Klee HJ. Rapid assay of foreign gene expression in leaf discs transformed by Agrobacterium tumefaciens: Role of T-DNA borders in the transfer process. Proc Natl Acad Sci U S A. 1986; 83:4428-32.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG and Fraley RT. A simple and general method for transferring genes into plants. Science. 1985; 227:1229-1231.

Houdebine LM. Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis. 2009; 32:107–21.

Hu YC. Baculoviral vectors for gene delivery: A review. Curr Gene Ther. 2008; 8: 54-65

Hubbard SJ, Eisenmenger F, Thornton JM. Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. Protein Sci.1994; 3:757-68.

Hull AK, Criscuolo CJ, Mett V, Groen H, Steeman W, Westra H, Chapman G, Legutki B, Baillie L, Yusibov V. Human-derived, plant-produced monoclonal antibody for the treatment of anthrax. Vaccine. 2005; 23:2082–2086.

Jefferis R. Glycosylation of recombinant antibody therapeutics. Biotechnol Prog. 2005; 21:11-6.

Jefferis, R. Glycosylation of antibody therapeutics: optimisation for purpose. Methods Mol. Biol. 2009; 483:223–238.

Jeong KJ, Jang SH, Velmurugan N. Recombinant antibodies: engineering and production in yeast and bacterial hosts. Biotechnol J. 2011; 6:16-27.

Johansen LK, Carrington JC. Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. Plant Physiol. 2001; 126:930-938.

Jung ST, Reddy ST, Kang TH, Borrok MJ, Sandlie I, Tucker PW, Georgiou G. Aglycosylated IgG variants expressed in bacteria that selectively bind FcgammaRI potentiate tumor cell killing by monocyte-dendritic cells. Proc Natl Acad Sci USA. 2010; 107:604–609.

Kaiser J. Is the drought over for pharming? Science. 2008; 320:473–5.

Kajiura K, Ryo Misaki, Kazuhito Fujiyama,. and Tatsuji Seki Lowe JA, Jones P. Biopharmaceuticals and the future of the pharmaceutical industry. Curr Opin Drug Discov Dev. 2007; 10:513–4.

Kajiura, H, Seki, T and Fujiyama, K *Arabidopsis thaliana* ALG3 mutant synthesizes immature oligosaccharides in the ER and accumulates unique *N*-glycans. Glycobiology. 2010; 20:736-751.

Kathuria S, Sriraman R, Nath R, Sack M, Pal R, Artsaenko O, Talwar GP, Fischer R, Finnern R: Efficacy of plant-produced recombinant antibodies against HCG. Hum Reprod. 2002; 17:2054-2061.

Kathuria SR, Nath R, Pal R, Singh O, Fischer R, Lohiya NK, Talwar GP. Functional recombinant antibodies against human chorionic gonadotropin expressed in plants. Current Science. 2002; 82:1452-1457.

Kaulfurst-Soboll H, Rips S, Koiwa H, Kajiura H, Fujiyama K, von Schaewen A. Reduced immunogenicity of Arabidopsis hgl1 mutant N-glycans caused by altered accessibility of xylose and core fucose epitopes. J Biol Chem. 2011; 286:22955–22964.

Kayser V, Chennamsetty N, Voynov V, Forrer K, Helk B, Trout BL. Glycosylation influences on the aggregation propensity of therapeutic monoclonal antibodies. Biotechnol J. 2011; 6:38–44.

Khoudi H, Laberge S, Ferullo JM, Bazin R, Darveau A, Castonguay Y, Allard G, Lemieux R and Vezina LP. Production of a diagnostic monoclonal antibody in perennial alfalfa plants. Biotechnol. Bioeng. 1999; 64:135–143.

Kim TG, Baek M, Lee EK, Kwon TH, Yang MS. Expression of human growth hormone in transgenic rice cell suspension culture. Plant Cell Rep. 2008; 27:885–91.

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc. Natl. Acad. Sci. 2003; 100:8013–8018.

Ko, K. and Koprowski, H. Plant biopharming of monoclonal antibodies. Virus Res. 2005; 111, 93–100.

Komarnytsky S, Borisjuk N, Yakoby N, Garvey A. and Raskin I. Cosecretion of protease inhibitor stabilizes antibodies produced by plant roots. Plant Physiol. 2006; 141:1185–1193.

Komarova TV, Kosorukov VS, Frolova OY. Plant-made trastuzumab (herceptin) inhibits HER2/Neu+ cell proliferation and retards tumor growth. PLoS One. 2011; 6:e17541.

Krishnamohan A, Balaji V, Veluthambi K. Efficient vir gene induction in Agrobacterium tumefaciens requires virA, virG, and vir box from the same Ti plasmid. J Bacteriol. 2001; 183:4079-89.

Lai H, Engle M, Fuchs A, Keller T, Johnson S, Gorlatov S, et al. Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. P Natl Acad Sci USA; 2010; 107:2419–24.

Lee JW, Forciniti D. Purification of human antibodies from transgenic corn using aqueous two-phase systems. Biotechnol Progr. 2010; 26:159–67.

Lee LY, Fang MJ, Kuang LY, Gelvin SB. Vectors for multi-color bimolecular fluorescence complementation to investigate protein-protein interactions in living plant cells. Plant Methods. 2008; 15:4-24.

Lee LY, Gelvin SB. T-DNA binary vectors and systems. Plant Physiol. 2008; 146:325–332.

Lentz EM, Segretin ME, Morgenfeld MM, Wirth SA, MJ Dus Santos, MV Mozgovoj. High expression level of a foot and mouth disease virus epitope in tobacco transplastomic plants. Planta 2010; 231:387–95.

Lessard PA, Kulaveerasingam H, York GM, Strong A, Sinskey AJ. Manipulating gene expression for the metabolic engineering of plants. Metab Eng. 2002; 4:67-79.

Li Y. Recombinant production of antimicrobial peptides in Escherichia coli: a review. Protein Expr Purif. 2011; 80:260-7.

Lico C, Chen Q, Santi L. Viral vectors for production of recombinant proteins in plants. J. Cell Physiol. 2008; 216:366–377.

Liénard D, Sourrouille C, Gomord V, Faye L. Pharming and transgenic plants. Biotechnol Ann Rev. 2007; 13:115–47.

Lim S, Ashida H, Watanabe R, Inai K, Kim YS, Mukougawa K, Fukuda H, Tomizawa K, Ushiyama K, Asao H, Tamoi M, Masutani H, Shigeoka S, Yodoi J, Yokota A. Production of biologically active human thioredoxin 1 protein in lettuce chloroplasts. Plant Mol Biol. 2011; 76:335-44.

Lohaus G, Pennewiss K, Sattelmacher B, Hussmann M, Hermann Muehling K. Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. PhysiolPlant. 2001; 111:457–465.

Lombardi R, Circelli P, Villani ME, Buriani G, Nardi L, Coppola V, Bianco L, Benvenuto E, Donini M, Marusic C. High-level HIV-1 Nef transient expression in *Nicotiana benthamiana* using the P19 gene silencing suppressor protein of Artichoke mottled crinckle virus. BMC Biotechnol. 2001; 9:96.

Lombardi R, Villani ME, Di Carli M, Brunetti P, Benvenuto E, Donini M. Optimisation of the purification process of a tumourtargeting antibody produced in *N. benthamiana* using vacuum-agroinfiltration. Transgenic Res. 2001; 19:1083–1097.

Longstaff M, Newell CA, Boonstra B, Strachan G, Learmonth D, Harris WJ, Porter AJ and Hamilton WD. Expression and characterisation of single-chain antibody fragments produced in transgenic plants against the organic herbicides atrazine and paraquat. Biochem. Biophys. Acta. 1998; 1381:147–160.

Loos A, Van Droogenbroeck B, Hillmer S, Grass J, Kunert R, Cao J, Robinson DG, Depicker A, Steinkellner H. Production of monoclonal antibodies with a controlled N-glycosylation pattern in seeds of Arabidopsis thaliana. Plant Biotechnol J. 2011; 9:179-92.

Lorence A, Verpoorte R. Gene transfer and expression in plants. Methods Mol Biol. 2004; 267:329-50.

Luckow VA, Summers MD. Signals important for high-level expression of foreign genes in Autographa californica nuclear polyhedrosis virus expression vectors. Virology. 1988; 167:56-71.

Ma JK, Lehner T, Stabila P, Fux CI and Hiatt A. Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. Eur. J. Immunol. 1994; 24:131–138.

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, van Dolleweerd C, Mostov K and Lehner T. Generation and assembly of secretory antibodies in plants. Science. 1995; 268**:**716–719.

Ma JK, Drake PM, Christou P. The production of recombinant pharmaceutical proteins in plants. Nat Rev Genet. 2003; 4:794-805.

Ma JK, Drake PM, Chargelegue D, Obregon P, Prada A. Antibody processing and engineering in plants, and new strategies for vaccine production. Vaccine. 2005; 23:1814-8.

Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y. Systemic Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol. 2005; 23:718-23.

Marusic C, Nuttall J, Buriani G, Lico C, Lombardi R, Baschieri S, Benvenuto E, Frigerio L. Expression, intracellular targeting and purification of HIV Nef variants in tobacco cells. BMC Biotechnol. 2007; 26: 7-12.

McCabe MS, Klaas M, Gonzalez-Rabade N, Poage M, Badillo-Corona JA, Zhou F. Plastid transformation of high-biomass tobacco variety Maryland Mammoth for production of human immunodeficiency virus type 1 (HIV-1) p24 antigen. Plant Biotechnol J. 2008; 6:914–29.

Merril CR, Dunau ML, Goldman D. A rapid sensitive silver stain for polypeptides in polyacrylamide gels. Anal Biochem. 1981; 110:201-207.

Mett V, Farrance CE, Green BJ, Yusibov V. Plants as biofactories. Biologicals. 2008; 36:354-8.

Mett V, Musiychuk K, Bi H, Farrance CE, Horsey A, Ugulava N, Shoji Y, de la Rosa P, Palmer GA, Rabindran S, Streatfield SJ, Boyers A, Russell M, Mann A, Lambkin R, Oxford JS, Schild GC, Yusibov V. A plant-produced influenza subunit vaccine protects ferrets against virus challenge. Influenza Other Respi Viruses. 2008; 2:33-40.

Miller TJ, Fanton MJ, Webb SR, inventors; Dow AgroSciences LLC, assignee. Stable immunoprophylatic and therapeutic compositions derived from transgenic plant cells and methods for production. United States patent 20090087448. 2009.

Misaki R, Kimura Y, Fujiyama K and Seki T. Glycoproteins secreted from suspension-cultured tobacco BY2 cells have distinct glycan structures from intracellular glycoproteins. Biosci Biotechnol Biochem. 2001; 65:2482–2488.

Morgenfeld M, Eugenia Segretin M, Wirth S, Lentz E, Zelada A, Mentarry A. Potato X coat protein fusion to human papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. Mol Biotechnol. 2009; 43:243–9.

Morrow KJ. Improving protein production processes. Gen Eng News. 2007; 27:50–41.

Nadai M, Bally J, Vitel M, Job C, Tissot G, Botterman J, et al. High-level expression of active human alpha1-antitrypsin in transgenic tobacco chloroplasts. Transgenic Res. 2009; 18:173–83.

Nandi S, Yalda D, Lu S, Nikolov Z, Misaki R, Fujiyama K, et al. Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. Transgenic Res 2005; 14:237–49.

Negrouk V, Eisner G, Hi Lee, Han K, Taylor D, Wong HC. Highly efficient transient expression of functional recombinant antibodies in lettuce. Plant Sci. 2005; 169:433–438.

Nose M, Wigzell H. Biological significance of carbohydrate chains on monoclonal antibodies. Proc Natl Acad Sci. 1983; 80:6632-6.

Nuttall J, Ma JK, Frigerio L. A functional antibody lacking N-linked glycans is efficiently folded, assembled and secreted by tobacco mesophyll protoplasts. Plant Biotechnol J. 2005; 3:497-504.

Nuttall J, Vine N, Hadlington JL, Drake P, Frigerio L and Ma JK. ER-resident chaperone interactions with recombinant antibodies in transgenic plants. Eur. J. Biochem. 2002; 269:6042–6051.

Orzaez D, Mirabel S, Wieland WH, Granell A. Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol. 2006; 140:3–11.

Owen M, Gandecha A, Cockburn B and Whitelam G. Synthesis of a functional anti-phytochrome single-chain Fv protein in transgenic tobacco. Biotechnology. 1992; 10:790–794.

Peckham GD, Bugos RC, Su WW. Purification of GFP fusion proteins from transgenic plant cell cultures. Protein Expres Purif. 2006; 49:183–9.

Pedrazzini E, Giovinazzo G, Bielli A, de Virgilio M, Frigerio L, Pesca M, Faoro F, Bollini R, Ceriotti A, Vitale A. Protein quality control along the route to the plant vacuole. Plant Cell. 1997; 9:1869-80.

Petersen K, Bock R. High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. Plant Mol Biol. 2011; 7:8526–30.

Petersen K, Schöttler MA, Karcher D, Thiele W, Bock R. Elimination of a group II intron from a plastid gene causes a mutant phenotype. Nucleic Acids Res. 2011; 39:5181-92.

Petruccelli S, Otegui MS, Lareu F, Tran Dinh O, Fitchette AC, Circosta A, Rumbo M, Bardor M, Carcamo R, Gomord V, Beachy RN. A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnol J. 2006; 4:511-27.

Platis D, Drossard J, Fischer R, Ma JK, Labrou NE. New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants. J Chromatogr. 2008; 1211:80-89.

Platis D, Labrou NE. Application of a PEG/salt aqueous two-phase partition system for the recovery of monoclonal antibodies from unclarified transgenic tobacco extract. Biotechnol J. 2009; 4:1320-1327.

Pogue GP, Vojdani F, Palmer KE, Hiatt E, Hume S, Phelps J, Long L, Bohorova N, Kim D, Pauly M, Velasco J, Whaley K, Zeitlin L, Garger SJ, White E, Bai Y, Haydon H, Bratcher B. Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. Plant Biotechnol J. 2010; 8:638-54.

Pogue GP, Lindbo JA, Garger SJ and Fitzmaurice WP. Making an ally from an enemy: plant virology and the new agriculture. Annu. Rev. Phytopathol. 2002; 40:45–74.

Predajňa L, Nagyová A, Subr Z. Simple and efficient biolistic procedure for the plant transfection with cDNA clones of RNA viruses. Acta Virol. 2010; 54:303-6.

Rademacher T, Sack M, Arcalis E, Stadlmann J, Balzer S, Altmann F, Quendler H, Stiegler G, Kunert R, Fischer R, Stoger E. Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. Plant Biotechnol J. 2008; 6:189-201.

Raju TS. Terminal sugars of Fc glycans influence antibody effector functions of IgGs. Curr. Opin. Immunol. 2008; 20:471–478.

Ramessar K, Rademacher T, Sack M, Stadlmann J, Platis D, Stiegler G, Labrou N, Altmann F, Ma J, Stoger E, Capell T and Christou P. Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. Proc. Natl Acad. Sci. 2008; 105:3727–3732.

Ramirez N, Rodriguez M, Ayala M, Cremata J, Perez M, Martinez A, Linares M, Hevia Y, Paez R, Valdes R, Gavilondo JV and Selman-Housein G. Expression and characterization of an anti-(hepatitis B surface antigen) glycosylated mouse antibody in transgenic tobacco (*Nicotiana tabacum*) plants and its use in the immunopurification of its target antigen. Biotechnol. Appl. Biochem. 2003; 38:223–230.

Rawlings ND, Barrett AJ, Bateman A. MEROPS: the peptidase database. Nucleic Acids Res. 2010; 38:D227-D233.

Rawlings ND, Morton FR, Kok CY, Kong J. and Barrett AJ. MEROPS: the peptidase database. Nucl. Acids Res. 2008; 36:D320–D325.

Rivard D, Anguenot R, Brunelle F, Le VQ, Vezina LP, Trepanier S. and Michaud D. An in-built proteinase inhibitor system for the protection of recombinant proteins recovered from transgenic plants. Plant Biotechnol. J. 2006; 4:359–368.

Rizzieri DA, Akabani G, Zalutsky MR, Coleman RE, Metzler SD, Bowsher JE, Toaso B, Anderson E, Lagoo A, Clayton S, Pegram CN, Moore JO, Gockerman JP, DeCastro C, Gasparetto C, Chao NJ and Bigner DD. Phase 1 trial study of 131I-labeled chimeric 81C6 monoclonal antibody for the treatment of patients with non-Hodgkin lymphoma. Blood. 2004; 104:642–648.

Rodríguez M, Ramírez NI, Ayala M, Freyre F, Pérez L, Triguero A, Mateo C, Selman-Housein G, Gavilondo JV, Pujol M. Transient expression in tobacco leaves of an aglycosylated recombinant antibody against the epidermal growth factor receptor. Biotechnol Bioeng. 2005; 89:188-94.

Rogers SG, Horsch RB and Fraley RT. Gene transfer in plants: Production of transformed plants using Ti plasmid vectors. Meth. Enz. 1986; 118:627-640.

Rosales-Mendoza S, Alpuche-Solis AG, Soria-Guerra RE, Moreno-Fierros L, Martinez-Gonzalez L, Herrera-Diaz A. Expression of an Escherichia coli antigenic fusion protein comprising the heat labile toxin B subunit and the heat stable toxin, and its assembly as a functional oligomer in transplastomic tobacco plants. Plant J. 2009; 57:45–54.

Rose PW, Beran B, Bi C, Bluhm WF, Dimitropoulos D, Goodsell DS, Prlic A, Quesada M, Quinn GB, Westbrook JD, Young J, Yukich B, Zardecki C, Berman HM, Bourne PE. The RCSB Protein Data Bank: redesigned web site and web services. Nucleic Acids Res. 2011; D392-D3401.

Ross KC, Zhang C. Separation of recombinant α-glucuronidase from transgenic tobacco by aqueous two-phase extraction. Biochem Eng J. 2010; 49:343–50.

Rybicki EP. Plant-made vaccines for humans and animals. Plant Biotechnol J. 2010; 8:620-37.

Ryll T. Antibody production using mammalian cell culture how high can we push productivity? AnnMtg Prog and Abstr. 2008; 101.

Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler,G, Fischer R, Katinger H, Stoeger E and Rademacher T. Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J. 2007; 21:1655–1664.

Saïda F. Overview on the expression of toxic gene products in Escherichia coli. Curr Protoc Protein Sci. 2007; 5:5-19.

Sainsbury F, Lavoie PO, D'Aoust MA, Vézina LP, Lomonossoff GP. Expression of multiple proteins using full-length and deleted versions of Cowpea mosaic virus RNA-2. Plant Biotechnol J. 2008; 6:82-92.

Sainsbury F, Liu L, Lomonossoff GP. Cowpea mosaic virus-based systems for the expression of antigens and antibodies in plants. Methods Mol. Biol. 2009; 483:25–39.

Sainsbury F, Lomonossoff GP: Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol. 2008; 148:1212-1218.

Sainsbury F, Sack M, Stadlmann J, Quendler H, Fischer R, Lomonossoff GP. Rapid transient production in plants by replicating and non-replicating vectors yields high quality functional anti-HIV antibody. PLoS One. 2010; 5:13976.

Sainsbury F, Thuenemann EC, Lomonossoff GP. pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol J. 2009; 7:682-693.

Sainsbury, F., Cañizares, M.C. and Lomonossoff, G.P. Cowpea mosaic virus-based expression vectors. In: Virus Expression Vectors. 2007; 339–355.

Saint-Jore-Dupas C, Faye L and Gomord V. From planta to pharma with glycosylation in the toolbox. Trends Biotechnol. 2007; 25:317–323.

Sands AT. The master mammal. Nat Biotechnol. 2003; 21:31-2.

Santi L. Plant derived veterinary vaccines. Vet Res Commun. 2009; 33:61-6.

Schahs M, Strasser R, Stadlmann J, Kunert R, Rademacher T and Steinkellner H. Production of a monoclonal antibody in plants with a humanized N-glycosylation pattern. Plant Biotechnol. J. 2007; 5:657–663.

Schaller A. A cut above the rest: the regulatory function of plant proteases. Planta. 2004; 220:183–197.

Schillberg S, Fischer R, Emans N. Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci. 2003; 60:433-45.

Schillberg S, Twyman RM, Fischer R. Opportunities for recombinant antigen and antibody expression in transgenic plants technology assessment. Vaccine. 2005; 23:1764–9.

Scotti N, Alagna F, Ferraiolo E, Formisano G, Sannino L, Buonaguro L. High-level expression of the HIV-1 Pr55gag polyprotein in transgenic tobacco chloroplasts. Planta. 2009; 229:1109–22.

Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol. 2007; 5:579–90.

Sharp JM and Doran PM. Characterization of monoclonal antibody fragments produced by plant cells. Biotechnol. Bioeng. 2001; 73:338–346.

Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem. 2002; 277:26733-40.

Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. Downstream processing of monoclonal antibodies application of platform approaches. J Chromatogr. 2007; 848:28–39.

Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A. Production of correctly processed human serum albumin in transgenic plants. Biotechnology NY. 1990; 8:217-21.

Simmons CW, VanderGheynst JS: Transient co-expression of post-transcriptional gene silencing suppressors and beta-glucuronidase in harvested lettuce leaf tissue does not improve recombinant protein accumulation in planta. Biotechnol Lett. 2007; 29:641-645.

Sleep D, Belfield GP, Ballance DJ, Steven J, Jones S, Evans LR, Moir PD, Goodey AR. Saccharomyces cerevisiae strains that overexpress heterologous proteins. Biotechnology NY. 1991; 9:183-7.

Smalle J, Vierstra RD. The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol. 2004; 55:555-90.

Sriraman R, Bardor M, Sack M, Vaquero C, Faye L, Fischer R, Finnern R. and Lerouge P. Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack corexylose and core-alpha(1,3)-fucose residues. Plant Biotechnol J. 2004; 2:279-287.

Stevens LH, Stoopen GM, Elbers IJW, Molthoff JW, Bakker HAC, Lommen A, Bosch D and Jordi W. Effect of climate conditions and plant developmental stage on the stability of antibodies expressed in transgenic tobacco. Plant Physiol. 2000; 124:173–182.

Stoger E, Ma JKC, Fischer R and Christou P. Sowing the seeds of success: pharmaceutical proteins from plants. Curr. Opin. Biotechnol. 2005; 16:167–173.

Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol. 2000; 42:583-90.

Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P, Fischer R. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol. 2000; 42:583-90.

Strasser R, Stadlmann J, Schähs M, Stiegler G, Quendler H, Mach L, Glössl J, Weterings K, Pabst M, Steinkellner H. Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. Plant Biotechnol J. 2008; 6:392-402.

Streatfield SJ, Howard JA. Plant-based vaccines. Int J Parasitol. 2003; 33:479–93.

Swartz JR. Escherichia coli recombinant DNA technology. In: Neidhardt FC, editor. Escherichia coli and Salmonella: Cellular and Molecular Biology. 2nd ed. Washington DC: American Society of Microbiology Press. 1996; 1693–711.

Tavladoraki P, Girotti A, Donini M, Arias FJ, Mancini C, Morea V, Chiaraluce R, Consalvi V, Benvenuto E. A single-chain antibody fragment is functionally expressed in the cytoplasm of both Escherichia coli and transgenic plants. Eur J Biochem. 1999; 262:617–624.

Tekoah Y, Ko K, Koprowski H, Harvey DJ, Wormald MR, Dwek RA and Rudd PM. Controlled glycosylation of therapeutic antibodies in plants. Arch. Biochem. Biophys. 2004; 426:266–278.

Terman DS, J.H. Bertram, Eur. J. Cancer Clin. Oncol. 1985; 21:1115.

Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. Appl Microbiol Biotechnol. 2006; 72:211–23.

Tissot G, Canard H, Nadai M, Martone A, Botterman J, Dubald M. Translocation of aprotinin, a therapeutic protease inhibitor, into the thylakoid lumen of genetically engineered tobacco chloroplasts. Plant Biotechnol J. 2008; 6:309–20.

Tomiya N, Lee YC, Yoshida T, Wada Y, Awaya J, Kurono M, and Takahashi N. Calculated two-dimensional sugar map of pyridylaminated oligosaccharides: elucidation of the jack bean β-mannosidase digestion pathway of $Man_9GlcNAc_2$. Anal Biochem. 1991; 193:90-100.

Torosantucci A, Chiani P, Bromuro C. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. PLoS One. 2009; 4:e5392.

Triguero A, Cabrera G, Cremata JA, Yuen CT, Wheeler J, and Ramirez NI. Plant-derived mouse IgG monoclonal antibody fused to KDEL endoplasmic reticulum-retention signal is N-glycosylated homogeneously throughout the plant with mostly high-mannose-type N-glycans. Plant Biotechnol. J. 2005; 3:449–457.

Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, Srinivasan K, Sodroski J, Moore JP, Katinger H. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol. 1996; 70:1100-8.

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. Trends Biotechnol. 2003; 21:570-8.

Valde´s R, Gomez L, Padilla S, Brito J, Reyes B, Alvarez T, Mendoza O, Herrera O, Ferro W, Pujol M, Leal V, Linares M, Hevia Y, Garcýa C, Mila L, Garcýa O, Sanchez R, Acosta A, Geada D, Paez R, Luis Vega J, Borroto C. Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants. Biochem Biophys Res Commun. 2003; 308:94–100.

van der Hoorn RAL. Plant proteases: from phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. 2008: 59:191–223.

Van Droogenbroeck B, Cao J, Stadlmann J, Altmann F, Colanesi S, Hillmer S, Robinson DG, Van Lerberge E, Terryn N, Van Montagu M, Liang M, Depicker A, De Jaeger G. Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. Proc Natl Acad Sci U S A. 2007; 104:1430-5.

van Engelen FA, Schouten A, Molthoff JW, Roosien J, Salinas J, Dirkse WG, Schots A, Bakker J, Gommers FJ, Jongsma MA, Bosch D and Stiekema WJ. Coordinate expression of antibody subunit genes yields highlevels of functional antibodies in roots of transgenic tobacco. Plant Mol. Biol. 1994; 26:1701–1710.

van Ree R, Cabanes-Macheteau M, Akkerdaas J, Milazzo JP, Loutelier-Bourhis C, Rayon C, Villalba M, Koppelman S, Aalberse R, Rodriguez R, Faye L, Lerouge P. Beta(1,2)-xylose and alpha(1,3)-fucose residues have a strong contribution in IgE binding to plant glycoallergens. J Biol Chem. 2000; 275:11451-8.

Van Rooijen G, Glenn KR, Shen Y, Boothe J, inventors; SemBioSys Genetics Inc., assignee. Commercial production of chymosin in plants. United States patent. 2008;7390936.

Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R. Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. Proc Natl Acad Sci. 1999; 96:11128-11133.

Vaquero C, Sack M, Schuster F, Finnern R, Drossard J, Schumann D, Reimann A, Fischer R. A carcinoembryonic antigen-specific diabody produced in tobacco. FASEB J. 2002; 16:408-10.

Verma D, Kanagaraj A, Jin S, Singh ND, Kolattukudy PE, Daniell H. Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. Plant Biotechnol J. 2010; 8:332–50.

Vézina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, Burel C, Lavoie PO, Bardor M, Gomord V. Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants. Plant Biotechnol J. 2009; 7:442-455.

Villani ME, Morgun B, Brunetti P, Marusic C, Lombardi R, Pisoni I, Bacci C, Desiderio A, Benvenuto E, Donini M: Plant pharming of a full-sized, tumour-targeting antibody using different expression strategies. Plant Biotechnol J. 2009; 7:59-72.

Villani ME, Roggero P, Bitti O, Benvenuto E, Franconi R. Immunomodulation of cucumber mosaic virus infection by intrabodies selected in vitro from a stable single-framework phage display library. Plant. Mol. Biol. 2005; 58, 305-316.

Vitale A, and Pedrazzini E. (Recombinant pharmaceuticals from plants: the plant endomembrane system as bioreactor. Mol. Interv. 2005; 5:216-225.

Viti F, Nilsson F, Demartis S, Huber A, Neri D. Design and use of phage display libraries for the selection of antibodies and enzymes. Methods Enzymol. 2000; 326:480-505.

Voinnet O. RNA silencing bridging the gaps in wheat extracts. Trends Plant Sci. 2003 ;8:307-9.

Voss A, Niersbach M, Hain R, Hirsch HJ, Liao YC, Kreuzaler F and Fischer R. Reduced virus infectivity in *Nicotiana tabacum* secreting a TMV-specific full-size antibody. Mol. Breed. 1995; 1:39-50.

Wagner GK, Pesnot T. Glycosyltransferases and their assays. Chembiochem. 2010; 11:1939-49.

Waheed MT, Thones N, Muller M, Hassan SW, Razavi NM, Lossl E, et al. Transplastomic expression of a modified human papilloma virus L1 protein leading to the assembly of capsomeres in tobacco: a step towards cost-effective second-generation vaccines. Transgenic Res. 2011; 20:271-82.

Wilken LR, Nikolov ZL. Factors influencing recombinant human lysozyme extraction and cation exchange adsorption. Biotechnol Progr. 2006; 22:745-52.

Wongsamuth R and Doran PM. Production of monoclonal antibodies by tobacco hairy roots. Biotechnol. Bioeng. 1997; 54:401-415.

Woodard SL, Wilken LR, Barros GOF, White SG, Nikolov ZL. Evaluation of monoclonal antibody and phenolic extraction from transgenic *Lemna* for purification process development. Biotechnol Bioeng. 2009; 104:562-71.

Wrotnowski C. Animal cell culture; novel systems for research and production. Gen Eng News 1998; 18:13-37.

Wu J, Yu L, Li L, Hu J, Zhou J, Zhou X. Oral immunization with transgenic rice seeds expressing VP2 protein of infectious bursal disease virus induces protective immune responses in chickens. Plant Biotechnol J. 2007; 5:570–8.

Xu J, Ge X, Dolan MC. Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. Biotechnol Adv 2011. doi:10.1016/j.biotechadv.2011.01.002.

Yano A, Maeda F and Takekoshi M. Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. J. Med. Virol. 2004; 73:208–215.

Yin J, Li G, Ren X, Herrler G. Select what you need: a comparative evaluation of the advantages and limitations of frequently used expression systems for foreign genes. J Biotechnol. 2007; 127:335-47.

Zeitlin L, Pauly M, Whaley KJ. Second-generation HIV microbicides: continued development of griffithsin. Proc Natl Acad Sci USA; 2009; 106:6029–30.

Zhang C, Baez J, Pappu KM, Glatz CE. Purification and characterization of a transgenic corn grain-derived recombinant collagen type I alpha 1. Biotechnol Progr 2009a; 25:1660–8.

Zhang C, Glatz CE, Fox SR, Johnson LA. Fractionation of transgenic corn seed by dry and wet milling to recover recombinant collagen-related proteins. Biotechnol Progr 2009b; 25:1396–401.

Zhang CM, Lillie R, Cotter J, Vaughan D. Lysozyme purification from tobacco extract by polyelectrolyte precipitation. J Chromatogr. 2005; 1069:107–12.

Zhou F, Badillo-Corona JA, Karcher D, Gonzalez-Rabade N, Piepenburg K, Borchers AM. High-level expression of human immunodeficiency virus antigens from the tobacco and tomato plastid genomes. Plant Biotechnol J. 2008; 6:897–913.

Zhu Z, Hughes KW, Huang L, Sun BL, Liu CM, Li Y, et al. Expression of human α-interferon cDNA in transgenic rice plants. Plant Cell Tissue Organ Cult 1994; 36:112-119.

Ziegelhoffer T, Raasch JA, Austin-Phillips S. Expression of Acidothermus cellulolyticus E1 endo-α-1,4-glucanase catalytic domain in transplastomic tobacco. Plant Biotechnol J. 2009; 7:527–36.

Ringraziamenti (Scritti di getto, 5 minuti prima della consegna, perdonate errori e forma)

Se una tesi di laurea è un traguardo, la tesi di dottorato è un punto di svolta. Una svolta che riguarda tutta la tua vita in quanto è il momento in cui le scelte che fai sono fondamentali, perchè iniziano tracciare un po' meglio le tue speranze, i tuoi pensieri, il tuo futuro. Mi trovo a consegnare una tesi con l'idea di lasciare questo ambito di lavoro. Ma tutto questo non è stato inutile. Questo dottorato è stata un'esperienza formativa, una sfida della vita che ho accettato forse senza neppure pensarci, non so, forse per un sentimento che potrei definire curiosità. Eppure ero fuori, avevo lasciato il laboratorio, ma qualcosa mi ha ritirato dentro, mi ha rincorso. Ricordo ancora la mattina che ho ripreso in mano la pipetta all'inizio del dottorato, Marcello era ancora in Giappone, mi sono detto …. Che dici ricominciamo? E mentre cercavo di dare una risposta a quella domanda sono arrivato a scrivere queste righe. Forse ora quella domanda è più chiara. Non era un ricominciamo….era un "lasciamoci trasportare" ……trasportare da cosa?.....da questo lavoro meraviglioso, perché la ricerca è così, la fai perché non te lo spieghi, perché pur se non hai certezze, se sai che fra 3 mesi dovrai pregare per avere un altro po' di contratto….non te lo spieghi, vai avanti, senza fermarti….poi quando ti ritrovi di fronte ad una banda che si accende dopo un mese di lavoro….allora ti spieghi perché l'hai fatto….e continui. Ma ora non più, la vita ti pone di fronte a delle scelte….e la campanella della prima ora suona. E tu devi decidere, oggi entro o no? Forse ho deciso di non entrare, oggi no. Forse per colpa di un sistema pericoloso che ti tiene dentro per il braccio sorridendo, ti prende a schiaffi, ma ancora ti sorride, e tu non puoi che sorridergli di rimando, aspettando di capire perché lo fai. Perché lo facciamo? Non lo so, ma so solo che quando sei dietro a un bancone di laboratorio con un obiettivo, non sai risponderti, anzi, non ci pensi proprio a farlo e diluisci in fretta il PBS perché devi finire i lavaggi. Tutto questo però non lo fai da solo, sei circondato da tante persone che ti aiutano, ti seguono, ti danno la forza di andare avanti quando tutto sembra impossibile. La mia prima parola di ringraziamento è per te; per te cha mi hai sorretto mi hai dato la forza di andare avanti, hai sostenuto le mie serate davanti ad un computer con lo stesso entusiasmo dall'inizio alla fine, senza farmi mai mancare il supporto morale, il sorriso, la voglia di andare avanti anche se c'erano pacchi di compiti in classe da correggere, ripetizioni da fare e correzioni da inserire. Tu mi hai messo davanti l'obiettivo ricordandomi il perché di quelle notti al computer, di quei fine settimana dietro un tavolino a scrivere e rileggere. Senza di te tutto questo non sarebbe stato possibile, senza di te non ce l'avrei fatta. Questa tesi è anche tua. Sei la ragione di tutto questo, la mia forza. Grazie Ilaria. E un ringraziamento alla mia famiglia Mamma papà Angela, sempre pronti a tendere una mano, ad accettare i fine settimana in cui rimanevamo a Roma senza vederci, a darmi quella speranza per costruire questo futuro, così difficile, in una società sempre più complicata. Pronti a ricevere anche se da lontano tutti i miei dubbi le mie paure. Grazie perché il vostro sostegno non morirà mai e so che potrò contare sempre su di voi. Ad Angela che in silenzio mi sostiene, mi incita, anche se non me lo dice, lo fa e basta e io lo so. E che dire di Marcello, un eccellente supervisore del mio lavoro, sempre pronto a suggerire, discutere, lasciar fare e consigliare. Un esempio di tutor che non mi ha fatto mancare mai il suo supporto, che ha passato ore e ore a correggere rileggere e perfezionare ogni mio singolo lavoro, dalle relazioni alla tesi. Grazie marc, da quello che sento degli altri tutor, non credo avrei potuto trovare di meglio per la mia formazione. Un ringraziamento anche a Carla, se sono rimasto nella ricerca è grazie a lei. Ha sempre creduto in me e sempre puntato su di me….non posso far altro che dire se sono stato a Londra e ho fatto queste esperienze è anche merito tuo. A Sole e Mari, due amiche di viaggio incredibili, due persone meravigliose sempre vive e piene di energia. A sole per avermi sostenuto sempre nella scrittura e correzione, ma soprattutto per aver messo la sua dolcezza in ogni discorso che addolciva il cuore di tutti….A mari perché mi è stata accanto sempre, per parlare confidarmi aprirmi e affidarle sempre i miei momenti difficili che spesso abbiamo superato insieme. Grazie! A Cri, sempre pronta ad accogliere le mie perplessità,

a preoccuparsi per me e a dirmi sempre la sua in modo asettico, senza mai essere faziosa, ma sempre onesta. A Chiara, la persona che mi ha formato di più in tesi e con la quale parlo sempre come se fosse la mia più cara amica. Sempre pronta ad ascoltare, consigliare e sorridermi anche quando tutto andava male. Nella tesi le avevo scritto una brutta premessa, ma dimenticala Chià, sei stata sempre grande. A Eugenio, nei miei confronti sempre gentile e disponibile, mi ha permessi di lavorare anche quando magari altri erano a casa….e per le nostre partite di calcetto, dove dimostri sempre il tuo valore, anche in campo! A Giampa; con lui ho riso, ho pianto e ho avuto modo di trovare sempre un amico per parlare….quasi mai di scienza….Una persona alla quale comunicare i danni fatti in un elisa o un western, senza mai paura che qualcuno lo sapesse. Una persona pronta con la sua umanità a regalarmi qualcosa. Bella zì! Ad Antonia, sempre aperta, disponibile, umana….per noi giovani del lab un pilastro, una colonna….senza di lei l'ENEA perderà tanto…..

Grazie a tutti voi…