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Production of tumour-targeting antibodies in hairy root cultures

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Will power is to the mind like a strong blind man
who carries on his shoulders a lame man who can see.
To Giulia and Andrea.

Table of Contents

Chapter 1. Summary.	5
Chapter 2. General Introduction.....	7
2.1 Overview on Plant Molecular Farming (PMF).	7
2.2 Different platforms for heterologous proteins production.....	9
2.3 Plants as bio-factories.	12
2.4 Plant species used for heterologous proteins production.....	13
2.5 Expression strategies used to produce heterologous proteins in plants. .	14
2.5.1 Stable transformation.	15
2.5.2 Transient expression systems.	18
2.6 Heterologous protein expression in plant cells and tissues.....	23
2.6.1 Plant cells suspension cultures.....	24
2.6.2 Moss cultures.	26
2.6.3 Microalgae cultures.....	27
2.6.4 Hairy root cultures.....	27
2.7 Challenges.	31
2.7.1 Maximizing heterologous protein yields.....	31
2.7.2 Downstream purification processing.	35
2.7.3 Glycosylation of heterologous proteins in plants.....	37
2.8 Plant-made antibodies.	40
2.8.1 Tumour- targeting antibodies.....	41
2.8.2 The tumour-targeting anti tenascin-C mAb H10.....	43
2.8.3 Anti-CD20 antibodies.	44
Chapter 3. Aim of the project.	65
Chapter 4. Hairy roots: a production platform for anti-cancer antibodies.....	67
.....	67
4.1 Abstract.	68
4.2 Introduction.....	69
4.2.1 Production of antibodies in hairy roots.	69
4.3 Materials & Methods.....	72
4.3.1 Generation of HRs expressing mAb H10.	72
4.3.2 Selection of HRs expressing mAb H10 by Western blot analysis.	72
4.3.3 HRs in liquid medium and antibody secretion.	73
4.3.4 Antibody purification from HR tissue and culture medium.....	74
4.3.5 ELISA assays.	75
4.3.6 Glycan analysis.	76
4.4 Results.....	77
4.4.1 Hairy roots generation in <i>N. tabacum</i> and glyco-engineered Δ XTFT <i>N. benthamiana</i>	77
4.4.2 Selection of HR clones expressing the full-size mAb H10.	78
4.4.3 Secretion of mAb H10 in the culture medium.....	82
4.4.4 Purification of functional tumor-targeting mAb H10 with a human-compatible glycosylation profile.....	85
4.5 Discussion.	89
4.6 References.	95

Chapter 5. Hairy roots: a production platform for anti-CD20 recombinant antibodies.....	101
.....	101
5.1 Abstract.....	102
5.2 Introduction.....	103
5.2.1 Non-Hodgkin's lymphomas.....	103
5.2.2 CD20 antigen as a target for immunotherapy.....	103
5.3 Materials & Methods.....	106
5.3.1 Generation of engineered Hairy Roots expressing 2B8-Fc Δ XTFT antibody.....	106
5.3.2 Western blot analysis.....	106
5.3.3 Antibody secretion in the culture medium.....	107
5.3.4 Antibody purification and size-exclusion chromatography.....	107
5.3.5 Antibody quantification by ELISA.....	108
5.3.6 Glycan analysis.....	109
5.3.7 Flow cytometry analysis.....	110
5.4 Results.....	112
5.4.1 Generation and selection of glyco-engineered HR clones expressing 2B8-Fc Δ XTFT.....	112
5.4.2 Comparison of 2B8-Fc Δ XTFT expression in selected hairy root clones.....	113
5.4.3 Secretion of 2B8-Fc Δ XTFT in the liquid culture medium.....	113
5.4.4 Antibody purification and size exclusion chromatography.....	116
5.4.6 2B8-Fc Δ XTFT binding to Daudi cells.....	118
5.5 Discussion.....	119
5.6 References.....	124
Chapter 6. Improving the secretion of recombinant proteins in hairy root cultures.....	127
6.1 Abstract.....	128
6.2 Introduction.....	129
6.2.1 Hairy Roots: limits and challenges.....	129
6.3 Materials & Methods.....	131
6.3.1 Cloning of the RFP gene and transformation of <i>A. rhizogenes</i>	131
6.3.2 Generation of HRs expressing the RFP and secretion in the culture medium.....	131
6.3.3 Screening of RFP positive HR clones.....	132
6.3.4 Confocal microscopy analysis.....	132
6.3.5 Fluorimetric analysis of culture medium.....	132
6.3.6 Western blot analysis of HR clones.....	133
6.3.7 ELISA assays.....	133
6.3.8 Statistical analysis.....	134
6.4 Results.....	135
6.4.1 Generation of HRs expressing RFP.....	135
6.4.2 Secretion of RFP in culture medium.....	136
6.4.3 RFP secretion in the culture medium by using different auxins.....	137
6.4.4 Improving RFP secretion in the culture medium using NAA and 2,4-D.....	139
6.4.5 Secretion of the tumour-targeting 2B8-Fc Δ XTFT in HRs culture medium using NAA and 2,4-D.....	141
6.5 Discussion.....	144
6.5 References.....	148

Chapter 7. Published work.	151
Chapter 8. Acknowledgements.	163

Chapter 1. Summary.

Large-scale production of biopharmaceuticals using traditional systems, such as yeasts, bacteria or CHO cells, present some limits in particular due to the high costs and biosafety issues. In the past twenty years a new production platform for biopharmaceuticals has been developed exploiting plant cells known as plant molecular farming. The use of whole plants as well as plant cells or tissue cultures have several advantages such as their intrinsic biosafety for the absence of human pathogens, the possibility of accumulating a large biomass, the secretion of the protein in the culture medium (easing the downstream purification processes) and the reduced production costs. In this context, Hairy Root cultures (HRs) represent an ideal platform for the production of heterologous proteins for their rapid growth under contained conditions in bioreactors and for the possibility of secreting the molecule of interest in the culture medium. The aim of the PhD project is to obtain *Nicotiana tabacum* or *Nicotiana benthamiana* hairy roots expressing two tumour-targeting antibodies and set up a protocol for the secretion of these antibodies in the culture medium. Moreover, we devised a novel strategy to obtain recombinant antibodies with a human compatible glycosylation pattern necessary for their use in tumour therapy. To this aim hairy roots clones expressing two tumour-targeting antibodies, the mAb H10 against the Tenascin-C tumour marker expressed in several cancer diseases (e.g. breast cancer, lung cancer, glioblastoma) and the recombinant 2B8-Fc Δ XTFT antibody against the CD20 antigen for the treatment of non-Hodgkin lymphomas were obtained from *N. tabacum* and the Δ XTFT *N. benthamiana* glyco-modified plant. Selected hairy root clones accumulated the mAb H10 in the culture medium with yields ranging from 2 to 3 mg/L. *N. tabacum*-derived mAb H10 beared plant-typical complex glycan structures while the one produced in Δ XTFT *N. benthamiana* showed mostly GnGn structures lacking xylose and fucose residues. Both antibodies exhibited comparable antigen binding activities and could be efficiently secreted in the culture medium and purified using a single-step affinity chromatography

protocol. In the case of the anti-CD20 2B8-Fc Δ XTFT, HRs expressing the recombinant antibody were obtained from the glyco-modified Δ XTFT *N. benthamiana*. Also in this case, the recombinant molecule accumulated in the culture medium at levels of about 8 mg/L. N-glycosylation profiles of 2B8-Fc Δ XTFT revealed GnGn structures lacking xylose and fucose and the antibody exhibited CD20 antigen-binding activities comparable to the same molecule produced in agroinfiltrated *N. benthamiana* leaves. These results show that HRs are an efficient and promising platform for the production of tumour-targeting antibodies with optimized glycosylation pattern to be used in therapy and diagnosis representing a valid alternative to traditional expression systems. Indeed, the secretion of heterologous proteins in the culture medium can vary among the different HR clones and the protocol used to induce the secretion of proteins can have a profound impact on the final yield. With the aim of improving our knowledge on the secretion process of heterologous proteins in HRs and to further optimize the protocols used for secretion we generated HRs expressing a secretory version of the red fluorescent protein (RFP). Different auxins were tested at different concentrations and results showed that the 2,4-D at 0.1 mg/L, improved the secretion allowing a higher accumulation of RFP in the liquid culture medium reaching the very high levels of $\sim$ 350 mg/L. In addition, to prove the efficacy of 2,4-D in the accumulation of molecules of interest in HR culture medium, we evaluated the secretion of the tumour-targeting 2B8-Fc Δ XTFT from transgenic Δ XTFT *N. benthamiana* hairy roots reaching the highest levels reported of 16.3 mg/L. To conclude we demonstrated that hairy roots are a valid platform for the production of glyco-optimized tumour-targeting antibodies and hairy roots expressing RFP are a valid model system to improve and study heterologous protein secretion. Future efforts need to be focused on devising further improved induction protocols to enhance protein secretion from roots in order to transform HRs into a more competitive system for heterologous protein production.

Chapter 2. General Introduction.

2.1 Overview on Plant Molecular Farming (PMF).

In the last twenty years, due to the increasing market demand of therapeutic proteins, a new production platform for biopharmaceuticals has been developed. The term plant biopharmaceuticals refer to proteins produced in plants or plant tissues. This approach using whole plants or plant tissues as 'bio-factories' is known as plant molecular farming (PMF). Biopharmaceutical world market, based on recombinant proteins production (e.g. antibodies, vaccines, antigens, hormones), was valued at approximately US\$ 199.9 billion in 2013, and it's estimated to reach US\$ 497.9 billion by 2020, representing an annual growth rate of 13,5% (Xu and Zhang, 2014). With fast-growing demand of recombinant proteins, the capacity of the current production systems (yeast, microbes, mammalian 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. Currently there are over 200 in-plant produced proteins (Xu and Zhang, 2014) and the majority are antibodies and antibody derivatives in addition to vaccines, human blood products and cytokines. Human growth hormone was the first pharmaceutically relevant protein produced in plants reported in 1986 (Barta *et al.*, 1986). Some years after, Human Serum Albumin (HAS) was produced in tobacco and potato (Sijmons *et al.*, 1990). The first full assembled monoclonal antibody was obtained in tobacco plants by crossing two plants expressing single immunoglobulin gamma and kappa chains (Hiatt *et al.*, 1989). In 1992, a hepatitis B virus surface antigen was reported as the first experimental vaccine (Mason *et al.*, 1992). The first edible plant-produced vaccine was *E. coli* enterotoxin (LT-B) produced in potatoes. These transgenic potatoes were fed fresh to mice and proven to be orally immunogenic (Haq *et al.*, 1995). Furthermore, sexual crosses between four transgenic *N. tabacum* plants that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A-G heavy chain, a

murine joining chain, and a rabbit secretory component resulted in plants that expressed all four protein chains simultaneously, as demonstrated by Ma *et al.*, (1995). It was after more than 10 years that the first plant-derived protein was commercialized in 1997. Not surprisingly, this was a non-pharmaceutical protein, which had to overcome fewer regulatory hurdles than biopharmaceuticals. Chicken avidin is a diagnostic reagent produced in maize by ProdiGene. One year later, an antibody to prevent dental decay obtained in tobacco started clinical trials (Ma *et al.*, 1998). Just very recently, a milestone was achieved by Protalix Biotherapeutics Inc. (Carmiel, Israel) that developed in carrot cells a biopharmaceutical drug for treating Gaucher's disease. ELELYSO™, the commercial name of the glucocerebrosidase enzyme, was the first plant-cell drug to receive the human use approval from FDA in 2012 (Mor *et al.*, 2015). The process for approval was accelerated by a guideline for the quality of biologically active substances produced in transgenic plants worked out by the European Medicines Agency (EMA). This gave the possibility to companies to invest in these manufacturing facilities, increasing the production of in-plant pharmaceuticals. For example, Bayer/Icon Genetics (Halle) started Phase I trial with 20 patients for vaccines against Non-Hodgkin's lymphomas. On the other continent, Medicago Inc. (Canada) has developed a plant-derived influenza vaccine (H1N1), while for an avian flu H5 pandemic vaccine the same company has already completed Phase II clinical trials. A wide range of recombinant proteins such as antibodies, enzymes and vaccines have been produced using plant-based expression systems and have demonstrated efficacy and safety in pre-clinical and clinical studies. (Yusibov *et al.*, 2011). The results of some clinical evaluations are detailed in the following table (Tab.1)

Product	Plant host	Expression system	Indication	Product development stage
<u>Vaccines</u>				
E.coli LT-B	potato	Transgenic	Diarrhea	Phase I
HBsAg	Lettuce	Transgenic	Hepatitis B	Phase I
Rabies virus GP/NP	Spinach	Transient (viral vector)	Rabies	Phase I

Personalized anti-idiotype scFVs	<i>N. benthamiana</i>	Transient (magnICON vectors)	Non-Hodgkin's lymphoma	Phase I (ongoing)
H5N1 Influenza HA VLP	<i>N. benthamiana</i>	Transient (Agobacterial binary vector)	H5N1 'avian' influenza	Phase II (Health Canada approved and enrolling volunteers)
<u>Antibodies</u>				
Anti-CD20	Duckweed	Transgenic	Non-Hodgkin's Lymphoma	Pre-clinical
Anti-streptococcus surface antigen I/II	Tobacco	Transgenic	Dental caries	Phase II; EU approved
Anti- α CCR5	<i>N. benthamiana</i>	Transient (magnICON vectors)	HIV	Pre-clinical
Anti-HIV gp120	<i>N. benthamiana</i>	Transient (Agobacterial binary vector)	HIV	Pre-clinical
Anti-HBsAg scFV	Tobacco	Transgenic	Hepatitis B	On market (in Cuba)
<u>Therapeutic and dietary proteins</u>				
Gastric lipase	Maize	Transgenic	Cystis fibrosis	Phase II (Marketed as analytical reagen)
Lactoferrin	Maize	Transgenic		Phase I (Marketed as analytical reagen)
Glucocerebrosidase	Carrot cell	Transgenic	Gaucher Disease	Phase III (Marketed in 2012)

Table.1 Plant-based vaccines, antibodies and therapeutic protein in clinical development on the market.

Table adapted from Yusibov, Streatfield and Kushnir, 2011.

2.2 Different platforms for heterologous proteins production.

The current systems employed for heterologous protein production are mainly

based on the use of bacteria, yeasts, insects, mammalian cells and genetically modified (GM) animals (Demain and Vaishnav, 2009). The most used organisms for heterologous protein production are bacteria such as *Escherichia coli* and the gram-positive *Bacilli* strains. These expression systems are widely used in laboratory practices because of their high growth rate on inexpensive substrates (Saïda *et al.*, 2007). In addition, these organisms present a well characterized genome, easy to manipulate with a large number of cloning vectors and mutant strains available. The major drawback is that bacteria systems do not allow post-translation modifications such as glycosylation, formation of disulphide bonds, proteolytic cleavage, phosphorylation, acetylation and myristoylation which are necessary for a correct protein folding, essential for the functionality of some classes of proteins. To overcome these disadvantages, yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* are also used combining the simple and inexpensive culture conditions of bacteria with the processing abilities of eukaryotic cells by increasing the likelihood of proper folding and post-translational modification of complex proteins. However, proteins with anti-fungal activity are difficult to produce in yeast cells. In addition, the glycosylation machinery in yeasts differs significantly from human cells, resulting in hyper-glycosylation of N-linked sugar chains which can mask the active sites of enzymes and reduce their activity, or create unusual epitopes which render the proteins immunogenic (Jayaraj and Smooker, 2009). Insect cells represent another efficient strategy for the expression of recombinant proteins. The expression system is based on baculovirus expression vectors, rod-shaped double strand DNA viruses able to infect insect cells (Hu *et al.*, 2005). These viruses are able to direct the production of proteins at high levels in insect cells and this natural ability has been exploited obtaining recombinant expression vectors (Vilcinskas and Kollwe, 2013). Insect cells have a post-translational modification machinery suitable for the production of complex proteins. However, a high protease activity and differences in the N-linked glycosylation pattern are serious drawbacks of this protein-production-system (Ferrer-Miralles *et al.*, 2009). 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). Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells are the most widely used mammalian cell cultures for recombinant protein production. Up to 39% of all approved recombinant biopharmaceuticals in the US and EU are produced in mammalian cell cultures (Ferrer-Miralles *et al.*, 2009). The advantages of more sophisticated protein processing in mammalian cells are offset by the need for expensive media and equipment and the increased risk of contamination with human pathogens, making mammalian cells suitable for recombinant therapeutic proteins with a high cost of goods. Genetically modified (GM) animals are an alternative production system for the expression of recombinant proteins, because they are 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). In the following table (Tab. 2) pros and cons of the different production systems are reported.

Heterologous system production	Advantages	Disadvantages
Bacteria	➤ Rapid expression ➤ Low production costs ➤ Simple process scale- up ➤ Well characterized genetics ➤ Moderately rapid expression	➤ No eukaryotic post-translational modification ➤ Some proteins are not properly folded
Yeasts	➤ Moderately rapid expression ➤ Low production costs ➤ Most protein folding and post-translational modification are possible	➤ Enhanced safety precautions are required ➤ Requires use of yeast secretion signal peptide for an efficient secretion ➤ Characteristic N-linked glycan structures of proteins are different when compared to the typical mammalian proteins

Insect cells	➤ Moderately rapid expression ➤ Most protein folding and post-translational modification are possible	➤ Characteristic N-linked glycan structures of proteins are different when compared to the typical mammalian proteins ➤ Expensive ➤ Difficult to scale-up
Mammalian Cells	➤ Moderately rapid expression ➤ Correct protein folding and compatible post-translational modifications	➤ Slow cell growth ➤ High production costs
Genetically modified animals	➤ Large scale expression in milk, blood and eggs ➤ Compatible protein folding and post-translational modification are possible	➤ High production costs ➤ Long time to obtain transgenic animals ➤ Enhanced safety precautions are required

Table 2. Advantages and disadvantages of the different production systems used for expressing heterologous proteins are detailed.

2.3 Plants as bio-factories.

In order to overcome the disadvantages detailed in the previous paragraph, the in-plant based approach is a valid alternative for heterologous protein production. Plants or plant tissues offer huge potential for time and labour savings while improving productivity and scalability. Different plant tissues like leaves, seeds, tubers and fruits can be used as bio-factories and, unlike transgenic animals, there is no need to maintain a founder herd, because transgenic plant lines can be stored as seeds, without the need of refrigeration (Fischer *et al.*, 2000). The accumulation of the recombinant protein in defined tissues such as vacuoles, in addition to the possibility of secreting of the protein in the culture medium make, in some cases, the downstream purification process easier (Ma *et al.*, 2003). Plants have a post-translation machinery able to fold and assemble efficiently complex proteins such as immunoglobulins and interleukins obtaining a fully active product. In contrast to mammalian cell cultures, plants aren't hosts for human pathogens and so, contamination of plant-produced recombinant proteins with human infectious agents is not considered a safety issue (Giddings *et al.*, 2000). Accordingly, these

advantages make plants an amenable candidate over the existing expression systems for the heterologous protein production.

2.4 Plant species used for heterologous proteins production.

Even though a wide range of plant species exists, it is of paramount importance selecting them based on the proteins to be produced. In fact every plant species has different biological features influencing proteins production, such as plant life cycle, product storage, biomass yield, downstream purification process, final product quality and production costs (Sharma and Sharma, 2009). Leafy crops as tobacco allow huge biomass, high efficient agrobacterium-mediated transformation and prolific seed production facilitating biomass scale-up. (Twyman *et al.*, 2003). Self-pollination abates the risks of transferring genetic material to other plants. In addition tobacco is a non-food crop, therefore it should not contaminate food chains (Ma *et al.*, 2003). The phenolic compounds released during extraction process that could make difficult the downstream processing in addition to the heterologous protein instability are the main disadvantages for leafy crops. Cereal and legume seeds overcome these drawbacks because they provide an environment suitable for protein accumulation and storage (Stoger *et al.*, 2002; Rademacher *et al.*, 2008). In addition, the recombinant protein is not exposed to phenolic compound improving downstream processing. Maize, rice and wheat are the most used cereal crops used for heterologous protein production while Alfalfa and soybean represent the most widespread legumes for this purpose having also the advantage fixing atmospheric nitrogen reducing the need of chemical fertilizers (Twyman *et al.*, 2003). The main drawback of using cereals or legumes for production of biopharmaceuticals relates to the possibility of contaminating with the heterologous gene, crops that are grown for food purposes, and the likelihood of an accidental contamination during seed collection and storage. On the other hand, the use of fruits and vegetables for heterologous protein production was regarded as a winning solution as they can be eaten raw. The employment of edible plants in underdeveloped countries is a major advantage

as they are a quick and low cost solution for vaccinations. Bananas are also a valid alternative as they are sweet and appreciated both by adults and children (Mason *et al.*, 2002). The major drawback of this approach is the difficulty to quantify the dose of antigen to be administered but also the bioavailability of the antigen itself and for this reason the validity of plant edible vaccines is still under debate.

2.5 Expression strategies used to produce heterologous proteins in plants.

Two main expression strategies are generally employed for the production of biopharmaceuticals in plants. One is based on the stable transformation of the nuclear/chloroplast genome the other on the transient expression mediated by recombinant viral or *Agrobacterium*-based vectors (Ko *et al.*, 2005; Komarova *et al.*, 2010). Concerning stable transformation, approaches are based on the use of biological (*agrobacterium*-mediated transformation) or physical (particle bombardment) methods to allow the penetration of cDNA through the nuclear envelope and inserting it into the plant genome. In addition, the gene can be stably inserted into the chloroplast genome obtaining high level of transgene expression. Transient expression, approaches mainly focus on the use of viral or *Agrobacterium*-based vectors. The agro-infiltration method involves the mechanical penetration of a suspension of *Agrobacterium tumefaciens* into the leaf tissues (as tobacco leaves), which allows the transferring of T-DNA to an high percentage of cells (Regnard *et al.*, 2010). The ability of plant viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) to be used as vectors to carry foreign gene into the plant cell without integrating in the host cell is described as the virus infection method (Porta and Lomonossoff, 2002). In addition to aforementioned methods, an innovative 'deconstructed' viral vector system combining the *Agrobacterium* ability to transform cells in addition to speed and high expression levels of viruses has been developed obtaining high expression level in addition to easy transformation process (Gleba *et al.*, 2005).

2.5.1 Stable transformation.

Stable genetic transformation of plants allows the insertion of a heterologous gene coding for a protein of interest, in the nuclear or chloroplast genome. The foreign 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 *A. tumefaciens*-mediated transformation and the 'gene gun' technology. In the following both nuclear and chloroplast transformations are detailed.

Nuclear transformation.

Nuclear transformation technology exploits the natural capability of *A. tumefaciens* to integrate a fragment of DNA (T-DNA contained in the Ti plasmid) in the genome of host cells (Escobar and Dandekar, 2003). Ti plasmid DNA has two borders (right and left) (Fig. 1) within which it is possible to replace the sequence of interest, without affecting its transformation ability (Lorence and Verpoorte, 2004). In this way the gene coding for a heterologous protein can be inserted in the nuclear genome leading to the expression of the protein of interest and can be inherited by subsequent generation of transgenic plants. The easiness of manipulation, the high efficiency of insertion and the possibility to transfer long DNA sequences (Krishnamohan *et al.*, 2001) are the main advantages reported for this transformation system. The extensive use of *Agrobacterium*-mediated transformation for tobacco leaf disks have made the regeneration of transgenic tobacco lines a routine laboratory procedure (Horsch and Klee, 1986). In fact, many heterologous protein have been produced in transgenic plants and plant tissue cultures using this approach (Hellwig *et al.*, 2004). 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 plant tissue with micro-particles 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

micro-particles and occasionally transferred and integrated into the genome (Predajňa *et al.*, 2010). Stable transformation mediated by *A. tumefaciens* or gene gun have as inconvenient the randomness with which the transgene integrates into the host genome and related 'position' effect that could vary the expression levels between individual transgenic plants. Multiple locus insertions may lead to transgene instability, gene silencing and low expression level of heterologous protein (Shih and Doran, 2009). Furthermore, several new tools of genome engineering have been introduced in recent years, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 system, for targeted mutagenesis in different plant species (Bortesi and Fischer, 2015). Disadvantages are related with the time and the cost in obtaining transgenic plants resulting higher compared to these needed for transient expression systems.

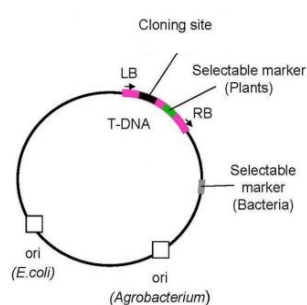


Figure 1: Schematic representation of Ti plasmid used for *Agrobacterium*-mediated expression.

Chloroplast transformation.

The gene of interest can also be incorporated into the chloroplast genome using gene gun technology (Cardi *et al.*, 2010). A mature leaf cell could contain from 50 to 100 chloroplasts and each of them can contain up to 100 copies of chloroplast genome. It is therefore likely that a higher yield of foreign protein could be produced from chloroplast transformation than the nuclear transformation. 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, with a homogeneous population of transformed plastids, is achieved after a sufficient number of cell divisions under

selective conditions (Scotti *et al.*, 2009). Among the disadvantages of this approach is the fact that post-translational modifications, which are necessary for the expression of folded and active mammalian proteins, are generally not allowed. Another drawback of this strategy is represented by the time-consuming processes necessary to achieve the plant homoplasmic chloroplast transformation. Plant stable transformation strategy was used to express several heterologous proteins of different nature (Tab. 1) such as a vaccine for rabies developed in spinach (Yusibov *et al.*, 2002), an anti-CD20 for treating non-Hodgkin's lymphomas expressed in duckweed (Yusibov *et al.*, 2011) and a single-chain antibody in transgenic tobacco used as anti-Hepatitis B antibody (Mason *et al.*, 1992).

Protoplast transformation.

Direct introduction of DNA into plant protoplasts facilitates a rapid analysis of transient gene expression, as well as the generation of stably transformed transgenic plants. The successful protoplast transformation depends on the efficiency of transferring DNA into protoplasts and protoplast purification. Because protoplasts are uniform and could be infected simultaneously with a number of plant viruses they are extensively used for studying virus infection, replication, cytopathology, protection and genetic engineering of virus-resistant plants (Murakishi *et al.*, 1984). There are a number of methods for introducing DNA into plant protoplasts such as electroporation, Polyethylenimine-mediated transfection but, the most commonly used technique is the polyethylene glycol (PEG) to mediate DNA uptake. In detail, Polyethylene Glycol (PEG) is able to alter the properties of the protoplast membrane and is efficient in promoting membrane fusion and the infection of plant protoplasts by viral nucleoprotein, nucleic acid and RNA-encapsulated liposomes (Mathur *et al.*, 1998). PEG treatment of protoplasts is a simple and efficient strategy, allowing a simultaneous processing of many samples, obtaining a cell population with high survival and division rates that can be readily adapted to a wide range of plant species and tissue sources (Mathur *et al.*, 1998).

2.5.2 Transient expression systems.

Obtaining a stable transgenic line takes several months in the best case, furthermore several generations are required to obtain homozygosity of the transgene (Yao *et al.*, 2015). Instead of integrating the sequence in the plant genome, transient expression approaches are based on epichromosomal expression of the heterologous protein. Transient or epichromosomal transformation differs from stable transformation as the exogenous sequence does not integrate into the genome of the plant and the transgene is not inherited by progeny. This strategy generally gives high protein yields in a very short period improving time of protein production (Leuzinger *et al.*, 2013). In fact, high amounts of heterologous protein can be obtained in a couple of days or weeks, which is not achievable via stable gene expression. This may be of critical importance in ensuring a rapid and effective response to a sudden outbreak of an infectious disease, such as the 2009 influenza A/H1N1 pandemic that would require a recombinant vaccine product to be made in sufficient quantities within a very short time period in order to meet the needs of a pandemic. Transient transformation can be obtained using: i) vectors or plasmids containing T-DNA gene cassettes derived from *A. tumefaciens* with a strong constitutive promoter (Fischer *et al.*, 2000), ii) vectors carrying the entire genome of appropriately modified plant viruses (first-generation vectors) and iii) vectors combining the two strategies and carrying 'simplified' genome of plant viruses (second-generation viral vectors) in T-DNA cassettes.

An efficient expression strategy is the *Agrobacterium*-mediated expression system based on T-DNA gene cassettes. The vectors used for transformation are made up of a T-DNA containing multiple cloning sites, a strong constitutive promoter (generally the CaMV 35S promoter) a selectable marker gene for transformed plant cells, selectable marker genes for bacteria and a vector backbone carrying plasmid replication components for *E. coli* and *A. tumefaciens* (Lee *et al.*, 2008). These vectors are delivered to the plant cells exploiting the ability of *Agrobacterium* in mediating the transferring of the gene the T-DNA bearing the gene of interest in the plant cells. This methodology is known as agroinfiltration. *Agrobacterium* containing solution is forced to enter in

the intercellular spaces either applying the solution with a needleless syringe or through vacuum process. For syringe agroinfiltration usually a small lesion is created with a needle in the epidermis on the lower page of the leaf (Fig. 2A) and then *Agrobacterium* is injected into the leaf with a needle-less syringe (Fig. 2B-2C) (Lai *et al.*, 2013).



Figure 2: Syringe agroinfiltration of *N. benthamiana* leaves was performed using *A. tumefaciens* harbouring the gene of interest (Lai *et al.*, 2013). A small lesion is created with a needle in the epidermis on the back side of the leaf (A) and then *Agrobacterium* is injected into the leaf through a syringe (B). As the *Agrobacterium* mixture enters the intercellular space of the leaf, the leaves turn into a darker green, indicating a successful infiltration (C).

For vacuum infiltration, plants are totally submerged in the *Agrobacterium* solution and vacuum is applied. Vacuum is then released and the *Agrobacterium*-containing culture is forced in the leaf intercellular spaces. The expression of the recombinant protein requires from 4 to 10 days depending on the vector used, the host organism, the *Agrobacterium* strain and the heterologous protein itself. A limitation of this technique is the possibility of post-transcriptional gene silencing (PTGS) mediated by 'RNA interfering' (Schwab and Voinnet, 2010). This defense mechanism works in plants as a response to viral infections to counteract the over-expression of pathogenic genes. In fact, during the infection there is the formation of aberrant double strand RNA molecules (dsRNAs) that are not present in 'normal' conditions that are recognized as exogenous and cut into fragments called small interfering RNAs (small interfering RNAs, SiRNA) which in turn trigger degradation of viral mRNA causing gene silencing. But in nature, plants and viruses have always co-evolved and, if the plant has found a mechanism to defend itself against viral infection, the virus has, on the other hand, developed a system to escape

silencing. By exploiting the viral genes encoding specific gene silencing suppressors, such as the p19 derived from some plant viruses (Tomato Bushy Stunt Virus, TBSV, Artichoke Mottled Crinkle Virus, AMCV), it is possible to significantly increase expression levels of the gene of interest (Scholthof *et al.*, 1995). In recent years, several biotechnology companies for the production of large-scale bio-pharmaceuticals, operating mainly in the United States, have built semi-automated systems that allow agro-infiltration of hundreds of plants in just a few minutes (Pogue *et al.*, 2010). In Figure 3, a representation of a vacuum chamber used at laboratory scale is shown.



Figure 3: Vacuum chamber used at laboratory scale for transient plants transformation.

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.

First generation vectors.

In the case of the first generation vectors, in order to obtain high expression levels, the heterologous gene is under the control of a sub-genomic promoter, both replacing the gene encoding 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). Virus-mediated expression is a way to rapidly accumulate high level of heterologous protein transiently and it has been used for in-plant heterologous protein expression (Tab. 1). Tobacco mosaic virus (TMV), potato virus X (PVX) Cauliflower mosaic virus (CaMV) and Cowpea mosaic virus (CPMV) are the most commonly used viral vector systems for transient production of bio-pharmaceuticals such as full-size antibodies and antibody fragments (Canizares *et al.*, 2005; Lico *et al.*, 2008). The major drawbacks of these viral vectors are the limitation in size of the heterologous gene that can be inserted in the viral genome and also the risk related with the possibility that these recombinant viruses may spread into the environment.

Second generation vectors.

Icon Genetics (Halle, Germany) overcame these disadvantages by developing an innovative TMV-based 'deconstructed' viral vector system, known as magnICON, aiming at over-expressing heterologous proteins. Combining the *Agrobacterium* and viruses advantages, MagnICON exploits the *Agrobacterium* ability to transform cells in addition to the speed and high expression levels of viruses (Gleba *et al.*, 2005). The 'deconstructed' strategy consisted in removing viral genes that were not strictly necessary to the production of the recombinant protein and replacing them with one or more gene of interests. An example of protein expressed using this strategy is represented by monoclonal antibodies (Giritch *et al.*, 2006). MagnICON split the TMV genome into three components (Fig. 4) which are co-infiltrated in the same plant leaves as a mix of three *Agrobacterium* cultures (Marillonnet *et al.*, 2004). In the magnICON system the

3' module contains the gene of interest with the nos terminator instead of the sequence of the viral Coat Protein (CP), the 5' module contains the *Arabidopsis* actin 2 (ACT2) promoter along with the *tobamovirus* polymerase and movement protein (MP) genes, while the recombinase module containing the PhiC31 integrase gene (from *Streptomyces* phage C31) acts to fuse the 3' and 5' modules together in the nucleus to allow the formation of a complete TMV-based vector where the gene of interest is under the control of the subgenomic promoter present within the MP sequence. The RNA is then able to replicate, spread from cell to cell but not throughout the entire plant because of the CP gene removal. 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). Another interesting strategy used for the expression of multiple polypeptides is represented by the CPMV-HT for 'hyper-translatable' Cowpea Mosaic Virus, where the regions encoding the viral movement protein, both coat proteins and the upstream start codons within the 5' leader sequence have been removed from RNA-2 of Cowpea Mosaic Virus (CPMV). The deleted RNA-2 still have the cis-acting sequences for enhancing translation and thus very high levels of heterologous proteins are generally obtained (Sainsbury and Lomonossoff, 2008).

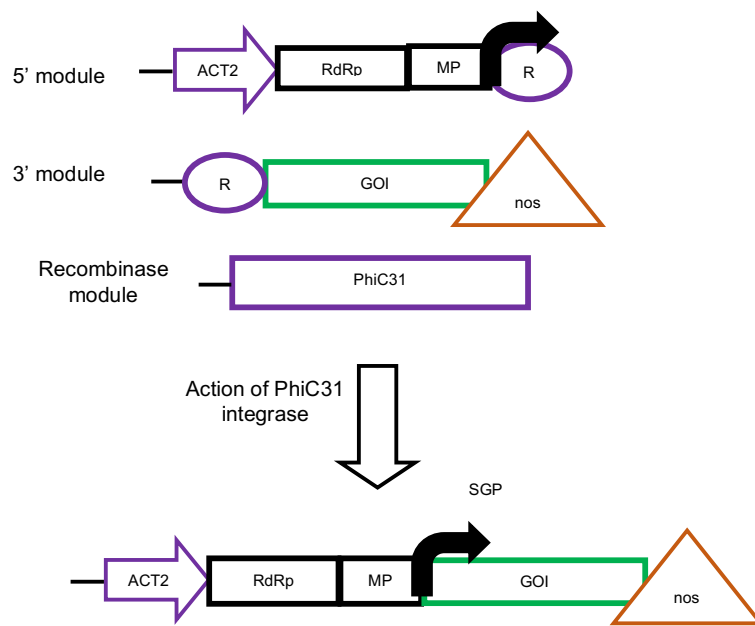


Figure 4: Simplified diagrams of deconstructed tobamovirus-based expression systems. The magnICON system is composed of three modules. The recombinase module allows the recombination of the 5' and 3' modules to form a complete replicon. ACT2: Arabidopsis actin 2 promoter, RdRp: tobamovirus RNA-dependent RNA polymerase, MP: tobamovirus movement protein, SGP: TMV subgenomic promoter directing transcription of the gene of interest (GOI), R: recombination site, nos: nos terminator, PhiC31: streptomyces phage 31 integrase.

2.6 Heterologous protein expression in plant cells and tissues.

Plant tissues and plant cell based platforms represent a valid system for the production of secondary metabolites but also heterologous proteins (Doran, 2000). Since 20 years, the use of plant cells or tissues as production-platforms of heterologous proteins is growing thanks to several advantages as the possibility of producing in sterile contained conditions generally obtaining constant yields of recombinant protein. In addition, plant cells and tissues allow post-translational modifications, have low production costs and are safer due to the lower risk of contamination by viruses, pathogens and toxins. Furthermore, the ability of plant cells and organs to propagate indefinitely without the need for sexual reproduction offers a solution to other problems relating to gene segregation and long-term transgene stability in agricultural crops (Doran,

2000). Alongside the protein of interest can be secreted into the culture medium easing the downstream product recovery and purification processes. The following paragraphs summarize the different types of plant cells and tissues as suspension cell cultures, moss cultures, microalgae cultures and hairy root cultures. The recent advances and commercial successes will be treated in addition to the perspectives and challenges that must be overcome to make these platforms competitive compared to other production systems.

2.6.1 Plant cells suspension cultures.

Since two decades undifferentiated plant cells suspension cultures are used for heterologous proteins production. Plant suspension cells are prepared by the agitation callus tissue in shaker flasks or fermenters to form single cells and small aggregates. Callus is an undifferentiated tissue obtained by cultivating explants on solid medium containing the appropriate mixture of plant hormones to maintain the undifferentiated state. If transgenic plants expressing the recombinant protein of interest are used as the source of callus tissue, further genetic manipulation is unnecessary, alternatively wild-type cell suspensions can be transformed with recombinant plasmids either by co-cultivation with *A. tumefaciens*, particle bombardment or through protoplast transformation. The most used plant cell lines are derived from *N. tabacum* such as BY2 or NT1 cells (Hellwig *et al.*, 2004). Other plant cell lines are used as production platform, among these some are derived from edible crop species such as rice, soybean, carrot and tomato (Xu and Zhang, 2014). Cultured plant cells require simple nutrients for growth and the purification and downstream process is much easier because they generally allow the secretion of the recombinant protein in the culture medium. The size and composition of the heterologous protein however may influence its secretion capacity (Schillberg *et al.*, 2013). The first human protein expressed in *N. tabacum cells* suspension culture initiated from transgenic plants was the human serum albumin (Sijmons *et al.*, 1990) but several other proteins such as cytokines, hormones, vaccine antigens have been produced. Antibodies remain the most expressed proteins because of their stability and easiness of purification. A yield of 20 g/L of the monoclonal

antibody M12 was produced in BY-2 cells in a 200 litres bioreactor (Raven *et al.*, 2015). Another example is the production of beta-glucocerebrosidase in carrot cells by the biotech Protalix Biotherapeutics for the treatment of Gaucher's disease marketed in 2013 (Mor, 2015). Technology developed by Protalix has made it possible to manufacture drugs inside plant cells. A different strategy to highly express heterologous proteins was recently developed using a platform based on plant suspension cell packs. The innovative strategy known as 'cookie method' basically involves making a cookie with cultured plant cells by suction of a suspension onto a membrane, the recombinant *Agrobacterium* is then drizzled into the cookie and the cell cookie preparation is incubated in a humid environment for a few days, until the desired level of protein has been reached. In the following, a table (Tab. 3) summarizing proteins of medical relevance produced in plant cell cultures.

Host cell	Protein	Indication	Yield
<i>N. tabacum</i> cells BY-2	Hepatitis B surface antigen (HBsAg)	Hepatitis B vaccine	6.5 µg/g FW
<i>N. tabacum</i> cells BY-2	Granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Production of white cells	Up to 250 µg/L
<i>N. tabacum</i> cells BY-2	IL-4	Immunoregulation	0.18 µg/L
<i>N. tabacum</i> cells BY-2	2G12 monoclonal α-HIV ab	Anti HIV antibody	12 mg/L
<i>N. tabacum</i> cells BY-2	IL-12	Immunoregulation	Up to 160 µg/L
<i>Rice cells Oryza sativa</i>	Human α1-antitrypsin	Emphysema	4.5-7.7 mg/L
<i>Rice cells Oryza sativa</i>	Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Production of white cells	2% of TSP
<i>Rice cells Oryza sativa</i>	hCTLA4Ig	Immunosuppressive agent	Up to 31.4 mg/L
<i>Rice cells Oryza sativa</i>	Human Growth Hormone	Growth Hormone	Up to 120 mg/L
Carrot cells <i>Daucus carota</i>	Taliglucerase alfa	Gaucher disease	
Soybean cells	Glycine max.	Hepatitis B vaccine	65 µg/g FW
Medicago cells <i>Medicago truncatula cv.</i>	EPO	Tissue protective	

Tomato cells <i>Lycopersicon esculentum</i>	hGM-CSF	Immunosuppressive and immunomodulator	Up to 45 µg/L
Siberian Ginseng cells <i>Acanthopanax senticosus</i>	Human lactoferrin	Immunosuppressive and immunomodulator	0.2-2.3 % of TSP
Korean Ginseng cells <i>Panax ginseng</i>	Human lactoferrin	Immunosuppressive and immunomodulator	3 % of TSP
Sweet potato cells <i>Ipomoea batatas</i>	Human lactoferrin	Immunosuppressive and immunomodulator	3.2 µg/mg of TSP

Table 3: Proteins of medical relevance produced in plant cell cultures. Adapted from Santos *et al.*, (2016).

2.6.2 Moss cultures.

In addition to undifferentiated cells of higher plants, moss suspension cell cultures have received increasing interest as new bio-production platform (Reski *et al.*, 2015). The ability of moss cells to photosynthesize in culture reduces the cost of culture nutrients. Furthermore, recombinant proteins can be secreted into the culture medium, which facilitates downstream processes and purification of the recombinant protein. Cultures of the moss, *Physcomitrella patens* (*P. patens*), are the most used for heterologous protein production. Some recombinant therapeutic proteins, such as glyco-optimized version of the antibody IGN311 (Orellana-Escobedo *et al.*, 2015) or a multi-epitope fusion protein from the human immunodeficiency virus (Rubio-Infante *et al.*, 2012) have all been produced in moss cultures. Bryo-Technology is a moss culture-based platform developed by a German company for producing high-quality proteins on large-scale. Some heterologous protein produced are Moss-GAA (Acid Alpha Glucosidase) for Pompe Disease and Moss-AGAL which is the alpha-galactosidase A enzyme used to treat Fabry Disease (*Patent-Pharmaceutical Protein Production in Moss - Greenovation Biopharmaceuticals*). This molecule is entering the phase I testing (Xu and Zhang, 2014). In addition, human beta-1,4-galactosyltransferase gene was expressed in a moss culture with a ‘humanized’ glycosylation pattern (Reski *et al.*, 2015).

2.6.3 Microalgae cultures.

Microalgae present a very simple structure: unicellular, colonial, or filamentous. The major advantage of algae is relative to the production of large amounts of biomass within a very short period due to their short life cycle. The major drawback is that recombinant proteins produced from algae do not undergo certain post-translational modifications, and as a result, may not be suitable for the production of some glycoproteins (Mathieu-Rivet *et al.*, 2014). However, a variety of therapeutic and diagnostic recombinant proteins as the bovine mammary-associated serum amyloid were expressed in the chloroplast of *Chlamydomonas reinhardtii* (Lu and Oyler, 2009). Another example is a single-chain antibody (HSV8-Isc) directed against glycoprotein D of the herpes simplex virus expressed in the chloroplast of *C. reinhardtii*. The protein was soluble, suggesting that it was correctly folded, and electrophoresis indicated the formation of the dimer by disulfide bond formation (Mayfield *et al.*, 2003). More recently a Human erythropoietin (Epo), used in the treatment of anemia, was fused to an export sequence and expressed in the nuclear genome of *C. reinhardtii*, revealing a close matching with the endogenous human Epo (Eichler-Stahlberg *et al.*, 2009). In addition to *C. reinhardtii*, other green algae cells were used for heterologous protein expression, such as halotolerant *Dunaliella salina* that is an edible alga. One of the major algae advantage, is that they are GRAS (generally recognized as safe) organisms. In fact, algae could produce inexpensive oral vaccines, making vaccination an accessible form of disease management for a whole host of third-world diseases. Furthermore, the oral delivery method and the option to store doses at room temperature would allow vaccines to be transported and administered to remote populations without the need for expensive refrigeration or highly trained medical personnel (Chebolu and Daniell, 2009).

2.6.4 Hairy root cultures.

Hairy roots cultures (HRs) are neoplastic tissues resulting upon wounding and transforming plant leaves with *A. rhizogenes* a gram negative soil bacterium of the family Rhizobiaceae. The pathogen enters into the plant cell after a wound,

introducing into the genome of the infected plant a T-DNA segment from its root-inducing (Ri) plasmid (White *et al.*, 1985). This T-DNA carries a set of genes encoding enzymes for the phytohormone auxin control, cytokinin biosynthesis and for opines genes inducing a new hormonal balance allowing the formation of proliferating roots, called hairy roots (HR), which emerge at the wounded site. In detail, among the 18 ORFs localized in the Ri T-DNA, four coincide with genetic loci (rol A, B, C, D) identified by transposon mutagenesis from the effects of their inactivation on the virulence properties of *A. rhizogenes* and responsible for the stable introduction of genetic material into host cells. The formation of HR from infected cells is more likely regulated by rolA, rolB and rolC. Among these, the rolB locus is thought to be the most important for hairy root induction (Cardarelli *et al.*, 1987). Hairy root based production systems, under contained conditions, offer substantial benefits, including fast economic biomass accumulation, genetic stability, and time efficiency in obtaining HRs expressing the protein of interest (Hu and Du, 2006). Hairy root cultures are generated both from transgenic plants expressing the protein of interest by infecting them with wild-type *A. rhizogenes* or by infecting wild-type plants with *A. rhizogenes* transformed with a plant expression vector carrying the gene of interest. In detail, plant leaf discs (Fig. 5A) after sterilization and wounding, are inoculated with a culture of *A. rhizogenes* carrying the gene of interest. The leaf discs are incubated in plates containing antibiotics such as cefotaxime in order to eradicate *A. rhizogenes*. Approximately after 20 days, hairy roots emerge (Fig. 5B, 5C) each representing a different clonal line (Fig. 5D).

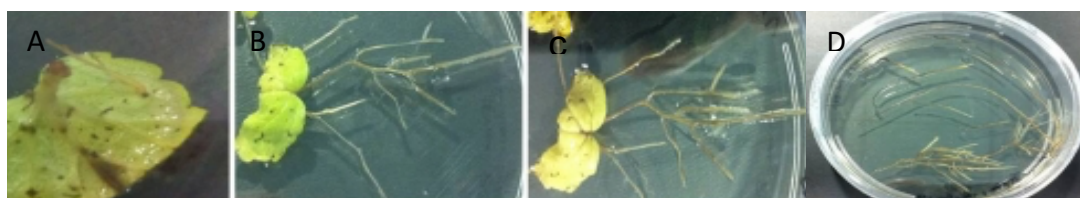


Figure 5: schematic representation of hairy roots generation.

Hairy roots offer several advantages compared to other systems such as fast

biomass accumulation, genetic stability, time efficiency, potential for continuous in-line extraction process, exposure to few proteolytic enzymes, possibility of producing a fully assembled heterologous protein with post-translational modifications and the secretion of the final product in the culture medium (Tab. 4) (Lonoce *et al.*, 2016).

Advantages of hairy roots
1. The potential for continuous in-line extraction process, and exposure to fewer proteolytic enzymes
2. The final product is secreted in the medium and therefore, potentially more homogeneous than a product extracted from plant tissues
3. Production in sterile contained bioreactors
4. Efficient downstream processing

Table 4: Advantages of hairy roots production system.

Among these advantages, the most interesting one is that hairy roots are able to secrete properly folded, functionally active recombinant proteins into the culture medium easing the purification process (Häkkinen *et al.*, 2014). Several approaches have been used to trigger the secretion of the heterologous protein in the medium such as the use of auxins that have been demonstrated to increase heterologous protein accumulation (Häkkinen *et al.*, 2014; Lonoce *et al.*, 2016; Madeira *et al.*, 2016). Some strategies are aimed to alter root cell wall permeability by adding Tween 20 in the liquid medium (*Patent US7350331 - Method for producing metabolites from plants cultivated in soil-less medium*). As a nitrogen source, in order to increase the protein synthesis, KNO₃ was used (Häkkinen *et al.*, 2014; Lonoce *et al.*, 2016; Luisa M. Madeira *et al.*, 2016). Once secreted, heterologous proteins could be attacked by proteases, the addition of protein stabilizers is a valid way to protract the half-life of the full protein. The most widely used are PVP or bovine gelatin in addition to protease inhibitors (J M Sharp and Doran, 2001; Lee *et al.*, 2002). Some of the strategies used to increase heterologous protein yield in hairy root cultures are detailed in the table below (Tab. 5).

Medium additive	Expression host	Expressed proteins	Yields	References
NAA 19 mg/L+ PVP 1.5 g/L + KNO ₃ 14 g/L	Tobacco HRs	mAb M12	5.9 mg/L	Suvi T. Häkkinen <i>et al.</i> , 2014
NAA 19 mg/L+ PVP 1.5 g/L + KNO ₃ 14 g/L	Tobacco HRs and <i>N.benthamiana</i> HRs	mAb H10	2.70 mg/L and 2.24 mg/L	Lonoce <i>et al.</i> , 2016
PVP 0.75 g/L or NaCl 100mM	Tobacco HRs	Thaumatococin	1.4 mg/L or 2.63 mg/L	Pham,Schäfer and Wink, 2012
PVP 1.5 g/L	Tobacco HRs	Guy's monoclonal antibody	1.4 mg/L	J.M. Sharp and Doran, 2001
KNO ₃ 0.5% + PVP 1.0 g/L	Tobacco HRs	Guy's monoclonal antibody	2.12 mg/L	Wongsamuth <i>et al.</i> , 1997

Table 5: Different approaches used to increase heterologous protein yield in hairy roots.

Several HRs have been established from numerous species of dicotyledonous plants such as *Brassica rapa*, *Solanum lycopersicum*, *N. benthamiana* and *N. tabacum* showing consistency in target gene expression over an extended period of time. Hairy roots can be established in solid and liquid cultures requiring mineral salts as nutrients, sucrose as carbon source and oxygen. A series of heterologous proteins, antibodies (Hayashi *et al.*, 2008; Häkkinen *et al.*, 2014; Lonoce *et al.*, 2016) and vaccine antigens (Skarjinskaia *et al.*, 2013)

have been expressed in HRs employing the aforementioned strategies. In particular, most of them were secreted in the culture medium easing the downstream purification processes. Among these, the highest yields were obtained in *N. tabacum* hairy roots expressing the monoclonal antibody M12 (5.9 mg/L) (Häkkinen *et al.*, 2014).

2.7 Challenges.

Despite several advantages, there are some bottlenecks limiting the efficacy of the production of heterologous proteins in transgenic plants or plant tissues. The problem of low yields intended both as a low-levels of expression or as heterologous protein instability is an issue for plant-based production systems. In addition, protein glycosylation pattern in plants is different compared with the human counterpart which could result immunogenic to humans. Lastly, extraction and purification processes can be costly and complex. Up to 80% of the costs for producing proteins in any system comes from the downstream processes. In the following paragraphs, all these drawbacks will be described and a description of the different approaches used to tackle them will be detailed.

2.7.1 Maximizing heterologous protein yields.

Low protein yields represent a major bottleneck for the production of heterologous proteins in plants. In order to obtain higher yields and overcome this issue, several approaches have been used. In detail, strategies are focused on molecular approaches aimed to improve transcription, translation, codon usage and to minimize protein degradation.

Regulation of transcription.

Transcription is regulated by specific signals placed contiguously (cis-acting) or not (trans-acting) to the gene-coding-sequence. Promoters belong to cis-acting regulators and the use of a strong promoter is a valid approach to improve heterologous protein transcription increasing the yield. A widespread method,

among dicotyledons as tobacco, is using constitutive promoters such as the cauliflower mosaic virus 35S (CaMV 35S) (Hiatt *et al.*, 1989; Ko *et al.*, 2005). Briefly, CaMV 35S is a strong promoter that guarantees high level of heterologous protein and ubiquitous expression in all stages of plant development. If, on one side, constitutive promoters enhance the yields of heterologous proteins, on the other side there is a high accumulation of this heterologous proteins in all plant tissues that could affect the growth and development of the plant. As an alternative approach, inducible promoters, which are activated by chemical or physical stimuli, are used for better monitoring the expression of heterologous proteins (Stöger *et al.*, 2000). Tissue-specific promoters are an additional valid alternative for controlling gene expression in a tissue-dependent mode or according to the plant developmental phase. Some example are specific promoters acting in the seeds, tubers, and roots (Masumura *et al.*, 2006; He *et al.*, 2008). In addition to cis-acting elements, an alternative approach is improving trans-acting elements such as transcription factors or by adding enhancer sequences upstream or downstream from the promoter that can also confer tissue specific or stage specific gene expression for further enhancing the heterologous protein yield (Obembe *et al.*, 2011).

Improve translation efficiency.

Another strategy is to stabilize the mRNA improving translation efficiency (Xu and Zhang, 2014). Eliminating cryptic introns and instability sequences is a way to improve the ribosome translation of the mRNA: in fact there are several internal sequences in the coding genes that are prone to form hairpin or secondary structures at the DNA level or double-stranded RNA that could affect mRNA amount (Kohli *et al.*, 2003). Furthermore, the use of stabilizing 5' and 3' untranslated regions (UTR) is a valid approach to increase heterologous protein expression. It has been demonstrated that the 5' and 3' UTR regions have a relevant role in translation initiation, stability and efficiency (Gutiérrez *et al.*, 1999). Replacing the structural 5' UTR with a plant virus UTR leads to an increase of the translation efficiency. The 5' -untranslated sequences of the

tobacco mosaic virus (TMV) are the most used sequences to improve proteins expression. Similar result were also obtained using the alfalfa mosaic virus (AMV) that was found to strongly enhance transgene expression (Datla *et al.*, 1993). The 3'UTR is the mRNA region immediately following the translation termination codon. Heterologous 3'UTR regions from plants or plant viruses have been used to stabilize recombinant transcripts. Examples are the *A. tumefaciens* nopaline synthase NOS terminator sequence and the Cauliflower mosaic virus 35S terminator.

Optimization of codon usage.

Codon optimization has tended to involve altering rare codons with synonymous codons used at a higher frequency, and has been implemented by many research groups using genes from various species in heterologous expression systems (Webster *et al.*, 2017). All the organisms, including plants, exhibits biased codon usage, and a winning strategy is adapting the coding sequence of the heterologous gene to that of the host plant in order to optimize translation efficiency (Obembe *et al.*, 2011). An example is the optimization of the gene sequence of the wheat cytochrome P450 to improve the expression of this heterologous protein in tobacco. Expression of the wild-type genes in tobacco led to very low protein expression levels due to the high level of GC content in wheat coding sequence, which is very different to tobacco. Adjusting the 50-terminus of the P450 gene sequence to resemble the codon usage of tobacco was sufficient to increase the levels of protein expression reaching a yield five times higher (Webster *et al.*, 2017). Another work concerns the optimization of bone morphogenetic protein 2 (BMP2) codon. BMP2 is important in the development of organs and tissues and plays a vital role in bone repair. The optimized codon of the human BMP2 gene and the native gene was fused with GUS reporter gene and expressed under the influence of the 35S promoter from Cauliflower Mosaic Virus. The optimized variant increased GUS activity by over two-fold compared to the native gene (Suo *et al.*, 2006). Optimizing a codon requires the use of a huge number of software packages that should consider several parameter as codon adaptation index (CAI), GC content,

Relative Synonymous Codon Usage (RSCU), protein structures, orthologs, mRNA free energy, restriction site elimination, elimination of repetitions, and deleterious sites (Webster *et al.*, 2017). Despite efforts, codon optimization alone is not sufficient to optimize synthetic gene design for protein expression. Other gene sequence factors need to be considered such as, mRNA secondary structure, cryptic splice sites, polyadenylation signals, and other regulatory elements may have to be avoided as they can cause undesirable processing of the mRNA (Webster *et al.*, 2017).

Protein degradation.

Protease activity in plant biofactories may lead to partial or complete hydrolysis of recombinant protein in leaf cells or in the leaf apoplast, dramatically affecting the yield of several recombinant proteins in plant systems (Jutras *et al.*, 2016). Targeting the heterologous protein to subcellular compartments such as vacuole or endoplasmic reticulum (ER) is an approach to prevent post-translational degradation (Desai *et al.*, 2010). ER resident proteins have a C-terminal sequence, usually KDEL or HDEL, which binds to receptors in the Golgi complex retrieving them back into the ER (Gomord *et al.*, 1997). The ER has very low hydrolytic activity, it is very plastic and it provides a neutral environment suitable for most proteins, thus it facilitates accumulation of high amounts of recombinant proteins which usually is 2 to 10-fold higher than the cytosol (Stöger *et al.*, 2000). Furthermore, high accumulation of recombinant proteins can be achieved into the cell wall and the intercellular space (apoplast). Some important recombinant secretory proteins, such as antibodies, can be secreted from plant cells and accumulated in the apoplast (Ma *et al.*, 1995). Co-expression of the heterologous proteins with a protease-inhibitor is another way to enhance the yield of the recombinant product (Jutras *et al.*, 2016). For example, the degradation of the recombinant full-length 2F5 antibody expressed in *N. tabacum* suspension cells was significantly reduced with the simultaneous expression of antisense RNAs against endogenous proteases, resulting in four-fold higher accumulation of the intact antibody heavy chain when compared to transformed wild type cells expressing the same

antibody. N-terminal sequencing data revealed that the antibody has two cleavage sites within the CDR-H3 and one site at the end of the H4-framework region that are found to be vulnerable to serine proteases (Mandal *et al.*, 2014).

Strategies	Approaches
Enhance transcription	➤ Improve cis-acting regulators ➤ Improve trans-acting regulators
Improve translation efficiency	➤ Design preferred genetic codon ➤ Optimize 5' and 3'-untranslated region
Minimize post-translation degradation	➤ Target nascent protein to subcellular compartments such as endoplasmic reticulum or apoplast ➤ Co-express with protease inhibitor

Table 6: Strategies and approaches used to increase heterologous protein yields in plant.

2.7.2 Downstream purification processing.

Until twenty years ago, in order to increase heterologous protein yields in plant, all approaches were based on increasing protein accumulation in tissues without considering the huge loss during downstream purification processes. Currently the focus has shifted on increasing the efficiency of downstream processing in order to obtain higher amount of protein and abate production costs (Gottschalk, 2008). It has been estimated that downstream processing cost corresponds to about 80% of total manufacturing costs. The major factors concurring in downstream steps efficiency are recombinant protein concentration, the purification method used and plant extract composition. The latter generally contains pigments, alkaloids, phenolics, polysaccharides, and proteases that could interact with the heterologous protein affecting final quality and efficiency of the purification process. For this reason, extraction needs to be optimized evaluating different parameters such as ultrafiltration, tissue homogenization, buffer composition, subcellular protein localization and chromatographic purification steps. The control of these conditions can improve the purification processes by reducing the impact of contamination of plant

compounds. A strategy was the combination of ultrafiltration and chromatography and it was employed to purify a recombinant human monoclonal antibody (anti-*Pseudomonas aeruginosa* serotype O6ad immunoglobulin G1) with a high degree of removal of impurities and the complete recovery of the antibody. The authors included a two-stage cascade ultrafiltration process followed by a two-step chromatographic procedure (Mayani *et al.*, 2013). The HIV-neutralizing human monoclonal antibody 2G12 expressed in transgenic tobacco was processed using an innovative approach made up of two different phases: a primary processing, essentially filtration and clarification and a second purification phase in which samples were loaded onto a protein A chromatography and the pooled eluates were subjected to a hydroxyapatite column to remove antibody aggregates and leached protein-A (Ma *et al.*, 2015). An innovative strategy was also proposed by Buyel *et al.*, (2015) accelerating and simplifying process development, including centrifugation, filtration, flocculation, and integrated methods that combine solid-liquid separation, purification and concentration, such as aqueous two-phase separation systems (Buyel *et al.*, 2015). An additional 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 and avoid tricky and expensive chromatographic steps. This method was also used for the expression of spider silk protein in the ER of tobacco and potato (Scheller *et al.*, 2004; Floss *et al.*, 2009). A possible approach to facilitate downstream processing and purification is the possibility of targeting the protein of interest to the secretory pathway and recovering it from the culture medium.

An example of this approach is the secretion from roots and root cultures. Rhizosecretion is the ability of roots to accumulate target proteins in the hydroponic medium providing an alternative manufacturing platform that simplifies the downstream purification procedure and increases protein yield (Gaume *et al.*, 2003). Plants naturally allow the secretion of molecules from the roots to the environment 'rhizosecretion'. Several of these molecules are 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). There are several examples in literature of transgenic *N. tabacum* plants that secrete from roots different recombinant proteins such as green fluorescent protein, bacterial xylanase, human placental alkaline phosphatase and the cyanobacteria anti HIV-1 Microcystin protein have been investigated (Drake *et al.*, 2003; Drake *et al.*, 2009). All the secreted proteins showed full biological functionality and higher accumulation in the medium than in the root tissues. The advantages given by this strategy are due to the possibility to produce heterologous proteins in a controlled environment with lower concentrations of plant contaminants (e.g. polyphenols, alkaloids) favouring the purification of a high quality product. Unfortunately, despite these advantages, heterologous protein yields obtained are generally low. Various strategies have been used to increase the production yields, 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) or to molecules that are able to stabilize the recombinant protein such as PVP or gelatin (Madeira *et al.*, 2016). As already presented before, secretion from roots has been successfully obtained also in contained hairy roots cultures.

2.7.3 Glycosylation of heterologous proteins in plants.

Stability, fully biological activity and solubility of a protein are influenced by its N-glycosylation profile (Bosch *et al.*, 2013). Different glycosylation patterns could for example compromise the capability of a protein to interact with components responsible of the stimulation of the human immune system. Concerning

antibodies, glycosylation is crucial for many fundamental biological processes, including complement activation and Fc γ receptor binding ability (Gomord *et al.*, 2005). The addition of the N-glycan chains contributes to the folding process of the protein and the following sugar modifications occurring in the ER have a signalling 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). From the ER, the nascent protein is transferred to the Golgi apparatus to continue its maturation. The major glycosylation differences between plants and mammals occurring in the Golgi apparatus are added by the enzymes β 1,2-xylosyltransferase and α 1,3-fucosyltransferase, absent in mammalian cells. Moreover, plant glycosylation lacks β 1,4-galactose, sialic acid complex-type glycans and the core α 1, 6-fucose (Gomord *et al.*, 2005). In plant proteins β 1,2-xylose is linked to a core mannose residue, and α 1,3-fucose is linked to the proximal N-acetylglucosamine involving further branching of the bi-antennary N- glycans (Fig. 6). Heterologous protein produced with these plant-typical residues could induce immunogenicity effect in humans (Gomord *et al.*, 2005). To overcome this issue, several strategies have been exploited such as the use of the retention C-terminal signals (HDEL or KDEL) forcing the protein retention in the ER. However, in some cases, ER retention in plant cells seems not to be complete since KDEL tagged antibodies could escape from the ER retrieval system during intracellular trafficking or to undergo a partial proteolytic cleavage of the KDEL tag (Floss *et al.*, 2008). As an alternative, α -glycosylated antibodies were used through mutation of the HC specific N- glycosylation site (Lombardi *et al.*, 2010), but the main drawback was their impaired binding to Fc receptors. The co-expression in plants of antibodies together with mammalian glycosyl-transferases was a valid alternative (Vezina *et al.*, 2009) that allowed to obtain heterologous proteins with a glycosylation pattern similar to that obtained in mammalian cells. A promising approach, was the expression of antibodies in plants in which genes coding for specific glycosyl-transferases were silenced

(Strasser *et al.*, 2008). In these *N. benthamiana* plants glycosyl-transferases (*FucT* and *XylT*) responsible for the addition of β 1,2-xylose or α 1,3-fucose were down-regulated using RNAi. The human anti HIV monoclonal antibody 2G12 produced in these glycol-engineered line showed a ‘human-compatible’ sugar profile lacking plant-specific sugars (Strasser *et al.*, 2008). Recently, inactivation of the β (1,2)-xylosyltransferase and the α (1,3)-fucosyltransferase genes in *N. tabacum* BY-2 cells was performed with the innovative CRISPR/Cas9 strategy, resulting in glycoproteins obtained without plant-specific glycans (Hanania *et al.*, 2017; Mercx *et al.*, 2017). Furthermore, plants do not have the machinery to synthesize the sugar nucleotide precursor CMP-sialic acid (CMP-N-acetylneuraminic acid) necessary for sialylation and innovative studies demonstrated how even entire glycosylation-associated biosynthetic pathways can be introduced (Loos and Steinkellner, 2014). The simultaneous overexpression of six mammalian genes enabled the *in planta* generation of activated sialic acid, the production of terminally galactosylated glycans and the transfer of sialic acid to these terminal galactoses (Castilho *et al.*, 2010).

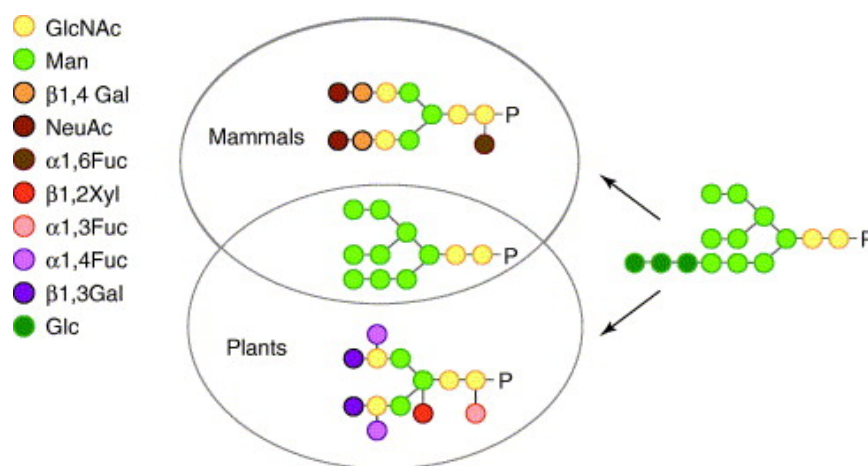


Figure 6: Different glycosylation pattern among plants and mammals. Image from (Gomord *et al.*, 2005).

2.8 Plant-made antibodies.

There are numerous reports about expression of antibody molecules in plants (Ahmad *et al.*, 2010) especially in *N. benthamiana*, *N. tabacum*, *A. thaliana*, *Daucus carota*, and *Oryza sativa* (Xu and Zhang, 2014). 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, single-chain antibodies and camelid heavy-chain antibodies (Fig. 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, for phytoremediation (Longstaff *et al.*, 1998; Drake *et al.*, 2002) and for the production of valuable immunotherapeutic agents (Ko, 2014). There are several advantages in producing antibodies in plants such as the possibility to direct the protein expression in specific cell compartments (Ma *et al.*, 2003). In addition antibodies expression and accumulation has been directed also to specific cell storage compartments such as seeds, that proved to be versatile targets for recombinant antibodies (Boothe *et al.*, 2010). Several functional recombinant antibody fragments were also expressed such as the antigen binding fragment (Fab), fragments consisting of only the variable regions of the L and H chains (Fv), that retain the antigen binding capacity, single-chain variable fragments (ScFv) consisting of the VL and VH domains linked by a flexible peptide and single-domain antibodies (VHH) from camelids (Capodicasa *et al.*, 2011).

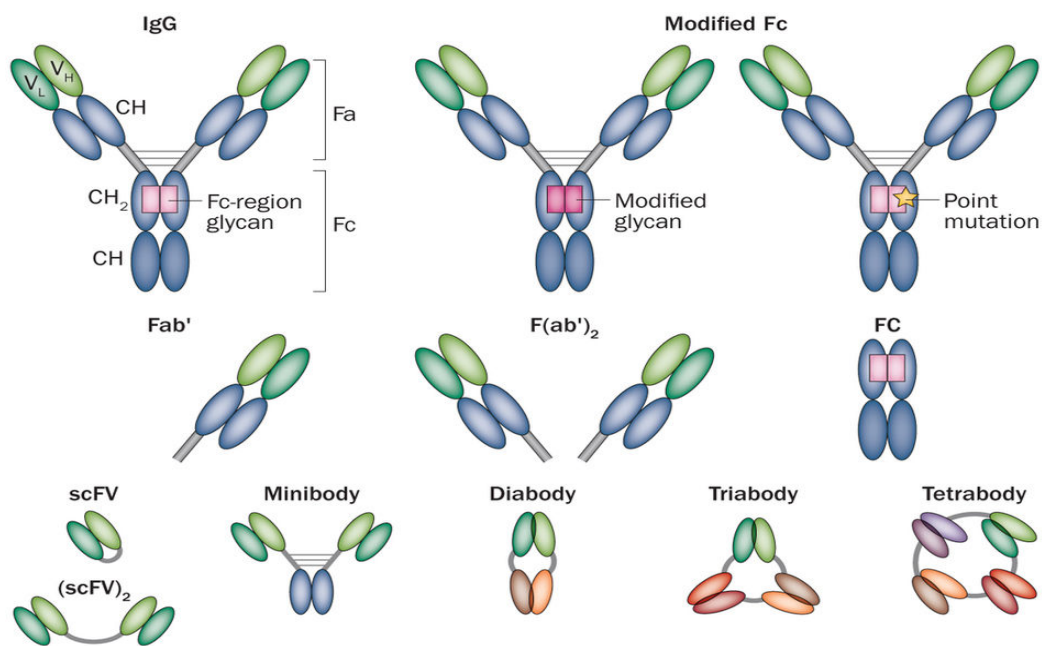


Figure 7: Several immunoglobulins have been expressed successfully in plants, including several classes of IgG , IgA and a chimeric IgA/G. A variation on the classical IgG tetrameric structure is the camelid heavy-chain antibody, which lacks a light-chain component and can be expressed as a single transgene. 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).

2.8.1 Tumour- targeting antibodies.

Monoclonal antibodies targeting different types of cancer have been efficiently expressed in plant systems. Carcinoembryonic antigen-specific diabody was one of the first plant-produced product obtained in tobacco (Vaquero *et al.*, 2002). The recombinant humanized antibody TheraCIM, targeting the Epidermal Growth Factor receptor (EGF-R), was transiently-expressed in an aglycosylated form in tobacco leaves by vacuum agroinfiltration, resulting active as its counterpart produced in mammalian cells. (Rodríguez *et al.*, 2005).

Another humanized monoclonal antibody that 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* (Komarova *et al.*, 2011). Furthermore, intraperitoneal administration of the antibody in mice, showed to efficiently retard the growth of xenografted Her2+ human ovarian tumors (Komarova *et al.*, 2011). Innovatively, plant-produced antibodies were developed for non-Hodgkin's lymphomas treatments essentially by using individualized idiotype vaccines created from patient's own cancer cells (Bendandi *et al.*, 2010). 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. The rapid and high level expression offered by plants, granted the possibility 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 7.

Target	Host plant	Expression method	Yields	Antibody format
Her2/neu (Trastuzumab)	<i>N. benthamiana</i>	TMV and PVX vectors	200/300 mg/Kg	Humanized murine IgG1
Her2/neu (Trastuzumab)	<i>N. benthamiana</i>	MagnICON viral-based	45 mg/Kg	Humanized murine IgG1
Human tumor specific mAb A5	<i>N. benthamiana</i>	TMV and PVX vectors	500 mg/Kg	Human IgG1
EGF-R (TheraCIM)	Tobacco	Vacuum agroinfiltration	Not reported	Humanized antibody
Tenascin-C (H10)	<i>N. benthamiana</i>	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	TMV vector	5-800 µg/ml	scFv
	<i>N. benthamiana</i>	Magninfection	0.6-4.8 g/Kg	IgG

Table 7: Examples of plant derived anti-cancer immunoglobulins (Adapted from Capodicasa *et al.*, 2011).

2.8.2 The tumour-targeting anti tenascin-C mAb H10.

The human recombinant mAb H10 antibody, isolated from a phage display library, is specific for the large isoform of tenascin-C (TNC-C) (Villani *et al.*, 2009). TNC, a glycoprotein of the extracellular matrix, comprises several alternatively spliced 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 tumour-associated antigen target for both therapy and diagnosis (Rizzieri *et al.*, 2004). The fully human mAb H10 was successfully produced in *N. tabacum* transgenic plants but also in *N. benthamiana* plants using leaf agroinfiltration strategy. Moreover, the use of the p19 silencing suppressor gene from artichoke mottled crinkle virus raised antibody expression levels to 75 mg/kg fresh weight (Villani *et al.*, 2009; Lombardi *et al.*, 2010). The mAb H10 was also expressed in a *N. benthamiana* RNAi down-regulated line in which β 1,2-xylosyltransferase and α 1,3-fucosyltransferase gene expression was silenced. The glyco-humanized antibody showed an high susceptibility to proteolytic cleavages, suggesting a role of the sugar moiety in protein stability (Lombardi *et al.*, 2012; Donini *et al.*, 2015). A characterisation of the antibody degradation in plants was also performed, this led to the identification of possible 'fragile' sites in the H10 individuating three proteolytic enzymes belonging to the cystein proteases family (Hehle *et al.*, 2015). In a recent work, the mAb H10 was agroinfiltrated in leaves of *N. benthamiana* also expressing

the human serine protease inhibitor α 1-antichymotrypsin or the cysteine protease inhibitor tomato cystatin SICYS8. SICYS8 had positive effects on the amount of fully-assembled antibody purified from leaf tissue confirming the potential of Cys protease inhibitors as convenient antibody-stabilizing expression partners to increase the quality of therapeutic antibodies in plant protein biofactories (Jutras *et al.*, 2016). Therefore, mAb H10 produced in plants may represent a valuable molecule in cancer treatment.

2.8.3 Anti-CD20 antibodies.

Non-Hodgkin lymphomas (NHLs) represent a serious threat, and some 70 thousands new cases have been diagnosed in USA in 2014. The majority of NHLs express the leucocyte antigen CD20, an integral transmembrane glycoprotein of 33–37 kDa, that represents a preferential target for immunotherapy (Marusic *et al.*, 2016). Among monoclonal recombinant antibodies employed in clinical studies, Rituximab (2B8) is an example of mouse/human chimeric antibody recognizing the CD20 expressed from B lymphocytes. Rituximab, approved in 1997 for B cells lymphoma treatment, contains Complementary Determining Regions (CDR) of the anti-CD20 murine antibody 2B8 fused to the constant regions of a human IgG1. After binding to the antigen, the anti-CD20 2B8 is capable to activate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which are mediated by the Fc γ receptors on effector cells, such as granulocytes, macrophages, and natural killer (NK) cells. Moreover, some antibodies can directly kill the target cell by triggering the apoptotic pathway. With the aim of producing an anticancer antibody in plants, recently the 2B8 was expressed in plants of *N. benthamiana* as recombinant antibody (single chain ScFv-Fc). Purification yields using protein-A affinity chromatography were in the range of 15–20 mg/kg of fresh leaf weight (FW). Glycopeptide analysis confirmed the presence of a highly homogeneous plant-type glycosylation and a flow cytometry on Daudi cells revealed that the purified 2B8-Fc showed a binding activity to the CD20 comparable to that of Rituximab and was effective in eliciting antibody-dependent cell-mediated cytotoxicity of human PBMC

versus Daudi cells, demonstrating its functional integrity. This was the first example of a recombinant molecule based on the therapeutic Rituximab antibody scaffold, whose expression in plants may be a valuable tool for NHLs treatment (Marusic *et al.*, 2016).

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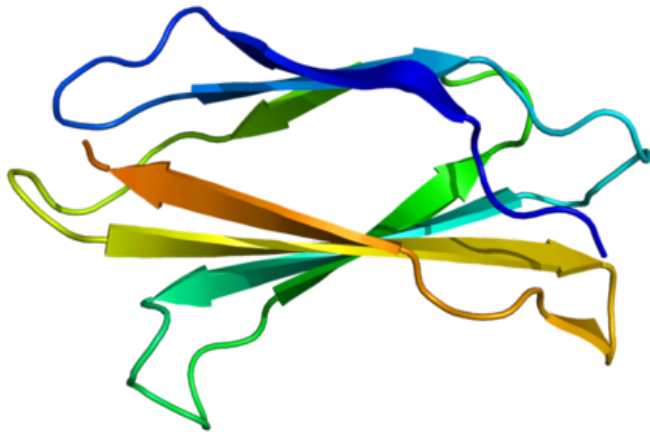
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Chapter 3. Aim of the project.

The present PhD thesis explored the potentiality of the production of tumour-targeting recombinant antibodies in hairy root cultures with a particular focus on the optimization of the secretion of the recombinant molecules in the culture medium, in order to facilitate the downstream purification processes, and on the modification of the protein glycosylation profile obtaining human-compatible immunotherapeutic reagents. To this aim, the first objective was generating *Nicotiana tabacum* and Δ XTFT *Nicotiana benthamiana* glyco-modified hairy roots expressing two tumour-targeting antibodies, the mAb H10 against Tenascin-C expressed in several cancer diseases (e.g. breast cancer, lung cancer, glioblastoma) and the recombinant 2B8-Fc antibody against the CD20 antigen found on the surface of non-Hodgkin lymphoma cancer cells. The use of *Agrobacterium rhizogenes* multiple gene co-transformation strategy allowed us to rapidly obtain hairy root clones expressing tumour-targeting antibodies that were successfully secreted in the culture medium and purified using a single step affinity chromatography purification protocol. Moreover, antibodies produced in Δ XTFT *N. benthamiana* hairy root lines showed a human compatible glycosylation profile. To optimize heterologous protein secretion in HRs medium, hairy roots lines expressing the red fluorescent protein were obtained and used as a model system to evaluate different auxins. Obtained results permitted us to significantly increase the yield of secreted 2B8-Fc antibody making HRs a competitive system for heterologous proteins production.

Chapter 4. Hairy roots: a production platform for anti-cancer antibodies.



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4.1 Abstract.

Hairy root cultures (HRs) derived from *A. rhizogenes* transformation of plant tissues are an advantageous biotechnological manufacturing platform due to the accumulation of recombinant proteins in an otherwise largely protein free culture medium. In this context, HRs descending from transgenic *N. tabacum* plants were successfully used for the production of several functional mAbs with plant-type glycans. Here, we expressed the tumor-targeting monoclonal antibody mAb H10 in HRs obtained either by infecting a transgenic *N. tabacum* line expressing H10 with *A. rhizogenes* or a glyco-engineered *N. benthamiana* line (Δ XTFT) with recombinant *A. rhizogenes* carrying mAb H10 heavy and light chain cDNAs. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields (2–3 mg/L). *N*-glycosylation profiles of antibodies purified from HR supernatant revealed the presence of plant-typical complex structures for *N. tabacum*-derived mAb H10 and of GnGn structures lacking xylose and fucose for the Δ XTFT-derived counterpart. Both antibody glyco-formats exhibited comparable antigen binding activities. All together, these data demonstrate that the co-infection of Δ XTFT *N. benthamiana* with recombinant *A. rhizogenes* is an efficient procedure for the generation of stable HRs expressing the tumor-targeting mAb H10 with a human-compatible glycosylation profile, thus representing an important step towards the exploitation of root cultures for the production of ‘next generation’ human therapeutic antibodies.

4.2 Introduction.

4.2.1 Production of antibodies in hairy roots.

Expression of therapeutic proteins in *in vitro* plant systems under contained conditions represents an advantageous manufacturing strategy in terms of uniform cultivation conditions, product quality and downstream purification process (Schillberg *et al.*, 2013). HRs represent a valid alternative thanks to their time efficiency in establishing transgenic HR lines, intrinsic genetic stability, fast biomass accumulation and manufacturing scalability (Talano *et al.*, 2012). HRs are normally generated by infecting transgenic plants expressing the recombinant protein with a wild-type *A. rhizogenes* strain or by transforming plant tissues with *A. rhizogenes* strains bearing a plant expression vector encoding the protein of interest (Huang *et al.*, 2013). Both approaches have been investigated and data showed that obtaining HRs by infecting leaf discs with *A. rhizogenes* strain, overcoming the need of generating a transgenic plant, is the easiest and fastest approach. Forty-days-old infected leaf discs lead to the formation of extensive secondary roots that can be cultivated under contained sterile conditions in hormone free media. Notably, recombinant proteins can be secreted in the culture medium facilitating the downstream purification processes (Guillon *et al.*, 2006). Roots have been successfully used to express a wide range of complex recombinant proteins, such as enzymes (Woods *et al.*, 2008), vaccine components (Skarjinskaia *et al.*, 2013), monoclonal antibodies (mAbs) (Wongsamuth and Doran, 1997) and anti-HIV microbicides (Drake *et al.*, 2013). Antibodies produced in *N. tabacum* HRs demonstrated that accumulation in the culture medium can be improved by the addition of hormones as well as protein stabilizing agents such as polyvinylpyrrolidone (PVP) (Drake *et al.*, 2013; Skarjinskaia *et al.*, 2013). Recently, an interesting study showed that components such as 1-naphthaleneacetic acid (NAA) can rise yields of the tumor targeting human IgG1 M12 produced in *N. tabacum* HRs by even 30-fold (~6 mg/L of culture medium) (Häkkinen *et al.*, 2014). Despite recent improvements, antibody yields obtained in HRs do not meet those reported for other plant expression systems, such as transgenic plants and *Agrobacterium*-based transient expression systems. In

fact, by agroinfiltration of *N. benthamiana* leaves using plant virus derived expression vectors, up to 500 mg/kg FW of the human tumor-specific mAb A5 were obtained (Giritch *et al.*, 2006). An important feature for many therapeutically interesting mAbs is proper Fc-glycosylation. It is now well established that certain glycan structures may alter the function of antibodies (Jefferis *et al.*, 2012). A prominent example is the lack of core fucose, which confers increased in vitro and in vivo activities (Giritch *et al.*, 2006; Villani *et al.*, 2009). Recently it was shown that a mAb produced in wild type *N. tabacum* HRs carries, as many other plant-produced glycoproteins, complex structures bearing plant typical β 1,2-xylose and α 1,3 fucose (Häkkinen *et al.*, 2014). A series of studies demonstrated that plants are particularly amenable to glycan engineering towards human-type structures as reviewed recently (Strasser *et al.*, 2008). A major achievement was the elimination of plant-typical fucose and xylose residues using RNA interference, as in the case of duckweed (*Lemna minor*) and Δ XTFT *N. benthamiana* transgenic line (Catania *et al.*, 2015; Schliemann *et al.*, 2015). However, whether mAbs with modulated N-glycan profiles can be produced in HRs was not investigated so far. In this study we have monitored different approaches for the generation of HRs producing the human tumour targeting mAb H10. This human IgG1 derived from a selected single-chain variable fragment (scFv) is directed against the C domain of the large isoform of the tumor-associated antigen tenascin C (TNC). TNC has been associated with a variety of tumors including breast, squamous cells, lung and prostate carcinomas, melanoma and malignant glioma (Giritch *et al.*, 2006; Villani *et al.*, 2009). Anti-TNC antibodies have been recently used with success for the therapy of patients with acute myeloid leukemia and metastatic breast cancer (Midwood *et al.*, 2011; Schliemann *et al.*, 2015). To express mAb H10, in HRs we used two plant binary expression vectors bearing heavy chain (HC) and light chain (LC) coding sequences. A signal peptide sequence deriving from an embryonic mouse immunoglobulin HC coding gene was added to direct the recombinant antibody to the secretory pathway (Villani *et al.*, 2009). Hairy roots were either obtained by infecting with wild-type *A. rhizogenes* transgenic tobacco cv. Petite Havana SR1 plants expressing mAb H10 or by the co-

infection of a glyco-engineered Δ XTFT *N. benthamiana* line with transformed *A. rhizogenes* strains bearing the plant expression vectors encoding antibody HC and LC sequences. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields. Purified antibodies were characterized for their glycosylation profile and functional activity *in vitro*. The goal of the present work was to express the tumor-targeting mAb H10 in glyco-engineered Δ XTFT *N. benthamiana* HRs with the aim of improving the quality of the final product.

4.3 Materials & Methods.

4.3.1 Generation of HRs expressing mAb H10.

N. tabacum cv. Petite Havana SR1 HRs were prepared from a T4 generation transgenic plant line (line 7.6) expressing mAb H10 HC and LC which was previously obtained by Villani and colleagues (Villani *et al.*, 2009). Briefly, wild-type *A. rhizogenes* A4 was grown in LB medium containing 50 mg/L rifampicin to OD600 = 0.5, at 28°C and 160 rpm. Bacteria were pelleted by centrifugation at 3000 ×g for 10 min and resuspended at OD600 = 1 in MS medium with 30 g/L sucrose and 100 µM acetosyringone, pH 5.8. Leaves were cut in segments (size: 1 cm × 1 cm), wounded with a sterile needle (10 pricks per 1 cm²) and inoculated by immersion in the *A. rhizogenes* suspension for 30 min, swirling in the dark. Leaves were dried and transferred to culture plates containing MS agar medium without phytohormones in the dark, for two days. Leaves were rinsed with sterile water and transferred to fresh MS agar medium containing 30 g/L sucrose and 0.5 g/L cefotaxime. Leaves were transferred onto fresh plates every 15 days. Roots emerging from leaves (typically after 40 days) were excised and transferred to new plates. *A. rhizogenes* was eradicated by transferring roots every 15 days onto MS agar plates using decreasing cefotaxime concentrations (0.25, 0.125, 0.05 g/L) until no antibiotic was added. *A. rhizogenes* eradication was confirmed by PCR using virC specific primers. Glyco-engineered ΔXTFT *N. benthamiana* plants (Strasser *et al.*, 2008) were used to obtain HRs expressing mAb H10. Leaf segments were infected with a co-culture of *A. rhizogenes* A4 strains transformed with the plant expression vectors p35S-HC or p35S-LC. Each strain was resuspended in inoculation medium to a final OD600 of 1. Next steps were carried out using the same protocol described for *N. tabacum* HRs generation with the exception that both solid and liquid MS culture media were supplemented with kanamycin (100 mg/L).

4.3.2 Selection of HRs expressing mAb H10 by Western blot analysis.

Root tissues (100 mg) were ground in liquid N₂ and homogenized in 100 µL of

phosphate-buffered saline pH 7.2 (PBS) containing a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany). After centrifugation at 20 000 ×g, 4°C for 30 min, the supernatant was recovered and analyzed by Western blotting. Samples were separated on 12 or 4–15% (Mini-PROTEAN® TGXTM Precast Gels, BIORAD, USA) SDS-PAGE and proteins were electrotransferred to a PVDF membrane (Millipore, Bedford, MA) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare, Freiburg, Germany) for 40 min at 0.4 mA/cm² and 30 V. The mAb H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and extracts from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative control. Membranes were incubated in PBS with 4% w/v non-fat milk, for at least 2 h. Protein detection was performed with anti-human γ chain (A8419 Sigma-Aldrich) and with anti-human λ chain secondary antibody (A5175; Sigma-Aldrich, Gillingham Dorset, UK) horseradish peroxidase conjugated diluted 1:5000 in 2% w/v non-fat milk in PBS. The membranes were washed three times with 0.1% Tween-20 in PBS, two times in PBS and developed using ECL Plus Western blotting detection system (ECL Plus; GE Healthcare). The ImageQuant™ LAS 500 (GE Healthcare) was used for chemoluminescence signal detection. Protein molecular mass markers were used in this analysis as reference (Color burst™, C1992 Sigma and Sharp Mass VI, Euroclone, Italy).

4.3.3 HRs in liquid medium and antibody secretion.

The HRs were grown in 250 ml Erlenmeyer flasks using 40 ml of MS medium supplemented with 3% sucrose. Starting inoculum weight was typically ~0.2 g of root FW and flasks, sealed with a cotton plug and aluminum foil, were orbitally shaken at 80 rpm, in the dark, at 26°C. HR growth was analyzed by measuring roots FW at different time points before and after induction. Data shown represent the average values of three independent experiments $\pm$ standard deviation (SD). Measurements were performed by gently removing medium and weighing flask and roots; fresh root net weight was then obtained by subtracting flask weight. Data were collected in triplicate at day 8, 15, 20, 25, 28, 30 and 33

from the initial inoculum. To evaluate antibody production, HRs were grown for three to four weeks, as described before, and secretion was triggered by the addition of 10 ml of MS culture medium supplemented with 14 g/L of KNO₃, 19 mg/L of NAA and 1.5 g/L of PVP (Molecular weight 40 000). Three days after the first induction, flasks were again supplemented with 19 mg/L of NAA and 1.5 g/L of PVP, and left for five days before collecting the spent culture medium for antibody purification. An aliquot of 20 µL of HR culture medium at different time points before (T0) and after induction (2, 4, 8 days; T2, T4, T8) was collected in order to analyze the amount of secreted antibody by Western blot analysis (as described before). mAb H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and culture medium from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative controls. Densitometric quantification of bands was performed by ImageQuant TL 7.0 Image analysis software (GE Healthcare). Induced (T8) and non-induced (T0) HRs from both *N. benthamiana* and *N. tabacum* were observed by stereomicroscopy 33 days after inoculum. Analysis was performed using a macro-photo microscope WILD/ Leica M420.

4.3.4 Antibody purification from HR tissue and culture medium.

Culture medium was separated from HRs by filtration using Miracloth™ (Millipore). Supernatant was centrifuged at 15 000 × g for 15 min, and filtered through 0.45 µm syringe filters (Millipore) ready for protein-A purification. Remaining roots (typically 4 to 11 g FW) were washed with PBS, ground in liquid nitrogen and homogenized using Ultra-Turrax homogenizer T25 equipped with a S25N-18G disperser (IKA, Staufen, German) in two volumes of 1x PBS. Extract was filtered using Miracloth™, centrifuged at 15000 × g for 15 min, passed through 0.45 µm syringe filters and subjected to affinity chromatography. Antibody purification was performed by affinity chromatography essentially as previously described (Lombardi *et al.*, 2010). Briefly, the clarified root extract or culture medium was loaded onto a protein-A affinity column (1 ml HiTrap™ rProtein A FF; GE Healthcare) previously

equilibrated with extraction buffer (1x PBS) at a flow rate of 1 ml/min. The column was washed with 10 ml of PBS (10 column volumes) and the antibody was eluted with 0.1 M citric acid (pH 3) and buffered with 1/5 volume of 1 M Tris-HCl, pH 8. Eluted fractions were analyzed by SDS-PAGE, followed by Coomassie staining. Reference molecular mass markers were used (Amersham, RPN5800). Antibody-containing fractions were dialyzed using slide-A-Lyzer™ Dialysis cassettes (Thermo Scientific) against 1x PBS. Antibody concentration was determined spectrophotometrically by measuring the absorbance at 280 nm (Gill and von Hippel, 1989).

4.3.5 ELISA assays.

For the quantification of mAb H10 secreted in HRs medium or in the root biomass, aliquots, taken eight days after induction (33 days after inoculum), were analyzed by quantitative ELISA. Briefly, the purified antigen, human TNC (CC065; Merck Millipore), was coated directly onto Nunc-MaxiSorp® 96 well plates at a concentration of 2 µg/ ml in PBS, and incubated at 4°C overnight. Plates were then blocked with 4% milk w/v in PBS, at 37°C, for 1 h. HR culture media were centrifuged at 15 000 × g for 15 min, at 4°C, the supernatants were recovered and serial dilutions were added to the wells (100 µL final volume) and incubated at 37°C, for 2 h. In the case of root biomass (for quantification of antibody in the roots), 0.2 g of roots were ground in liquid nitrogen in two volumes of 1x PBS, clarified by centrifugation 15 000 × g for 15 min, at 4°C, and serial dilutions were added to the wells and incubated at 37°C, for 2 h. After washing, the anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was added at 1:5000 diluted in 2% milk w/v PBS, and incubated at 37°C, for 1 h. As an internal standard for quantification, we used serial dilutions of purified mAb H10 positive control starting from a concentration of 3 µg/ml. Each HR sample was assayed in triplicate and mAb H10 concentrations were interpolated in the linear portion of the standard curve. For the functional characterization of the HR purified antibodies, the ELISA assay was performed essentially as described before. Purified antibodies in triplicate samples were added to TNC coated plates at serial dilutions starting from a concentration of 3

µg/ml. The anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was used as secondary antibody. After 30 min, enzymatic activity was measured at 405 nm (TECAN-Sunrise, Austria) using 2,2-azino-di-3-ethylbenz-thiazoline sulfonate as substrate (ABTS, KPL).

4.3.6 Glycan analysis.

The N-glycosylation profile was determined by LC-ESI-MS as previously described by Stadlmann and colleagues. In brief, purified IgG was separated by reducing SDS-PAGE, and bands corresponding to the HC and LC were excised from the Coomassie stained gel. Upon S-alkylation and tryptic or tryptic/GluC digestion, fragments were eluted from the gel with 50% acetonitrile and separated on a reversed-phase column (150 × 0.32 mm BioBasic-18, Thermo Scientific) using a gradient of 1–80% acetonitrile. Glycopeptides were analyzed with a Q-TOF Ultima Global mass spectrometer (Waters). Spectra were summed and deconvoluted for the identification of glycoforms. Glycans were annotated according to the proglycan nomenclature (www.proglycan.com).

4.4 Results.

4.4.1 Hairy roots generation in *N. tabacum* and glyco-engineered Δ XTFT *N. benthamiana*.

Hairy roots of both *N. tabacum* and Δ XTFT *N. benthamiana* expressing mAb H10 were obtained using two different strategies. As shown in the Figure 1A, as first approach a transgenic *N. tabacum* line expressing mAb H10 was infected with wild-type *A. rhizogenes* strain generating *N. tabacum* HRs expressing H10. In addition (Fig. 1B), a Δ XTFT *N. benthamiana* plant was infected with a co-culture of *A. rhizogenes* carrying the heavy and light chain coding gene sequences (Fig. 2A) obtaining Δ XTFT *N. benthamiana* HRs expressing mAb H10.

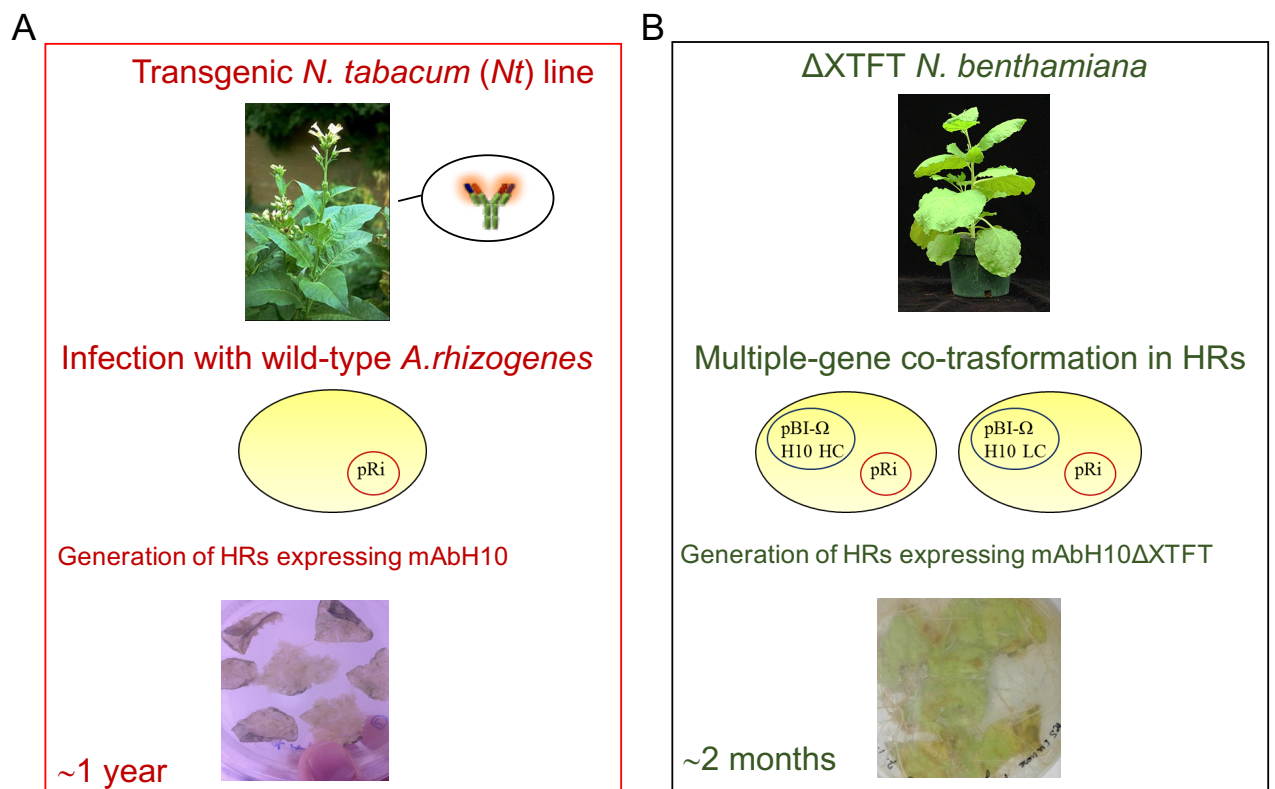


Figure 1: Schematic representation of the two different approaches used to produce hairy root expressing mAbH10.

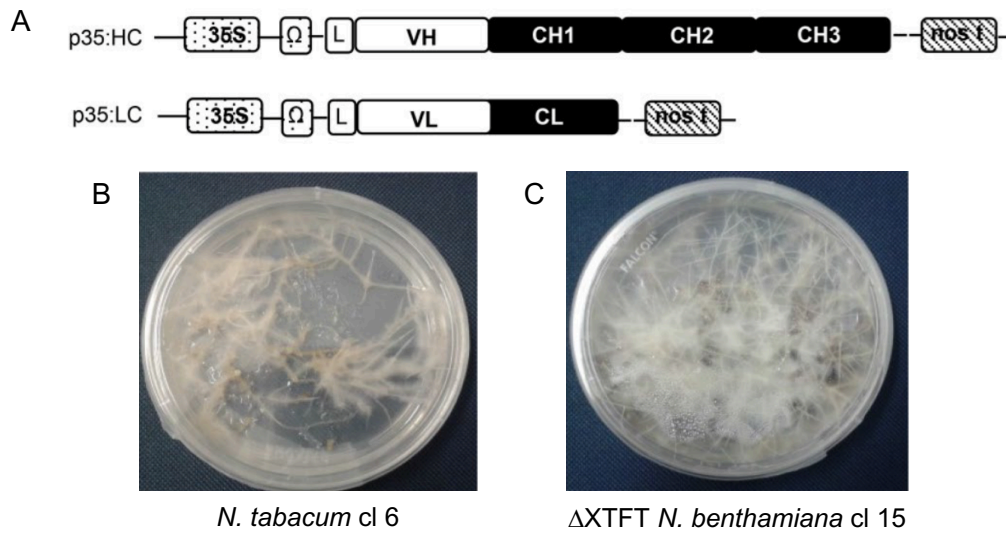


Figure 2: Schematic representation of the two constructs used for the leaf discs infection and hairy root generation. Both HC and LC coding sequences were under the control of the cauliflower mosaic virus 35S promoter (35S) and the translation enhancer sequence of tobacco mosaic virus (Ω). L: signal peptide sequence derived from an embryonic mouse immunoglobulin HC coding gene. Solid HRs expressing the mAb H10 antibody; (B) *N. tabacum* clone 6 and (C) Δ XTFT *N. benthamiana* clone 15.

4.4.2 Selection of HR clones expressing the full-size mAb H10.

All Δ XTFT *N. benthamiana* HR clones were screened for mAb expression by non-reducing Western blot analysis using an anti-HC and anti-LC antibodies. Only five clones, corresponding to 20%, showed a band at 150 kDa indicating the presence of the full size mAb H10 and the three clones 11, 15 and 16 exhibited the highest mAb H10 levels (Fig. 3). In the case of HRs derived from *N. tabacum* transgenic line, 14 clones were positive (about 60% of the clones), with clones 3, 4, and 6 showing the highest antibody levels (Fig. 4). The best selected clones were: clone 15 for Δ XTFT *N. benthamiana* (Fig. 2C) and clone 6 for *N. tabacum* (Fig. 2B). HR protein extracts were separated by gradient gel under reducing and non-reducing conditions and then subjected to Western blot analysis using both anti-HC and LC antibodies. In the anti-HC blotting under non-reducing conditions, both *N. tabacum* and Δ XTFT *N. benthamiana* clones showed a similar band pattern, with the expected band migrating at high molecular mass (~150 kDa) corresponding to the assembled mAb. Additional lower bands were also observed: an intense band (at about 100 kDa) and very

faint ones (at about 135, 75 and 50 kDa) that probably relate to antibody assembly intermediates (HC dimers/monomers) or putative degradation fragments. In the corresponding analysis under reducing conditions, a 50 kDa band associated with intact HC was visible as well as a faint band at about 25 kDa indicating specific degradation of the heavy chain (Fig. 5). The anti-LC blot under non-reducing conditions (Fig. 5) showed a 150 kDa band in both clones, together with additional lower faint bands at about 135, 100 and 75 kDa, which were already observed in the anti-HC blot. A very strong band at about 45 kDa was visible, which also occurred in the control (mAbH10 purified from agroinfiltrated *N. benthamiana* leaves). This may correspond to the homodimeric form of free LC. Under reducing conditions, the 25 kDa band corresponding to the LC was detected in both clones. It must be noted that both anti-HC and LC polyclonal antibodies seem to better recognize the antigens in their monomeric form, when they are separated under reducing conditions. This was already evidenced in previous works (Stadlmann *et al.*, 2008; Villani *et al.*, 2009). The eradication of *A. rhizogenes* from HRs, a genomic analysis of DNA extracted from clone 15 for Δ XTFT *N. benthamiana* and clone 6 for *N. tabacum* HRs was assessed by PCR using *A. rhizogenes* specific vir C oligonucleotides (Fig. 6). An expected amplification product of 425 bp was observed only in the C+ positive control (*A. rhizogenes* colony). No amplification product was observed on both HR clones indicating the complete eradication of *A. rhizogenes* by cefotaxime treatment. As internal control of DNA amplification EF primers that amplify the plant elongation factor gene were used. The amplification of 660 bp fragments was observed in the two HR clones as well as in the positive control (C+: DNA extracted from wild-type *N. benthamiana* leaves). As negative controls no DNA was added in the PCR reaction using virC or EF primers.

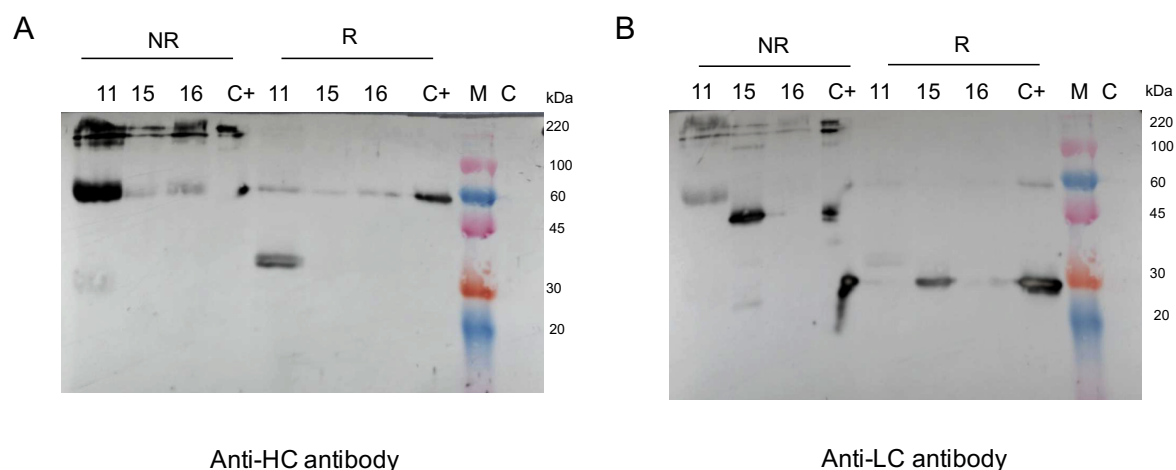


Figure 3: Western blot analysis on the best expressing Δ XTFT *N. benthamiana* hairy root clones using both anti-heavy and anti-light chain antibodies. Twenty μ l of root extract was loaded on each well. C+: mAbH10 purified from Agroinfiltrated *N. benthamiana* leaves (100ng) was used as positive control. C-: extract from HRs expressing an unrelated protein (antimicrobial peptide).

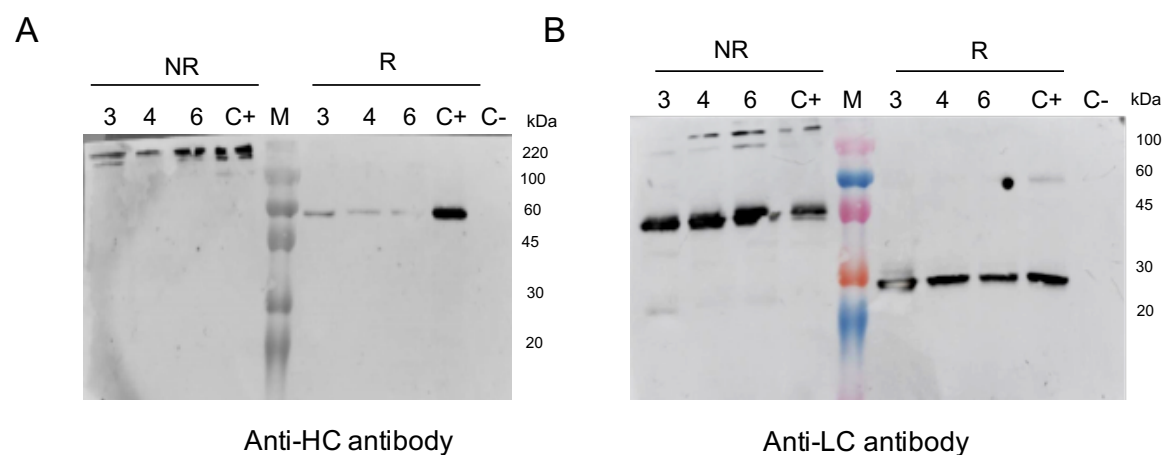


Figure 4: Western blot analysis on the best expressing *N. tabacum* HR clones using both anti-heavy and light chain antibodies. Twenty μ l of root extract was loaded on each well. C+: mAbH10 purified from Agroinfiltrated *N. benthamiana* leaves (100ng) was used as positive control. C-: extract from HRs expressing an unrelated protein (antimicrobial peptide).

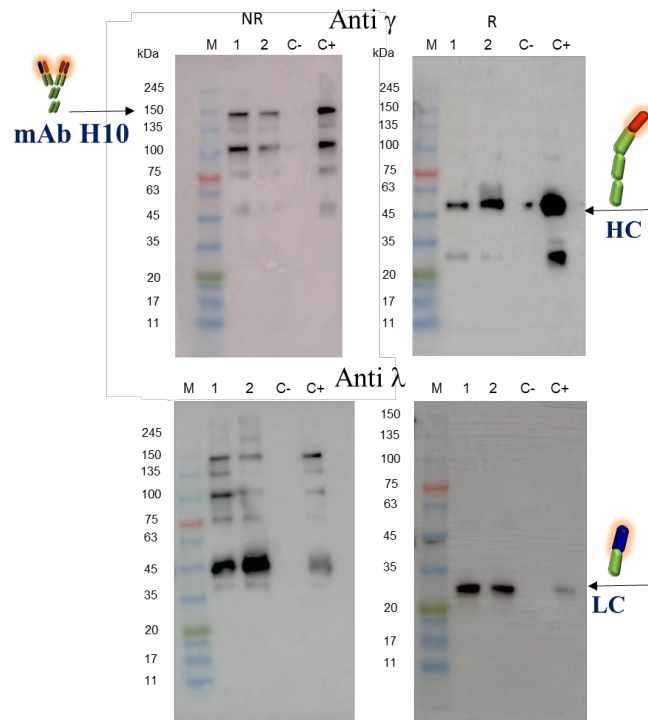


Figure 5: HR clones expressing mAb H10. *N. tabacum* clone 6 (1) and Δ XTFT *N. benthamiana* clone 15 (2) were analyzed by gradient gel under reducing (R) and non-reducing (NR) conditions then subjected to Western blot analysis using both anti-HC (anti γ) and LC (anti λ) antibodies. Extraction was performed using 0.1 g of root tissue for all samples and 20 μ L of each extract were loaded on 4–15% SDS-PAGE gradient gel. C+, mAb H10 purified from agro-infiltrated *N. benthamiana* leaves. C-, HR clone expressing an unrelated protein (antimicrobial peptide); M, molecular mass markers.

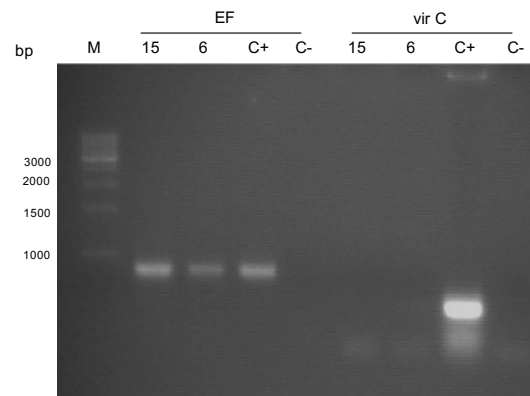


Figure 6: PCR performed on DNA extracted from HRs expressing the mAb H10 to assess the eradication of *A. rhizogenes*.

4.4.3 Secretion of mAb H10 in the culture medium.

Hairy root cultures of Δ XTFT *N. benthamiana* (clone 15) and *N. tabacum* (clone 6) in liquid MS medium were established (Fig. 7). Secretion of mAb H10 in the culture medium was induced by the addition of KNO₃ as nitrogen source, the plant growth regulator NAA and PVP as protein stabilizing agent (J M Sharp and Doran, 2001). Growth curves of HRs were obtained by measuring root fresh weight (FW) at different time points before (T0) and after induction (days 2, 4 and 8; T2, T4, T8) (Fig. 8). Antibody secretion was induced at day 25 and 28 post-inoculation and flasks were left for another five days prior collecting the culture medium for antibody purification (T8) (33 days after inoculum). The two clones showed a significant difference in their growth, with Δ XTFT *N. benthamiana* (clone 15) reaching about 11 g FW/40 ml (275 g FW/L) 25 days post-inoculation, compared to about 4 g FW/40 ml (100 g FW/L) in *N. tabacum* (clone 6). Moreover, we recorded a decrease in growth for both clones after the start of induction at day 25. Microscopy observations at day 33 (T8) showed a general enhanced fragility of roots with swollen tips and root hair proliferation, compared to non-induced ones (T0) (Fig. 9). The accumulation of mAb H10 in culture medium was assayed by Western blot (Fig. 10) using an anti-HC (γ chain) antibody. Samples were collected at different time points before (T0) and after induction of antibody secretion (T2, T4, T8), and separated by non-reducing SDS-PAGE. Very faint bands were observed before the induction (T0) at about 150 kDa, while stronger bands were detected in the following days (T2, T4, T8). Maximum mAb H10 levels were typically reached at day 8 for both *N. tabacum* and Δ XTFT *N. benthamiana* (T8). The amount of active antibody in HR culture medium was quantified by ELISA (Fig. 11). HR supernatant was recovered at day 8 after induction and serial dilutions were added to the ELISA wells coated with human TNC. Results showed a similar concentration of functional antibody in the medium of both HR clones, with 2.70 ± 0.32 mg/L in *N. tabacum* (mAb H10Nt) and 2.24 ± 0.19 mg/L in Δ XTFT *N. benthamiana* (mAb H10 Δ XTFT). Antibody quantification in the root biomass was performed at day 8 after induction by adding serially diluted root extracts to TNC-coated ELISA wells. Results showed a slightly higher concentration of

functional antibody in *N. tabacum* clone 6 ($48.7 \pm 7.7 \mu\text{g/g FW}$) compared to $\Delta\text{XTFT } N. benthamiana$ clone 15 ($31.3 \pm 5 \mu\text{g/g FW}$). Total antibody in the HRs was calculated as the total amount of antibody (secreted antibody + antibody in root biomass) in the system, taking into account the amount of biomass present and the residual volume of each HR culture (initial medium volume of 40 ml). Results showed higher levels of mAb in $\Delta\text{XTFT } N. benthamiana$ clone 15 ($0.47 \pm 0.03 \text{ mg}$) compared to *N. tabacum* clone 6 ($0.3 \pm 0.08 \text{ mg}$). The amount of antibody in the medium (considering HRs with 40 ml of starting volume) corresponded to about 20% of the total antibody (secreted + antibody in root biomass) in $\Delta\text{XTFT } N. benthamiana$ and about 35% in *N. tabacum*. Tobacco HR clones were grown and stably propagated for 10 months on solid MS culture medium and were used as starting inoculum to establish different batches of HRs in liquid medium. During this period, we did not observe significant variations of mAb H10 yields in different culture batches.

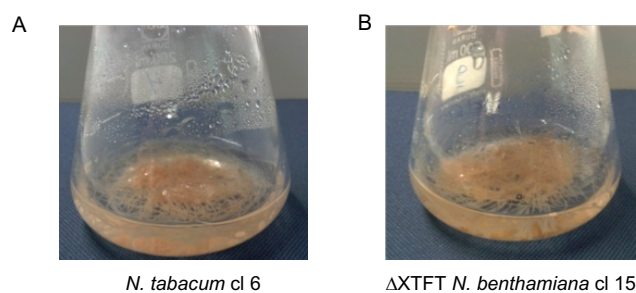


Figure 7: Examples of HR cultures of $\Delta\text{XTFT } N. benthamiana$ clone 15 and *N. tabacum* clone 6 in liquid medium.

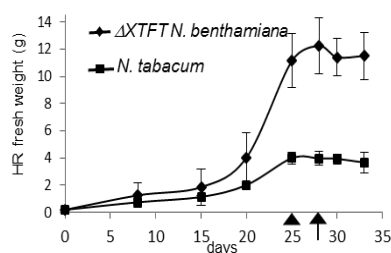


Figure 8: Growth curve of $\Delta\text{XTFT } N. benthamiana$ clone 15 and *N. tabacum* clone 6 was performed by measuring root fresh weight at different time-points before and after the induction of mAb H10 secretion in the culture medium. The secretion of mAb H10 was triggered by the addition of KNO_3 , NAA and PVP (days 25 and 33 after inoculum indicated by arrows). At day 33 medium was collected for purification analysis (T8).

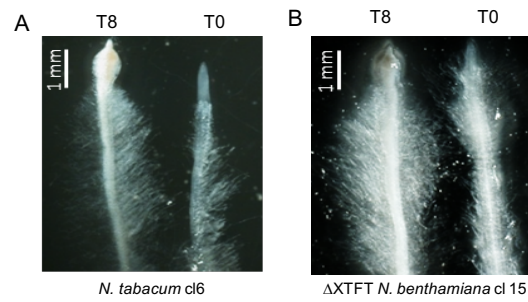


Figure 9: Stereo-microscopic analysis showing phenotypic differences between induced (T8) and non-induced (T0) HRs in both *N. tabacum* and Δ XTFT *N. benthamiana*. Induced HRs show typical swollen root tips and enhanced root hair proliferation.

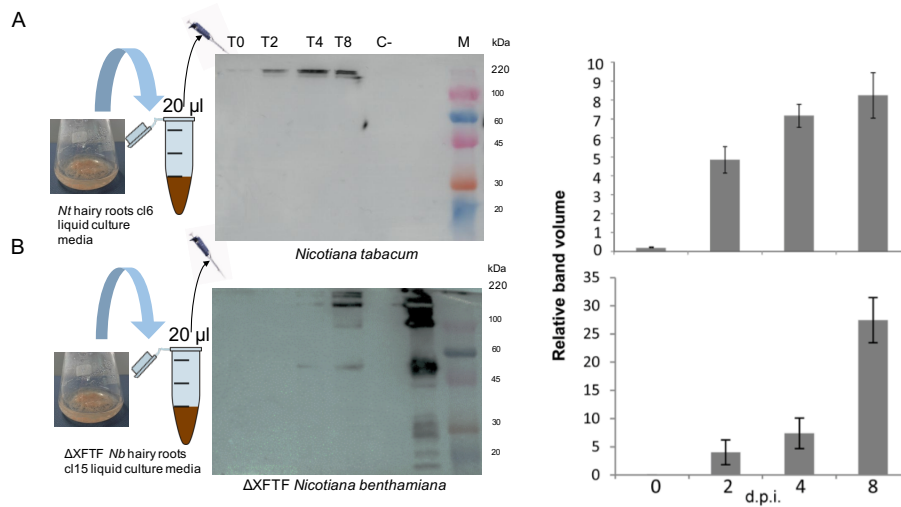


Figure 10: Antibody secretion in the culture medium of *N. tabacum* clone 6 and Δ XTFT *N. benthamiana* clone 15 was assayed by Western blot analysis using an anti-HC antibody at different time-points before (T0) and after induction of antibody secretion (T2, T4, T8). C+, mAb H10 purified from agro-infiltrated *N. benthamiana* leaves. C-, HR clone expressing an unrelated protein (antimicrobial peptide); M, molecular mass markers. Right panels show accumulation of mAbH10 evaluated by densitometry analysis of band volume.

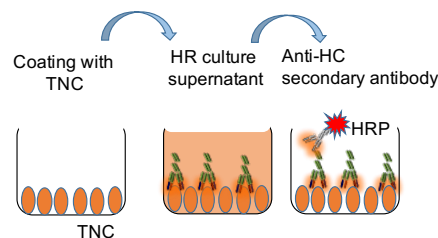


Figure 11: Schematic representation of ELISA used to quantify antibody mAb H10 in liquid culture medium.

4.4.4 Purification of functional tumor-targeting mAb H10 with a human-compatible glycosylation profile.

Culture medium was separated from HRs by filtration/centrifugation and clarified supernatant was directly subjected to protein-A affinity chromatography. A picture showing the columns used for leaf tissues purification and purification from hairy root culture medium is reported in Figure 12. The column used for purification from hairy roots is not pigmented and could be reused several times with no observed efficiency decrease. Eluted fractions were pooled, dialyzed against PBS and separated by SDS-PAGE under reducing or non-reducing conditions (Fig. 13). Reducing SDS-PAGE analysis of the purified antibodies revealed, as expected, two major bands at about 50 kDa (HC) and 25 kDa (LC), together with an additional degradation product (~23 kDa). A control mAb H10 purified from agro-infiltrated *N. benthamiana* leaves showed the same pattern of bands observed for the HR derived antibodies. In the case of non-reducing SDS-PAGE, a band at about 150 kDa corresponding to the assembled antibody was present in both tobacco and HR purifications. Additional faint (at about 100 kDa) and stronger bands (at about 40 kDa) demonstrated the presence of degradation products/assembly intermediates. The observed pattern of bands was similar to that of mAb H10 control from agro-infiltrated leaves. Average yields from three independent purifications were 1.6 ± 0.4 mg/L for mAb H10Nt from *N. tabacum* HRs and 1.5 ± 0.19 mg/L for mAb H10 Δ XTFT from Δ XTFT *N. benthamiana* HRs, corresponding to about 60% recovery of the total secreted antibody. The antibody was also purified from the biomass of the induced HRs. Clarified root extracts were directly loaded onto a protein-A column and the eluted fractions were separated by SDS-PAGE under reducing conditions. Average yields from three purifications were 16.2 ± 1.7 μ g/g FW for mAb H10Nt from *N. tabacum* HR and 12.4 ± 1.5 μ g/g FW for mAb H10 Δ XTFT from Δ XTFT *N. benthamiana* HR (Tab. 1). The integrity of both antibody versions was estimated by their ability to bind the antigen human TNC. ELISA plates were coated with recombinant C domain of human TNC (Fig. 14), and incubated with purified antibodies in serial dilutions. Both antibodies recognized the antigen in a concentration dependent manner, thus demonstrating functional activity in a

similar range. Glycan analyses of H10Nt by LC-ESI-MS revealed the presence of a single glycan species, i.e. plant-typical GnGnXF structures (Fig. 15 lower panel). In contrast, H10 Δ XTFT carried GnGn structures lacking xylose and core a 1,3-fucose (Fig. 15 upper panel).



Figure 12: Protein-A affinity chromatography column used to purify mAb H10 from HR culture medium (left) appears white compared to the pigmented column used for agroinfiltrated leaves (right).

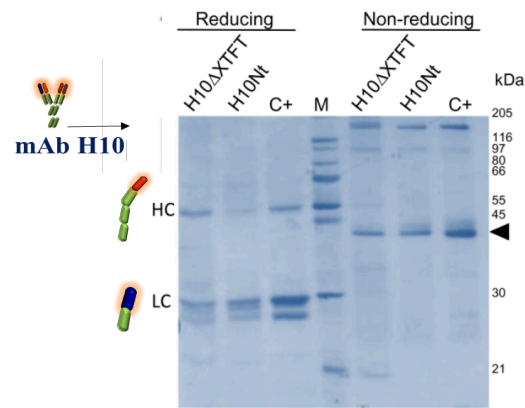


Figure 13: Purification of HR produced antibodies. Purified antibodies were loaded on SDS-PAGE gels under reducing and non-reducing conditions. The bands corresponding to mAb H10 Δ XTFT from *N. benthamiana* and mAb H10 Nt from *N. tabacum* were revealed using Coomassie blue staining. C+, mAb H10 purified from agro- infiltrated leaves of *N. benthamiana*. M, molecular mass standards. HC and LC are indicated as well as a major degradation fragment at about 40 kDa (black triangle).

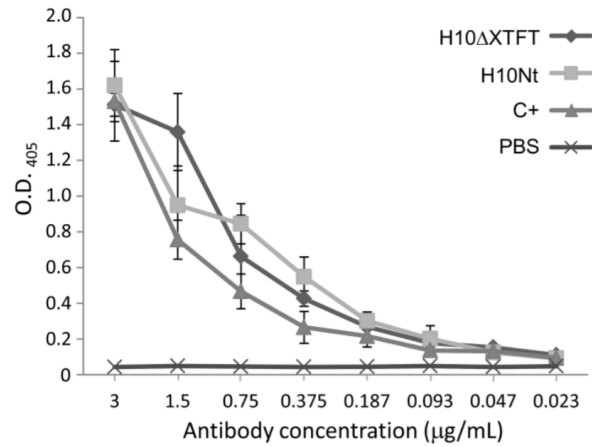


Figure 14: Antigen-binding analysis of mAb H10Nt and mAb H10 Δ XTFT. Purified antibodies were added to TNC coated plates at serial dilutions (data represent mean $\pm$ SD of three experiments). C+, mAb H10 purified from agro-infiltrated *N. benthamiana* leaves. C-, PBS.

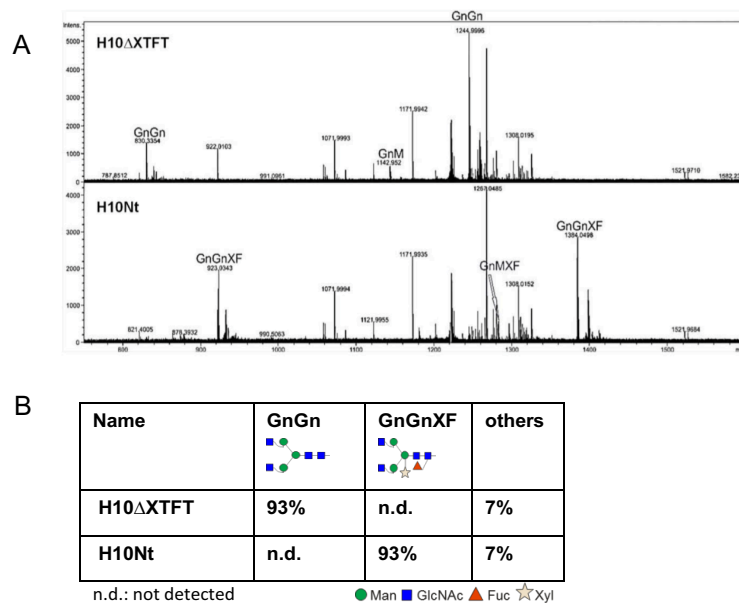


Figure 15: (A) Liquid chromatography-electrospray mass spectrometry (LC-ESI-MS) of tryptic glyco-peptides of mAb H10 purified from hairy roots culture medium of Δ XTFT *N. benthamiana* (H10 Δ XTFT) and *N. tabacum* (H10Nt). Doubly and triply charged ions of *N*-glycosylated variants of the expected tryptic fragments are indicated. (B) Relative amounts (%) of *N*-glycans in the purified mAbs H10 Δ XTFT and H10Nt.

Antibody	Purification yield from medium (mg/L)	Purification yield from roots ($\mu\text{g/g FW}$)
H10 Δ XTFT	1.5 ± 0.19	12.4 ± 1.5
H10Nt	1.6 ± 0.4	16.2 ± 1.7

Table 1: Yields of purified mAb H10 from *N. tabacum* (H10Nt) and Δ XTFT *N. benthamiana* (H10 Δ XTFT) from hairy roots culture medium and biomass.

4.5 Discussion.

In literature, there are only a few examples of the successful expression of monoclonal antibodies in HRs. All were developed from stable *N. tabacum* transgenic plants (J M Sharp and Doran, 2001; Skarjinskaia *et al.*, 2013). In this study, we evaluated the production of a tumour targeting mAb in tobacco HRs by using two different approaches. Hairy roots were either generated 'classically' by *A. rhizogenes* infection of a transgenic *N. tabacum* line expressing the antibody or by co-infection of glyco-engineered Δ XTFT *N. benthamiana* with a mix of recombinant *A. rhizogenes* strains bearing the antibody *hc* and *lc* genes. We showed that the percentage of clones expressing detectable amounts of recombinant protein was higher in *N. tabacum* (60% of the clones were positive in Western blot analysis) than in co-transformed Δ XTFT *N. benthamiana* (20% of the clones were positive). This result simply demonstrates that co-infection of leaf discs with a mix of *A. rhizogenes* carrying antibody HC and LC coding sequences, although at a lower efficiency, can be used for the rapid establishment (approx. two months) of stable HR clones expressing functional mAbs. A previous work based on the same multiple gene co-transformation strategy, i.e. transgenes located in different plasmids and transformed in different batches of *A. rhizogenes*, was used to co-express GUS and GFP in *N. tabacum* hairy roots. In this case a co-transformation efficiency of 65% was obtained, while a higher value (82%) was obtained when the reporter genes were inserted in two different T-DNAs within the same binary vector (Huang *et al.*, 2013). Comparison of the two best mAb expressors in liquid culture showed a faster growth (nearly three-fold) in the *N. benthamiana* clone, compared to the *N. tabacum* one. Starting from an inoculum size of ~0.2 g in a 40 ml culture, the FW biomass value of Δ XTFT *N. benthamiana* HRs was 11 g (275 g/L) after 25 days, while in the case of *N. tabacum* it was lower (100 g/L), but comparable to that already described by other groups (Häkkinen *et al.*, 2014). It has been previously demonstrated that transgene expression in HRs is strongly influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA), affecting not only protein expression but

also hairy root growth and morphology (Kim, Veena and Gelvin, 2007; Huang *et al.*, 2013). This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic HRs expressing monoclonal antibodies in different plant species (Wongsamuth and Doran, 1997; Häkkinen *et al.*, 2014). A major advantage of HRs is the possibility to secrete the recombinant proteins in the culture medium. Previous literature showed that the addition of protein stabilizing agents (PVP and gelatin) and KNO_3 in the medium improved antibody recovery (Drake *et al.*, 2013; Skarjinskaia *et al.*, 2013). Moreover, some authors also highlighted the importance of the auxin NAA in increasing rhizosecretion of recombinant proteins (Drake *et al.*, 2009). An optimized protocol was recently developed leading to high yield accumulation of a mAb in *N. tabacum* HRs, which is based on KNO_3 and NAA to induce rhizosecretion and PVP as protein stabilizing agent (Häkkinen *et al.*, 2014). We used a similar protocol to compare mAb H10 secretion in the two best expressing HR clones and the concentration of secreted antibody was similar in both *N. tabacum* (2.7 mg/L) and ΔXTFT *N. benthamiana* (2.24 mg/L) with levels comparable to those previously reported in literature (Häkkinen *et al.*, 2014). Maximum antibody accumulation was typically achieved eight days post-induction (T8) and, most interestingly, antibody accumulation in the medium before induction was hardly detectable (T0) (Fig. 10). Overall, our results showed that the amount of functional antibody secreted in the medium by *N. benthamiana* clone 15 represents about 20% of total antibody. This amount is slightly lower but comparable to that of *N. tabacum* clone 6 (35% of total antibody), indicating that there is no major difference in trafficking ability of the two mAb H10 glyco-variants. Significant variations in the amount of secreted antibody in three different *N. tabacum* HR clones expressing mAb M12 were reported in a previous work with levels ranging from 25 to 57% of the total antibody yield (Häkkinen *et al.*, 2014). Microscopy analysis of roots after induction revealed a peculiar phenotype with the formation of swollen root tips, root hair proliferation and elongation. This phenomenon was already described by Häkkinen and colleagues who observed the formation of lateral root primordia in the swollen root tip, associated with

morphological changes in the inner cortex and pericycle cells (Häkkinen *et al.*, 2014). A similar root morphology was also revealed in hydroponic tobacco cultures rhizosecreting a human monoclonal IgG after NAA induction (Madeira *et al.*, 2016). Antibody purification from culture medium was performed using a two-step protocol in which the medium was clarified by filtration/centrifugation and directly passed through a protein A column with no need for pH adjustment or DNase treatment. Purification yield of mAb H10 from liquid medium cultures of Δ XTFT *N. benthamiana* and *N. tabacum* HR clones were similar, in the range of 1.5 mg/L. It is interesting to note that the amount of antibody purified from the HR spent medium represents about 60% of the total secreted antibody while only 30% of the total antibody in the roots was recovered after protein A purification of root extracts. Purification yields from culture medium are slightly lower than those described by Häkkinen and colleagues which reported yields of 5.9 mg/L for mAb M12, reflecting possible lower antibody degradation and higher accumulation levels in the culture medium (Häkkinen *et al.*, 2014). It is important to note that the performance of the protein-A affinity column used for antibody purification from the medium remained unaltered even after several purification cycles, thus indicating that the filtered HR culture medium can be directly used in the downstream processing without the need of further clarification steps. As expected, mAb H10 purification yields in HRs are sensibly lower compared to those obtained by using transient expression systems based on *N. benthamiana* agroinfiltration (Lombardi *et al.*, 2012); the latter yielded up to 75 mg/kg FW of the antibody. Nevertheless, HRs still represent an advantageous system in terms of manufacturing and downstream processing for the contained root growth in sterile conditions and the secretion of the recombinant protein in the culture medium. Degradation of recombinant proteins in the HR culture medium represents a major problem and previous studies demonstrated that the addition of PVP or gelatin can enhance antibody stability (Drake *et al.*, 2013; Skarjinskaia *et al.*, 2013). In particular, the addition of PVP had stabilizing effects on HR-secreted mAb M12 increasing accumulation by two times (Häkkinen *et al.*, 2014). Previous studies reported that the susceptibility of recombinant antibodies to plant proteolysis is largely dependent

on their primary sequence including the variable domains (Gill and von Hippel, 1989; Cox *et al.*, 2006). Degradation profile of mAb H10 has been extensively studied in different plant species as well as production systems. This molecule was shown to be particularly susceptible to proteolytic cleavage in the proximity of the interdomain antibody sequences (Donini *et al.*, 2015). In particular, a previous study from our group demonstrated that mAb H10 purified from leaves of the transgenic tobacco line 7.6 was almost completely degraded leading to the formation of Fab fragments (Villani *et al.*, 2009). Reducing SDS-PAGE analysis of purified mAb H10 from Δ XTFT *N. benthamiana* and *N. tabacum* HRs, and from agro-infiltrated *N. benthamiana* leaves revealed a similar pattern with two major bands, corresponding to the HC and LC and a band at a lower molecular mass associated with HC degradation as a result of a proteolytic event close to the hinge region (Villani *et al.*, 2009). Identical patterns for the three purified antibodies were also observed in non-reducing conditions, with a major degradation product at about 40 kDa. In a previous work, a similar degradation product was described and characterized as a functional antibody Fab fragment deriving from specific cleavage in the antibody hinge region (Hehle *et al.*, 2015). Identical band patterns for the two HR clones were also observed in Western blot analysis of root extracts under non-reducing conditions using an anti-LC antibody (Fig. 2). A strong signal at about 45 kDa was obtained suggestive for the formation of LC homodimers. The occurrence of LC dimers in *N. benthamiana* had been already described in a recent work in which dimerized monoclonal camelidae variable heavy chain domain antibodies (VHH) were obtained through the C-terminal fusion of a k light chain domain (Giersberg *et al.*, 2010). Non-reducing analysis using anti-HC antibody revealed the presence of an intense band at about 100 kDa and a fainter one at about 50 kDa possibly representing HC dimers and monomers, respectively. The presence of free LC and HC polypeptides in HR clones indicated that they are not synthesized in stoichiometric ratios. Since HC and LC coding genes were located in separate plasmids and transformed in different batches of *A. rhizogenes*, further used for hairy roots co-transformation, it is very likely that transgenes were inserted in these two clones in different regions of genomic

DNA and possibly in different copy numbers, thus accounting for the differential accumulation of monomeric HC and LC. It is interesting to note that no significant differences in band patterns were observed between the two HR secreted antibodies or mAb H10 produced in *N. benthamiana* leaves, indicating that equivalent proteolytic events take place in both species and production systems. A similar evidence was observed for analogous proteolytic activity of *A. thaliana* or *N. tabacum* rhizosecretomes on a human IgG (Lallemand *et al.*, 2015). Plant-derived mAbs differ from their mammalian counterpart in their Fc *N*-glycan composition. Non-human glycosylation may represent a limitation in their therapeutic application since plant typical complex glycan structures moieties may cause immunogenic reactions (Bencúrová *et al.*, 2004; Lallemand *et al.*, 2015) and may also interfere with Fcγ receptors binding (Forthal *et al.*, 2010; Gomord *et al.*, 2010). This has a negative impact on immunotherapeutic mAbs, since their effector functions, such as antibody dependent cell cytotoxicity (ADCC), rely on the interaction with Fcγ receptors (Jez *et al.*, 2012). Recent studies demonstrated that antibodies lacking plant specific xylose and fucose have enhanced binding to Fcγ receptors and improved biological activity (Loos *et al.*, 2015). With the aim of improving mAb H10 immunotherapeutic potential we have demonstrated here that it is possible to obtain HRs from ΔXTFT *N. benthamiana* producing mAbs with a human-compatible glycosylation pattern. Häkkinen and colleagues previously characterized the glycosylation profile of mAb M12 obtained in *N. tabacum* HRs, showing that the secreted antibody carries mainly GnGnXF plant typical complex- type glycans (Häkkinen *et al.*, 2014). In this work, we have shown that mAb H10*Nt* has a similar profile to that observed for mAb M12 (GnGnXF), while mAb H10ΔXTFT carries predominantly GnGn structures (>90%). To our knowledge, this is the first example of a functional glyco-modified mAb obtained from HRs lacking typical plant-type sugars, thus representing a step towards the exploitation of root cultures for the production of 'next generation' human therapeutic antibodies.

In conclusion, we have shown here that it is possible to rapidly establish HRs secreting full size mAbs with a targeted glycosylation profile by using a simple *A. rhizogenes* infection protocol of ΔXTFT *N. benthamiana* leaf segments. As

reported in literature, maximum antibody secretory yields in HRs are in the range of few milligrams per liter of culture medium. To our view, this is the major limitation of this production system, especially if compared to the high-yield methods (hundreds of milligrams per kg of FW) based on transient expression systems using whole plants. For this reason, efforts have to be focused on devising novel expression vectors as well as cultivation and induction protocols to enhance recombinant protein production and secretion, making stably transformed HRs a competitive production system of glyco-modified antibodies for cancer immunotherapy.

4.6 References.

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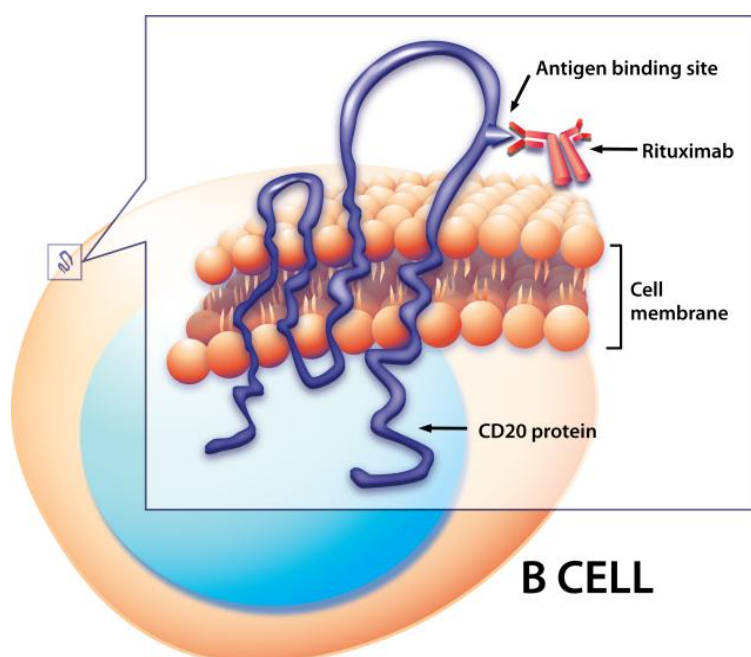
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Chapter 5. Hairy roots: a production platform for anti-CD20 recombinant antibodies.



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5.1 Abstract.

Immunotherapy is a cancer treatment designed to boost the body's natural defenses. There are several types of immunotherapies such as T-cell therapy, cancer vaccines or the use of cancer targeting monoclonal antibodies. Antibody-based pharmaceuticals have been successfully exploited for the therapy of non-Hodgkin lymphomas (NHLs), which represent a very serious threat nowadays. The majority of NHLs express the leucocyte antigen CD20, an integral transmembrane glycoprotein of 33-36 kDa, making it a suitable target for immunotherapy (Calcagno *et al.*, 2012). Rituximab, a mouse/human chimeric anti-CD20 antibody, is the first antibody-based drug approved by FDA for the treatment of patients affected by B-cell lymphoma. In the current work, a recombinant antibody, derived from rituximab, the 2B8-Fc (Marusic *et al.*, 2016) has been produced in transgenic Δ XTFT *N. benthamiana* hairy root cultures (HRs). As previously described, hairy roots are a valid production platform offering several advantages such as low costs, ease of production, scalability and limited risk of contamination by human pathogens (Stoger *et al.*, 2014). Three selected glyco-engineered Δ XTFT *N. benthamiana* hairy root clones accumulated the 2B8-Fc Δ XTFT in the culture medium at comparable levels, reaching yields of about 8 mg/L. The best performing clone was used to purify the antibody from the culture medium obtaining yields of 5.57 mg/L. N-glycosylation profiles of HR purified glyco-optimized 2B8-Fc Δ XTFT revealed GnGn structures lacking xylose and fucose and the antibody exhibited CD20 antigen binding activities comparable to the same molecule produced in agroinfiltrated *N. benthamiana* leaves. This is the first example of the expression of an engineered anti-CD20 antibody with a scFv-Fc format in hairy roots. *In vitro* analysis demonstrated the functionality of this molecule representing an important step towards the use of HRs as a production platform of recombinant anti-CD20 biopharmaceuticals with a human-compatible glycosylation profile.

5.2 Introduction.

5.2.1 Non-Hodgkin's lymphomas.

Lymphoma is a general term used to define blood cancers that develop mainly in the lymphatic system and are represented by a group of several related tumours. There are approximately 30 different types of lymphomas that are divided into two main categories: Hodgkin's lymphomas (HL) and non-Hodgkin's lymphomas (NHLs), which are the most commonly diffused (representing approximately 80%). Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of solid tumours arising from lymphocytes at different stages of development, representing a major health problem for both developed and developing countries (Perry *et al.*, 2016). It has been estimated that 72.580 new cases of non-Hodgkin Lymphomas have been diagnosed in the USA in 2016 (Armitage *et al.*, 2017). NHLs usually occur in adults and can be further classified by growth rate; fast-growing subtypes are defined as aggressive, slow-growing NHLs are defined as indolent. The two most common forms of NHL are diffuse large B cell lymphoma (DLBCL), an aggressive type accounting for 30% of NHL cases, and follicular lymphoma (FL), an indolent type that accounts for 25-30% of cases. NHLs are often cured by combined therapies including chemotherapy and radiotherapy and, rarely, surgery and transplants. Chemotherapy's efficiency is limited by non-specific toxicity to normal tissues and by the development of resistance to therapeutic agents. Moreover, a percentage of patients treated with chemotherapy and radiotherapy develop secondary malignancies due to the lowering of immunosurveillance induced by conventional antitumour agents and radiation and to the therapies transformative actions. Approximately 50% of NHL patients either relapse or become refractory to conventional therapy diagnosis (Polito *et al.*, 2014). For these patients with poor outcomes, the use of new therapeutic strategies, such as immunotherapy, is required.

5.2.2 CD20 antigen as a target for immunotherapy.

In combination with chemotherapy and radiotherapy, the use of monoclonal antibodies (mAbs) for anti-cancer therapy has been widely explored. The

specificity of immunotherapy is based upon characteristics (surface antigens) that are completely independent from the parameters that allow for the differential toxicity of chemotherapy and radiotherapy. Most NHLs' express the leukocyte antigen CD20 that is a highly expressed transmembrane surface antigen with a molecular weight of approximately 35 kDa. The function of CD20 is not completely understood; however, this antigen seems to be involved in the regulation of B cell growth and differentiation via intracellular signaling or by acting as a calcium channel in association with antigen stimulation of the B cell receptor. Extensive studies were carried out on the CD20 molecule due to its properties such as: i) the antigen is expressed on nearly 90% of B cell NHLs and on normal B cells, but neither on B cell precursors and plasma cells nor on other tissues; ii) the antigen is highly expressed on the cell surface; iii) CD20 is not normally shed from the cell; iv) CD20 is internalized after antibody binding. Anti-CD20 mAbs cause cell death through different pathways. After binding to the antigen, these mAbs can activate complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC), which are mediated by the Fcγ receptors on effector cells, such as granulocytes, macrophages, and natural killer (NK) cells. Moreover, some antibodies can directly kill the target cell by triggering the apoptotic pathway, thus having a direct cytotoxicity. These effects make anti-CD20 antibodies essential tools in lymphoma therapy, both alone and in combination with chemotherapy. Clinically active anti-CD20 mAbs can be divided into two groups based on the triggered cell death pathway. Type I antibodies as rituximab (RTX), ofatumumab (OFA), velutuzumab, ocrelizumab, ocaratuzumab, and rhumAb v114 induce CD20 translocation into lipid rafts with consequent ADCC, CDC, and weak apoptosis. Type II mAbs (tositumomab and obinutuzumab) do not redistribute CD20 on the cell surface and induce strong ADCC and apoptosis but weak CDC (Polito *et al.*, 2014).

5.2.3 Hairy roots as a production platform for an anti-CD20 antibody.

Rituximab (mAb C2B8), a mouse-human chimeric mAb, was the first antibody-based drug approved for the treatment of patients with B-cell lymphomas (Reff

et al., 1994; Calcagno *et al.*, 2012). This mAb is composed of a human immunoglobulin (IgG₁) Fc region and human kappa constant region with variable regions derived from the 2B8 murine IgG. The primary cell death mechanism induced by rituximab (RTX) is CDC, but it can also lead to ADCC and apoptosis. In addition, RTX is able to raise the T cell response against malignant clones. RTX treatment improves response rates in several types of B cell NHLs, including DLBCL, FL, and mantle cell lymphoma (Polito *et al.*, 2014). In a previous work, the recombinant anti-CD20 2B8-Fc antibody, obtained in our laboratory, has been efficiently engineered and produced in *N. benthamiana* plants. The *in planta* codon optimized sequence was cloned in a plant binary vector (pBI- Ω) under the control of the CaMV 35S promoter and the omega (Ω) translational enhancer sequence from TMV and electroporated into *A. tumefaciens* and then used to agroinfiltrate *N. benthamiana* plants (Marusic *et al.*, 2016). The purified antibody showed a plant typical glycosylation profile bearing β 1,2-xylose and α 1,3-fucose residues but retained the capacity to bind to CD20 and to induce ADCC in DAUDI cells. In the present study, we have exploited advantages offered by hairy roots for the production of the anti-CD20 recombinant 2B8-Fc antibody. Our goal was to obtain Δ XTFT *N. benthamiana* hairy roots efficiently secreting 2B8-Fc Δ XTFT with a human compatible glycosylation profile in the culture medium.

5.3 Materials & Methods.

5.3.1 Generation of engineered Hairy Roots expressing 2B8-Fc Δ XTFT antibody.

A. rhizogenes A4 strain carrying pBI-2B8-Fc expression vector (described in Marusic *et al.*, 2016) was used to transform Δ XTFT *N. benthamiana* leaf segments obtaining HR cultures as described in the previous chapter (Chapter 4). Briefly, *A. rhizogenes* A4 carrying pBI-2B8-Fc was grown in YEB medium to OD600 = 0.5, at 28 °C and 160 rpm. Bacteria were pelleted by centrifugation at 3000 x g for 15 min and resuspended at OD600 =1 in MS medium with 30 g/L sucrose and 100 μ M acetosyringone, pH 5.8. Leaf segments (1cm x 1cm) were wounded with a sterile needle and inoculated by immersion in the *A. rhizogenes* suspension for 30 minutes, swirling in the dark. They were dried and transferred to culture plates containing MS agar medium in the dark for 2 days and then transferred again to fresh MS agar medium containing 30 g/L sucrose with 0.3 g/L cefotaxime and 100 g/L kanamycin. Roots emerging from leaves were excised and transferred to new plates. For the complete eradication of *A. rhizogenes*, hairy roots were transferred in plates with decreasing cefotaxime concentrations (0.25 g/L, 0.125 g/L, 0.05 g/L) until no antibiotic was added.

5.3.2 Western blot analysis.

Hairy Root tissues from plates or liquid cultures were ground in liquid nitrogen and homogenized in phosphate-buffered saline pH 7.2 (PBS) containing a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany) in a 1:1 weight/volume ratio as described for mAb antibody H10 (Chapter 4, Lonoce *et al.*, 2016). To assay the presence of the antibody secreted in HRs, the culture medium was collected at different time-points before (T0) and after induction (day 2 and 8; T2, T8) and filtered using a Miracloth™ (Millipore). The filtered medium was then centrifuged at 13000 x g, 16 °C for 15 min, Proteins were separated on 10% SDS-PAGE under reducing and non-reducing conditions. Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare, Freiburg, Germany) for

70 min at 0.4 mA/cm² and 30 V. Membranes were incubated in a blocking solution of PBS with 4% (w/v) non-fat milk, for 16-18 hours. Protein detection was performed with anti-human γ chain horseradish peroxidase conjugated (A8419 Sigma-Aldrich) diluted 1:5000 in 2% (w/v) non-fat milk in PBS. The ImageQuant[™] LAS 500 (GE Healthcare, Germany) was used for chemiluminescence signal detection. Sharp Mass VI (Euroclone, Italy) or PinkK plus (GeneDireX, USA) were used as protein molecular weight markers.

5.3.3 Antibody secretion in the culture medium.

For growth in liquid medium, about 0.2 g of Δ XTFT *N. benthamiana* hairy root segments were seeded in 200 ml Erlenmeyer flasks using a starting volume of 5 ml of MS medium supplemented with 3 % sucrose and increasing up to 30 ml after 10 days when roots reached a biomass of about 3-4 g. Flasks were orbitally shaken at 80 rpm ($\varnothing$ 19 mm), in the dark, at 24-26 °C for 4-5 weeks. Liquid medium was replaced every ten days, by adding fresh MS with sucrose. Heterologous protein secretion was triggered 28 day after inoculum (T0) by the addition of MS culture medium supplemented with 14 g/L of KNO₃, 19 mg/L of NAA and 1.5 g/L of PVP (Mw 40000) as previously described in chapter 4 (Lonoce *et al.*, 2016). Three days after the first induction, flasks were again supplemented with 19 mg/L of NAA and 1.5 g/L of PVP, and left for 5 days before collecting the spent culture medium for 2B8-Fc Δ XTFT analysis and purification (T8). An aliquot of HR culture medium (50 μ l) was collected at different time points, before (T0) and after induction (T2,T8) in order to analyze the accumulation of 2B8-Fc Δ XTFT by Western blot analysis or ELISA. To assay the presence of residual antibody molecules in HR tissue, protein extracts obtained as previously described were also analyzed by Western blot and ELISA. 2B8-Fc purified from wild type (WT) agro-infiltrated *N. benthamiana* leaves was used as a positive control (Marusic *et al.*, 2016).

5.3.4 Antibody purification and size-exclusion chromatography.

Liquid culture medium (typically 30 ml) was separated from roots by filtration using Miracloth[™] (Millipore). Supernatant was centrifuged at 13000 x g, 16 °C

for 15 min, and filtered through 0.45 µm syringe filters (Millipore) ready for protein-A affinity chromatography that was carried out essentially as described in chapter 4 (Lonoce *et al.*, 2016). Briefly, the clarified culture medium was loaded onto a protein-A affinity column (1 ml HiTrap™ rProtein A FF; GE Healthcare) previously equilibrated with extraction buffer (PBS 1X) at a flow rate of 1 ml/min. The column was washed with 10 ml of PBS (10 column volumes) and the antibody was eluted with 0.1 M of citric acid (pH 3) and buffered with 1/5 volume of 1 M Tris-HCl, pH 8. Antibody concentration in the eluted fractions was determined spectrophotometrically by absorbance at 280 nm (Gill and von Hippel, 1989). Purified scFv-FcΔXTFT molecules were analysed by size-exclusion chromatography on a calibrated Superdex™ 200 5/150 GL column (GE Healthcare) eluted in PBS at flow rate of 0.3 ml/min, using an AKTA FPLC P920 instrument (GE Healthcare) thermostated at 20 °C, essentially as described before (Lombardi *et al.*, 2010). 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), according to manufacturer instructions. Plant purified 2B8-Fc was used as a positive control. Peaks representing different protein fractions were collected using a fraction collector (FRAC-920) and further analyzed.

5.3.5 Antibody quantification by ELISA.

The amount of 2B8-FcΔXTFT, in the culture medium or in root tissue, was measured by quantitative ELISA. In detail, Nunc-MaxiSorp® 96 well plates were coated with an anti-human γ chain antibody (H10700) 1:1000 dilution in PBS 1X and incubated overnight at 4 °C. Plates were then blocked with 4% milk (w/v) in PBS, at 37 °C, for 2 h and serial dilution of samples (in PBS 1X) were added to the wells (100 µl final volume) and incubated at 37 °C, for 1 h. After washing, the anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was added at 1:5000 diluted in 2% milk (w/v) PBS, and incubated at 37 °C, for 1 h. Enzymatic activity was measured at 405 nm on a microplate reader (TECAN-Sunrise, Groedig, Austria) using 2,2-azino-di-3-ethylbenz-thiazoline sulphonate as substrate (ABTS, KPL). Serial dilutions of purified 2B8-Fc were used as a

positive internal standard for quantification, starting from a concentration of 500 ng/well while PBS 1X was used as negative control. Each sample was assayed in triplicate and 2B8-Fc Δ XTFT amount was interpolated in the linear portion of the standard curve.

5.3.6 Glycan analysis.

Bands from SDS-PAGE under reducing conditions were excised from the gel, triturated, *in-gel* reduced, S-alkylated and digested with trypsin (Rec. Proteomics Grade, Roche) (D'Ambrosio *et al.*, 2008). Gel particles were extracted with 25 mM NH₄HCO₃/acetonitrile (1:1 v/v) and digests were concentrated. Peptide mixtures were either desalted using μ ZipTipC₁₈ pipette tips (Millipore) before MALDI-TOF-MS analysis or directly analysed by nanoLC-ESI-LIT-MS/MS (see below). To isolate glycopeptides, tryptic digests from 2B8-Fc Δ XTFT were also solved in 80% acetonitrile, 2% formic acid and loaded on NuTip Gel loader capillary HILIC columns (Glygen, Columbia, MD). After washing with 80% acetonitrile, 2% formic acid, glycopeptides were first eluted with 10 μ l of 2% formic acid and then with 5 μ l of 50% acetonitrile, 2% formic acid; pooled fractions were analysed by MALDI-TOF-MS, as described below. Protein digests were analysed by nLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (ThermoFinnigan, USA) equipped with a Proxeon nanospray source connected to an Easy-nLC (Proxeon, Denmark) (Salzano *et al.*, 2013). Peptide mixtures were resolved on an Easy C₁₈ column (100 x 0.075 mm, 3 μ m) (Proxeon) using a linear gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid, at a flow rate of 300 nl/min, for 60 min. Spectra were acquired in the range *m/z* 400-2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the 3 most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 1 min). The mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively. During MALDI-TOF-MS analysis, whole tryptic digests or their glycopeptide fractions were mixed 1:1 with 2,5-dihydroxy-benzoic acid (10 mg/ml in 50% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) and loaded on the instrument target. Spectra were acquired in the *m/z* range 500-5000 on a

Bruker Ultraflextreme MALDI-TOF-TOF instrument (Bruker Daltonics) operating in reflectron mode. Instrument settings were: pulsed ion extraction = 100 ns, laser frequency = 1000 Hz, number of shots per sample = 2500-5000 (random walk, 500 shots per raster spot). Mass spectra were calibrated externally using nearest neighbour positions loaded with Peptide Calibration Standard II (Bruker Daltonics), with quadratic calibration curves. MS/MS spectra were acquired in LIFT mode. Data were elaborated using the FlexAnalysis software (Bruker Daltonics). nLC-ESI-LIT-MS/MS data were searched with MASCOT (version 2.2.06) (Matrix Science, UK) against a database containing the 2B8-Fc sequence. As searching parameters, we used a mass tolerance value of 2 Da for precursor ion and 0.8 Da for ion fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2.0, Cys carbamidomethylation as fixed modification, and Met oxidation and N-terminal Glu/Gln cyclization as variable modifications, respectively. A MASCOT score > 30 and a significant threshold ($p < 0.05$) were required for peptide assignment. In the case of glycopeptides, MALDI-TOF mass signals were assigned to specific components using the GPMW 4.23 software (Lighthouse Data, Denmark). This software generated a mass/fragment database output based on protein sequence, protease selectivity, nature of the amino acids susceptible to eventual glycosylation and the molecular mass of the modifying groups. Searching parameters were set as mentioned above; mass values were matched to protein regions using a 0.02% mass tolerance value. MALDI-TOF-TOF searching parameters were set with tolerances of 100 ppm and 0.5 Da for MS and MS/MS data, respectively.

5.3.7 Flow cytometry analysis.

CD20-expressing Daudi human cells, purchased by ATCC (American Type Culture Collection, code CCL213), were stained with 2B8-Fc Δ XTFT purified from HRs or *N. benthamiana* agroinfiltrated leaves. Cells labeled with only the secondary antibody were used as a control. Binding to CD20 was revealed with a goat anti-human IgG polyclonal Ab (Invitrogen H10700), which in turn was detected with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG polyclonal Ab (Sigma Aldrich F7367). Fluorescence signals were collected

in log mode using a FACSCalibur (BD Biosciences). Analyses of cell populations were performed on events gated according to forward scatter/side scatter light parameters as previously described (Marusic *et al.*, 2012).

5.4 Results.

5.4.1 Generation and selection of glyco-engineered HR clones expressing 2B8-Fc Δ XTFT.

Δ XTFT *N. benthamiana* leaf discs were infected with *A. rhizogenes* A4 strain carrying the anti-CD20 recombinant 2B8-Fc encoding sequence (Fig. 1) generating 93 independent hairy root clones. Hairy root clones (Fig. 2A) were grown in solid MS plates and then screened for 2B8-Fc Δ XTFT expression by Western blot analysis in non-reducing conditions (Fig. 2B). Positive clones exhibited the expected band at ~110 kDa corresponding to 2B8-Fc Δ XTFT dimers (marked with a triangle) and a second band at about 55 kDa probably corresponding to the non assembled monomeric antibody chain (marked with an asterisk). A fainter band at about ~95 kDa suggests a possible degradation fragment of 2B8-Fc Δ XTFT (marked with an asterisk). About 30% of the analyzed hairy root clones were positive. Clones 14, 27 and 63 showing the highest expression levels were chosen for further analysis.

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Figure 1: Schematic representation of the p35:2B8-Fc construct that is under the control of the Cauliflower Mosaic Virus 35S promoter (35S) and the translation enhancer sequence of Tobacco Mosaic Virus (Ω). L: signal peptide sequence derived from an embryonic mouse immunoglobulin HC coding gene.

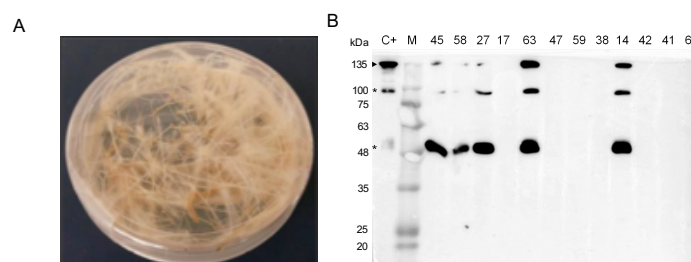


Figure 2: Hairy roots expressing 2B8-Fc Δ XTFT growing on MS solid medium (A). Example of the screening of some hairy root clones (including clones 14, 27 and 63) by Western blot using an anti- γ antibody in non reducing conditions (B). Twenty μ l of extract were loaded on wells. 2B8-Fc purified from agroinfiltrated WT *N. benthamiana* leaves (200ng) was used as positive control (C+).

5.4.2 Comparison of 2B8-Fc Δ XTFT expression in selected hairy root clones.

To compare the expression levels of 2B8-Fc Δ XTFT in the selected clones (14, 27, 63), an ELISA assay was performed (Fig. 3). Root extracts were added in serial dilutions to the wells coated with an anti-HC antibody. Results showed comparable levels of 2B8-Fc Δ XTFT accumulation in clones 14 and 63, while clone 27 displayed a slightly lower (but not statistically significant) antibody amount. As positive control (C+), was used 2B8-Fc antibody purified from WT agro-infiltrated *N. benthamiana* leaves.

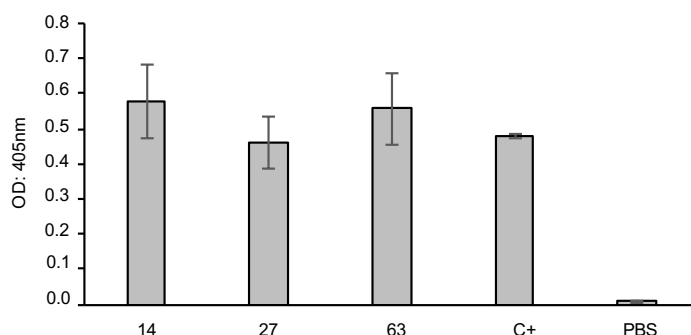


Figure 3: Comparison of 2B8-Fc Δ XTFT expression in different Hairy Root clones was performed by ELISA. Roots extracts were added to anti-HC coated plates. An anti-human γ chain HRP-conjugated antibody was used for detection. As positive control (C+), 50 ng of 2B8-Fc purified from agro-infiltrated *N. benthamiana* leaves was used. PBS was used as negative control. Results were obtained from triplicate HR extracts $\pm$ SD of the mean.

5.4.3 Secretion of 2B8-Fc Δ XTFT in the liquid culture medium.

Cultures of Δ XTFT *N. benthamiana* clones 14, 27, and 63, were established in liquid medium (Fig. 4A, B and C). The hairy root secretion-based production system used in this work, involved the addition of potassium nitrate (KNO₃) as nitrogen source, the plant growth regulator naphthaleneacetic acid (NAA) and polyvinylpyrrolidone (PVP) as protein stabilizing agent in order to induce the accumulation of recombinant antibody in the culture medium (Lonoce *et al.*, 2016). Induction of antibody secretion was performed at day 28. Briefly, the day before induction (day 28) was called T0, followed by T2 and T8 (day 31 and 36) corresponding to the time after the first and the second induction. Samples of

liquid media were collected before (T0) and after induction (T2, T8) and analyzed by non-reducing Western blot (Fig. 5). The selected clones (27, 14 and 63) showed, at different time-points, a band pattern similar to the 2B8-Fc purified from WT *N. benthamiana* leaves. All clones showed a very faint band at ~ 55 kDa before induction (T0) corresponding to the monomeric antibody and an increasing amount of assembled antibody (the higher band marked with an asterisk) at ~ 110 kDa with the highest accumulation 8 days after induction (T8). A strong degradation product at about 95 kDa and an additional minor band of ~ 30 kDa was observed in clones 14 and 63 which also showed higher 2B8-Fc Δ XTFT accumulation. To assay any residual amount of 2B8-Fc Δ XTFT inside root tissue, hairy roots were well rinsed with PBS after induction and total protein extraction was performed. Results showed a major band corresponding to the monomer, while the higher band corresponding to the assembled 2B8-Fc Δ XTFT is barely visible (Fig. 5 27R, 14R, 63R). Due to the higher accumulation of 2B8-Fc Δ XTFT and good morphology and growth rate of roots, clones 14 and 63 were chosen for further analysis. The amount of 2B8-Fc Δ XTFT, in HR liquid culture medium, was quantified by ELISA. HR supernatants collected 8 days after induction (T8) were added in serial dilutions to the wells coated with an anti-HC antibody. Results showed a similar concentration of 2B8-Fc Δ XTFT in both clones (14 and 63), reaching yields of 7.10 ± 0.38 mg/L and 7.76 ± 0.28 mg/L, respectively. Growth curves were obtained by measuring root fresh weight (FW) at different time points before and after induction (Fig. 6). Hairy roots obtained by infecting with non transformed *A. rhizogenes* (WT) were used for comparison. Antibody secretion was induced at day 28 and 31 post-inoculation and 5 days after, medium was totally collected for purification (36 days after inoculum; T8). The two clones exhibit comparable growth with a peak 28 days after inoculum. At this time, clones 14 (13.0 ± 0.8 g) and 63 (12.6 ± 0.5 g) accumulated a comparable amount of biomass matching that of WT HRs (11.7 ± 0.7 g). It can be noted that no appreciable increase of biomass was recorded after hairy root induction.

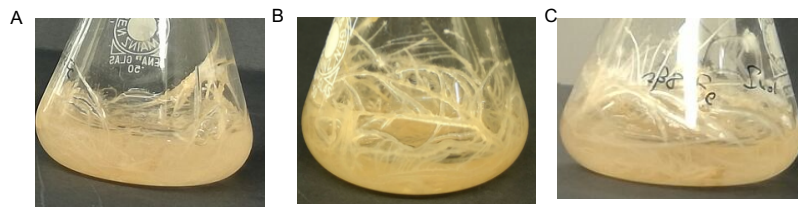


Figure 4: Δ XTFT *N. benthamiana* HR liquid cultures of clones 14 (A), 27 (B) and 63 (C) expressing 2B8-Fc Δ XTFT.

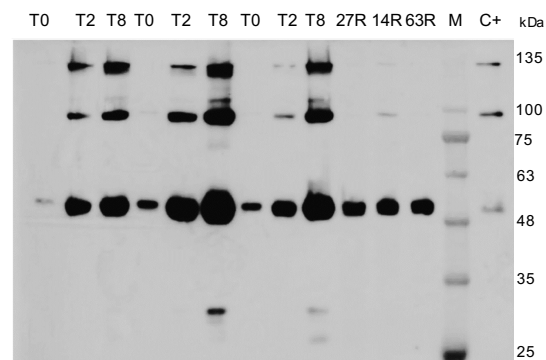


Figure 5: Analysis of 2B8-Fc Δ XTFT accumulation in the liquid medium was assayed by Western blot analysis at different time points before (T0) and after induction (T2, T8). The amount of 2B8-Fc Δ XTFT in roots after induction was also assayed (14R, 27R, 63R). A total of 20 μ l of culture medium or root extracts for each sample were loaded on wells. As positive control (C+), 2B8-Fc (20ng) purified from agro-infiltrated WT *N. benthamiana* leaves was used.

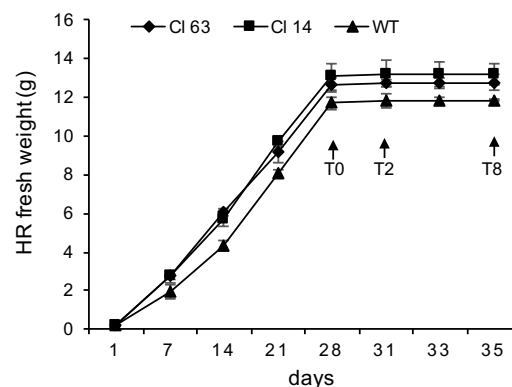


Figure 6: The growth rate of Δ XTFT *N. benthamiana* HR clones 14 and 63 was measured as HR fresh weigh, expressed, at different time points (days) before (T0) and after induction (T2, T8).

5.4.4 Antibody purification and size exclusion chromatography.

Antibody purification from liquid culture medium of clone 63 was performed by flowing the clarified liquid culture medium through a protein-A affinity chromatography column. Eluted fractions were pooled, dialyzed against PBS and separated by SDS-PAGE under reducing and non-reducing conditions (Fig. 7). The samples exhibited in non reducing conditions (NR) a major band at about 110 kDa corresponding to the dimeric 2B8-Fc Δ XTFT and additional fainter bands at ~95 kDa and ~55 kDa (marked with an asterisk), suggesting possible degradation fragments of 2B8-Fc Δ XTFT or the presence of non assembled monomers as previously observed in the Western blot analysis. In reducing conditions (R) a major ~55 kDa band corresponding to the monomeric 2B8-Fc Δ XTFT was detected in addition to a faint degradation fragment at ~30 kDa. To further assess assembly and integrity of purified antibody, size exclusion chromatography using a Superdex S-200 column was performed. Analysis revealed that more than 70% of the purified 2B8-Fc Δ XTFT had the expected molecular mass of ~110 kDa, corresponding to a major peak eluted at 2.13 ml, similar to that obtained for plant-purified 2B8-Fc used as a control (2.12 ml) (Fig. 8). An additional peak at 2.83 ml, indicated by an arrow is also visible possibly corresponding to the minor degradation products observed in the coomassie gel. The purification yield of 2B8-Fc Δ XTFT from liquid medium cultures of clones 14 and 63 were 4.65 ± 0.9 mg/L and 5.57 ± 1.3 mg/L, respectively.

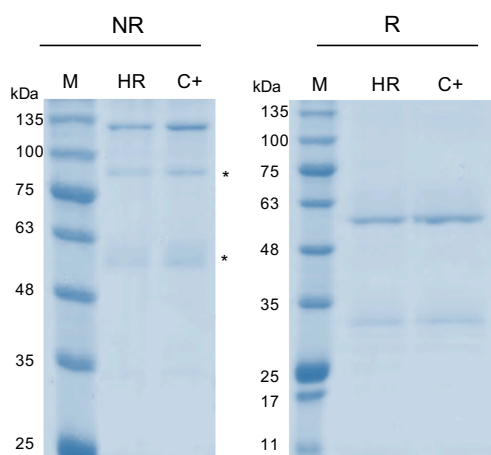


Figure 7: 2B8-Fc Δ XTFT antibody was purified from the culture medium by protein-A affinity chromatography column. Purified antibody was loaded on 10% SDS-PAGE gels under reducing (R) and non-reducing conditions (NR) and revealed with Coomassie Stain. As positive control (C+), 2B8-Fc purified from agro-infiltrated WT *N. benthamiana* leaves (1 μ g) was used. M: molecular weight standard.

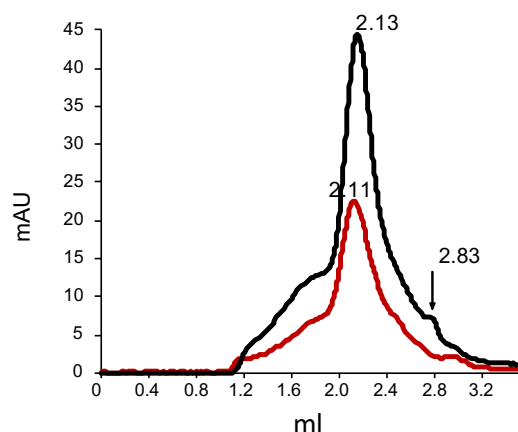


Figure 8: Chromatogram obtained from size-exclusion chromatography analysis of purified 2B8-Fc Δ XTFT clone 63. The red line corresponds to the chromatogram of 2B8-Fc purified from WT *N. benthamiana* leaves used as a control. Elution volumes of the peaks are indicated (ml).

5.4.5 Glycosylation analysis.

Glycan analysis of purified 2B8-Fc Δ XTFT was performed by LC-ESI-MS and compared to that of 2B8-Fc from WT *N. benthamiana* agroinfiltrated leaves. The analysis revealed the presence of predominant (~92%) GnGn structures lacking β 1,2 xylose and core α 1,3-fucose for 2B8-Fc Δ XTFT. In contrast, the presence of a major plant-typical GnGnXF glycan species (~74%), was revealed in 2B8-Fc (Fig. 9).

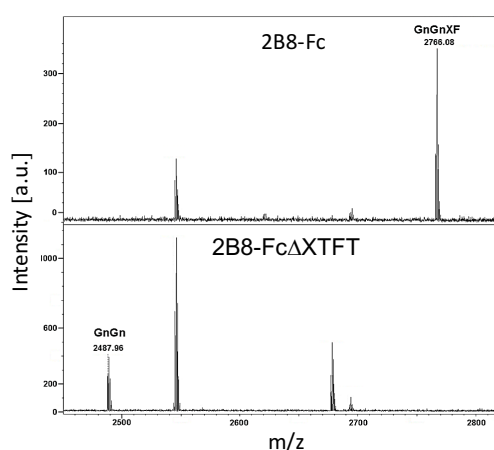


Figure 9: Glycan analysis of 2B8-FcΔXTFT purified from HR culture medium (upper panel) and 2B8Fc from agroinfiltrated *N. benthamiana* leaves (lower panel) by liquid chromatography-electrospray mass spectrometry (LC-ESI-MS) of tryptic glycopeptides. Doubly and triply charged ions of N-glycosylated variants of the expected tryptic fragments are indicated.

5.4.6 2B8-FcΔXTFT binding to Daudi cells.

Binding of 2B8-FcΔXTFT purified both from HRs and agroinfiltrated ΔXTFT *N. benthamiana* leaves to the CD20 antigen on the surface of human Daudi cells was analysed by flow cytometry. Both plant produced 2B8-FcΔXTFT bound to CD20 at comparable levels, even if the antibody purified from leaves showed slightly higher functional activity as indicated by mean fluorescence intensity values (MFI) (Fig. 10).

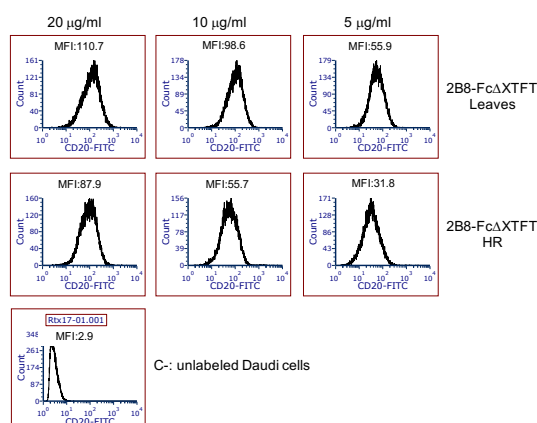


Figure 10: Binding of recombinant antibodies to CD20 expressed on Daudi cells. Cells were labelled with 2B8-FcΔXTFT purified from both HRs medium and from agroinfiltrated ΔXTFT *N.*

benthamiana leaves and analyzed by flow cytometry. Binding to CD20 was revealed with a goat anti-human IgG polyclonal Ab, which in turn was detected with a FITC-conjugated rabbit anti-goat IgG polyclonal Ab and quantified by mean fluorescent intensity values (MFI). C-: as negative control, cells labelled with only the secondary antibody were used.

5.5 Discussion.

The anti-CD20 immunotherapy represents one of the most important advances in the treatment of lymphoproliferative disorders in the last 30 years. Prior to its introduction, there had been only modest improvement in the treatment outcome of diseases such as follicular (FL) and diffuse large B-cell lymphoma (DLBCL). However, the use of rituximab, particularly in combination with various chemotherapy/radiotherapy regimes, has significantly improved all aspects of the survival statistics for these patients (Polito *et al.*, 2014). In addition, rituximab is approved, or being investigated for the treatment of many other hematologic disorders ranging from other malignancies, such as chronic lymphocytic leukemia (CLL), to autoimmune disorders, such as immune and thrombotic thrombocytopenic purpura and rheumatoid arthritis (Lim *et al.*, 2010). In this work we evaluated the production of an anti-CD20 2B8-Fc Δ XTFT recombinant antibody with a secretory scFv-Fc format in glycol-engineered Δ XTFT *N. benthamiana* hairy root cultures. The scFv-Fc format has several advantages: it can be easily engineered for plant transient expression, it generally retains its full antigen-binding activity and it maintains the Fc portion of human IgG1 immunoglobulins, which can activate different arms of human immune responses and can be efficiently expressed in plants or plant tissues at high levels (Marusic *et al.*, 2016). Hairy roots were generated by infecting glyco-engineered Δ XTFT *N. benthamiana* leaf discs with an *A. rhizogenes* strain bearing the 2B8-Fc coding sequence. Among 93 clones analyzed by Western blot analysis 30% were positive for 2B8-Fc Δ XTFT, slightly more than the 20% of positive clones obtained in a previous work for the mAb H10 (Lonoce *et al.*, 2016). The difference can be explained both with the higher number of analyzed clones and with the fact that for H10, a multiple gene co-

transformation strategy had been used, decreasing the probability and efficiency of transformation (Huang *et al.*, 2013). Non reducing Western blot analysis on positive clones exhibited an expected band at about ~110 kDa revealing the presence of 2B8-Fc Δ XTFT dimers but also a ~55 kDa band probably corresponding to the monomeric intact antibody chain in addition to a ~95 kDa band suggesting a possible antibody degradation product. Antibody degradation in hairy roots was already observed for the mAb H10 (Lonoce *et al.*, 2016) but minor differences were found with the same molecule expressed in agroinfiltrated leaves. Also in the case of 2B8-Fc Δ XTFT we noticed a similar degradation profile of the molecule produced in agroinfiltrated *N. benthamiana*, with a strong band at ~55 kDa corresponding to the monomer indicating a non complete assembly of 2B8-Fc Δ XTFT into its dimeric form (Marusic *et al.*, 2016). The positive clones were analyzed for recombinant protein accumulation by ELISA and hairy root clones 14, 27 and 63, which showed highest expression levels were chosen for further characterisation. For HR growth and secretion of the recombinant protein in liquid cultures we essentially followed a previously described protocol (Häkkinen *et al.*, 2014; Lonoce *et al.*, 2016) specifically devised for antibody production but with some minor changes. In particular, the main difference concerned the total amount of culture medium used for growth (30 ml of medium in a 200 ml flask instead of 40 ml) and also the frequency with which it was replaced during growth. In fact, by using less volume the transferred root were not completely submerged in the medium possibly permitting a better aeration of the tissues. Moreover, by refreshing the medium every ten days we noticed that roots had a healthy morphology without the appearance of a brownish color in the root tips typically observed with the previous protocol. To trigger the secretion of the antibody PVP, NAA and KNO₃ were added by using the same protocol previously described in chapter 4 (Lonoce *et al.*, 2016). As already observed in the case of HRs expressing mAb H10, 2B8-Fc Δ XTFT barely accumulated in the medium before induction and the highest antibody amount was typically detected 8 days after induction (chapter 4, Lonoce *et al.*, 2016). As expected, a residual amount of 2B8-Fc Δ XTFT was detected inside the root tissue, indicating that antibody secretion was not

completely efficient. The non complete secretion of the heterologous protein in the liquid culture medium has been already shown in other works; for example the mAb H10 was secreted at 20% of the total amount present in roots (Lonoce *et al.*, 2016) and the M12 in a range between 25 and 57%. For this reason, the development of more efficient protocols for antibody secretion would be of great importance to increase heterologous protein accumulation HR culture medium. Secreted 2B8-Fc Δ XTFT showed a very similar pattern to that observed in total root extracts, with major bands corresponding to 2B8-Fc Δ XTFT monomers (~55 kDa) and the assembled dimeric form (~110 kDa) but also degradation bands at ~95 and ~30 kDa. Most interestingly, analysis of the antibody trapped in the roots after the induction showed a single strong band at about ~55 kDa probably corresponding to 2B8-Fc Δ XTFT non assembled monomers while no bands corresponding to the assembled dimeric antibody or degradation fragments were observed. This probably indicated that the antibody is not fully assembled and is still retained in the root cells for further maturation processes. The total amount of 2B8-Fc Δ XTFT in the liquid culture medium for both clones 14 and 63 was 7.10 mg/L and 7.76 mg/L, respectively. These figures are higher than those previously reported for mAbH10 (2.24 mg/L) and this result could be explained by the the fact that the recombinant 2B8-Fc Δ XTFT behaves better for secretion in the culture medium but also that a larger number of clones were screened. In fact as already previously discussed, it is well known that genes are randomly inserted in different regions of the genomic DNA so, the positional effect plays a relevant role both in hairy root growth and morphology and in protein expression (Lonoce *et al.*, 2016). Even though, we observed no major differences between growth curves of 2B8-Fc Δ XTFT HR clones and those previously reported for mAb H10 (Lonoce *et al.*, 2016) indicating that biomass accumulation is probably not directly correlated to the final recombinant protein yield. Overall, the yield of 2B8-Fc Δ XTFT in the culture medium was remarkable, comparable to the highest yields reported in literature for a HR produced antibody as shown by Häkkinen and colleagues (5.9 mg/L for mAb M12) (Häkkinen *et al.*, 2014). Purification of the antibody from the culture medium by protein-A affinity chromatography reported yields of 5.57 mg/L, higher than

those previously obtained for mAb H10 (chapter 4, Lonoce *et al.*, 2016). Interestingly, the antibody recovered after purification represented about 70% of that secreted in the liquid medium for both clones, much higher compared with the 30% obtained for M12 (Häkkinen *et al.*, 2014) and slightly better than the 60% of recovery obtained for the mAb H10 (Chapter 4, Lonoce *et al.*, 2016). The non-reducing Coomassie stained gel of the purified 2B8-Fc Δ XTFT revealed a major band corresponding to the assembled dimeric antibody with only a minor fraction of the monomeric form at about 55 kDa. This result is in contrast to what observed in the Western blot analysis of the HR culture medium in which instead the 55 kDa band was the strongest one and could be explained by the fact that protein-A column preferentially binds to the Fc of the dimeric assembled antibody. An additional band at ~30 kDa was also visible in the reducing Coomassie stained gel confirming the presence of a specific degradation product as already shown in the Western blot analysis of HR culture medium of clones 14 and 63. the purified antibody was also assayed for its capacity to bind CD20 antigen expressed on Daudi cells. Flow cytometry analysis showed that 2B8-Fc Δ XTFT purified from HRs bound to CD20 with slightly lower efficiency compared to 2B8-Fc Δ XTFT purified from agroinfiltrated Δ XTFT *N. benthamiana* leaves. This result could be explained by the lower degradation observed in the purified 2B8-Fc Δ XTFT from leaves (Marusic *et al.*, 2016).

We demonstrated that the generation of HR clones from glyco-engineered Δ XTFT *N. benthamiana* is a general strategy to obtain tumour targeting recombinant antibodies of different formats with a human compatible glycosylation profile. This strategy has been successfully applied to obtain both the anti-Tenascin C human monoclonal antibody mAb H10 (Lonoce *et al.*, 2016) and the anti-CD20 recombinant 2B8-Fc Δ XTFT. Glycan analysis performed by LC-ESI-MS of 2B8-Fc Δ XTFT revealed the absence of β 1,2-xylose and α 1,3-fucose residues as previously reported for mAb H10.

It was previously demonstrated that the functionality and therapeutic potential of anti-CD20 antibodies relies on their capacity of activating a strong ADCC response and that this is strongly influenced by the glycosylation of the antibody

Fc. In particular, it was shown that fucose residues of the Fc of rituximab have a negative impact on ADCC activity (Gasdaska *et al.*, 2012). Therefore, future experiments will explore the immunotherapeutic potential of the glyco-optimized 2B8-Fc Δ XTFT by specifically characterizing its ADCC activity.

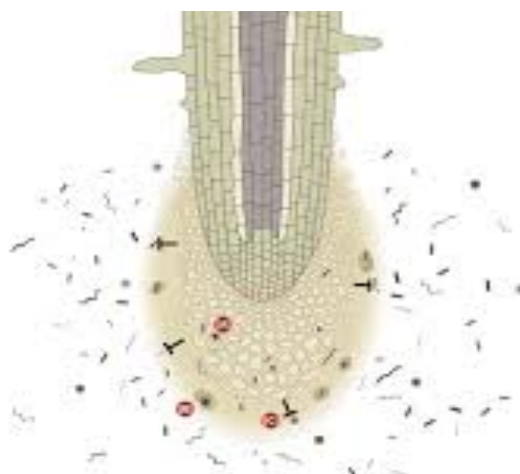
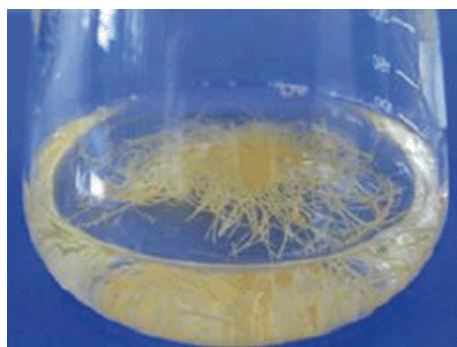
To conclude, we have demonstrated for the first time the possibility of obtaining a functional anti-CD20 recombinant scFv-Fc antibody in HRs. The screening of about 100 independent HR lines allowed the identification of clones with high expression levels. The glyco-optimized 2B8-Fc Δ XTFT was readily purified from the culture medium despite more efficient protocols need to be developed specially to obtain higher accumulation levels in the medium. The latter will be discussed in chapter 6.

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Chapter 6. Improving the secretion of recombinant proteins in hairy root cultures.



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6.1 Abstract.

Hairy root cultures represent an ideal platform for the production of heterologous proteins due to their rapid growth under contained conditions in bioreactors and for the possibility of secreting the molecule of interest in the culture medium. We demonstrated the possibility of accumulating the tumour-targeting mAb H10 and 2B8-Fc antibodies in glyco-engineered *N. benthamiana* hairy root cultures (HRs) by triggering active secretion of the recombinant protein with the plant auxin (1-Naphtalenacetic acid, NAA). Despite secretion of the recombinant antibodies has proved to be a valid strategy, to maximize purification recovery and to reduce production costs, the main limitation was related to the low yield of heterologous protein (about 2.5-7 mg/L). In order to evaluate the effects of different chemical compounds on recombinant protein secretion and to set-up improved protocols for the accumulation in the culture medium, we produced *N. benthamiana* HR cultures expressing a secretory version of the Red Fluorescent Protein (RFP). We demonstrated that RFP was accumulated in the apoplast and secreted from roots after the addition of NAA by measuring the relative fluorescence of the culture medium. These HRs were then used as a model system to easily screen for different auxins able to induce RFP secretion in the medium. Four different auxins were tested at different concentrations and results revealed that 2,4-Dichlorophenoxyacetic acid (2,4-D) at the concentration of 0.1 mg/L showed the highest accumulation of RFP in the culture medium (~350 mg/L). Moreover, to verify the efficacy of 2,4-D in the accumulation of valuable heterologous proteins, we evaluated the secretion of the tumour-targeting 2B8-Fc Δ XTFT from the transgenic Δ XTFT *N. benthamiana* HR line previously obtained. Data showed that accumulation level (~16 mg/L) of 2B8-Fc Δ XTFT with 2,4-D was almost doubled compared to those obtained using NAA. In conclusion, we demonstrated that of HRs expressing RFP are a valid model system to improve and study the secretion of heterologous proteins in HR cultures.

6.2 Introduction.

6.2.1 Hairy Roots: limits and challenges.

Expression of therapeutic proteins in *in vitro* plant systems under contained conditions represents an advantageous manufacturing strategy in terms of uniform cultivation conditions, product quality and downstream purification process (Schillberg *et al.*, 2013). Hairy root cultures represent a valid alternative thanks to their time efficiency in establishing transgenic HR lines, intrinsic genetic stability, fast biomass accumulation and manufacturing scalability (Talano *et al.*, 2012). Despite recent improvements, heterologous protein yield obtained from HRs is low compared with that obtained with other systems such as *Agrobacterium*-mediated transient expression. An example is the expression of the anti HIV microbicide cyanovirin-N (CV-N) in *Althaea officinalis* L. HRs in which expression levels were very low (0.02 µg /ml/24h) (Drake *et al.*, 2013). In order to overcome these disadvantages current efforts made by several research groups are aimed to: i) increase the expression of the heterologous protein; ii) enhance protein secretion from roots; iii) stabilize the heterologous protein in the culture medium. An example, was the strategy used to increase the accumulation of the sweetener protein thaumatin in *N. tabacum* HR lines by fusing the calreticulin signal to the N-terminus of the thaumatin encoding gene in order to retain the protein in the Endoplasmic Reticulum (ER). The expression levels of recombinant thaumatin were about 12-fold higher when the signal sequence was present. This was probably due both to the oxidized state of the ER that provides optimal conditions for disulfide bond formation, also due to the abundance of protein disulfide isomerases. Moreover, to improve stability and secretion of thaumatin in the medium the addition of polyvinylpyrrolidone (PVP) and sodium chloride led to an increase of protein amounts up to 2.63 mg/L (Pham *et al.*, 2012). There are several examples showing the increase of antibody accumulation in the culture medium by adding plant hormones. One is represented by the two monoclonal antibodies (mAb) Guy's 13 and 4E10, that were secreted from roots of transgenic hydroponic plants. It has been demonstrated that rhizosecretion rates increased significantly by adding the

NAA reaching levels of 58 $\mu\text{g/g}$ root dry weight/24h for mAb Guy's 13 and 10.43 $\mu\text{g/g}$ root dry weight/24h for mAb 4E10. In addition, the plant growth regulator indole-butyric acid 6-benzylaminopurine (IBA) was also tested for its ability to induce rhizosecretion of the recombinant antibodies but did not show significant effects (Drake *et al.*, 2009). Recently, data concerning the secretion of the full-size mAb M12 from HR cultures showed that the addition of NAA at different concentrations caused an increased in antibody accumulation in the culture medium. These results suggested that auxins are very important not only to promote root growth but also in inducing secretion of recombinant proteins (Häkkinen *et al.*, 2014). Interestingly, a recent work showed that the treatment of roots expressing mAb M12 with NAA over a prolonged period caused an altered root phenotype characterized by root hair proliferation, elongation and formation of nodular structures. This phenotype was more pronounced in roots induced with a higher concentration of NAA (Madeira *et al.*, 2016). The same phenomenon was also shown in HRs expressing the mAb H10 (Chapter 4, Lonoce *et al.*, 2016). Given these evidences, it is important to assay different auxins at different concentrations in order to find the best protocol for heterologous protein secretion from HRs. To this aim, in the present work, we generated HRs expressing a secretory version of the RFP to be used as a model system to study protein secretion from roots. Fluorescent proteins (FPs) present several advantages such as allowing the specific and targeted labeling of proteins in organelles, cells and tissues for imaging applications *in vivo* (Shcherbakova *et al.*, 2012). Red fluorescence protein is commonly used to study the localization of a protein of interest using confocal microscopy analysis. For example, the use of a membrane-tagged protein fused to the RFP facilitated the imaging of tomato roots and the localization of the protein in the root cap (Ron *et al.*, 2014). Another strategy used RFP as a tag for visual selection of transformed roots allowing rapid screening of positive clones (Lin *et al.*, 2011). In this work we describe a new protocol based on a comparison of different auxins to trigger the secretion in HRs expressing RFP, in order to improve the yield of heterologous proteins in the culture medium.

6.3 Materials & Methods.

6.3.1 Cloning of the RFP gene and transformation of *A. rhizogenes*.

The RFP coding sequence fused with the apoplast secretory signal peptide from phaseolin (SP) was amplified by PCR using as template the *rfp1* gene (gently provided by Prof. Lorenzo Frigerio, University of Warwick, UK) and as primers the following oligonucleotides: RFP-for (5'-ATCGAGGATCCATGATGAGAGCAAGGGTTC CACTCC-3') and RFP-rev (5'-TCCCCCGGGTTAGGCGCCGGTGGAGTGGC-3') adapted from Hunter *et al.*, (2007)., The primers RFP-for and RFP-rev carried the BamHI and the XmaI restriction sites respectively (underlined) to allow the cloning into the pGEM-Nos plasmid, carrying the nopaline synthase terminator (Nos-ter) from *A. tumefaciens*. Using the BamHI/EcoRI restriction sites (in order to include the nos-terminator) the fragment SP-RFP-Nos was cloned into the binary vector pBI- Ω (previously described by Marusic *et al.*, 2007). The pBI-RFP construct, once verified by DNA sequencing, was electroporated into *A. rhizogenes* A4.

6.3.2 Generation of HRs expressing the RFP and secretion in the culture medium.

A. rhizogenes A4 strain carrying pBI-RFP expression vector was used to transform *N. benthamiana* leaf segments obtaining HR cultures essentially as described in Chapter 4 (Lonoce *et al.* 2016). To obtain cultures in liquid medium about 0.2 g of *N. benthamiana* HR tissue was seeded in 150 ml Erlenmeyer flasks, using a starting volume of 5 ml of MS medium supplemented with 3 % sucrose increasing up to 30 ml after 20 days. Flasks were orbitally shaken at 80 rpm in the dark, at 24-26 °C for 4-5 weeks. Liquid medium was replaced every ten days, by adding fresh MS with sucrose. Heterologous protein secretion was triggered at day 20 (after inoculum) by the addition of MS culture medium supplemented with 19 mg/L of NAA. Two days after the first induction, flasks were again supplemented with 19 mg/L of NAA and left for 8-11 days before collecting the culture medium. An aliquot of HR culture medium (50 μ l) was collected at different time points, before (day 0, T0) and after induction (T2, T8,

T10) in order to analyze the accumulation of RFP by Western blot analysis and fluorimetric analysis. In the following tests HR cultures were similarly induced with indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) in different concentrations as 0,1 mg/L, 0.5 mg/L, 1 mg/L, 5 mg/L and 10 mg/L. An aliquot of HR culture medium (50 μ l) was collected at different time points, before (T0) and after induction (T2, T8, T10) in order to analyze the accumulation of RFP by fluorimetric analysis.

6.3.3 Screening of RFP positive HR clones.

Leaf discs infected with *A. rhizogenes* carrying pBI-RFP were left on MS plates for about 30 days, and emerging HRs were analyzed using a mercury vapor lamp (Osram Sylvania, 68846), exciting at 550-635 nm to visualize RFP expression. Positive HR clones (appearing with a red-purple color), were placed on MS plates containing 3% sucrose, 100 mg/L of kanamycin and 0.3 g/L of cefotaxime. For the complete eradication of *A. rhizogenes*, HRs were transferred in plates with decreasing cefotaxime concentrations (0.25 g/L, 0.125 g/L, 0.05 g/L) until no antibiotic was added.

6.3.4 Confocal microscopy analysis.

Hairy root samples were excised from the plates, mounted on a microscope slide in water and analyzed by a Zeiss LSM 880 confocal microscope, using either a $\times 10$ $\times 20$ $\times 40$ or $\times 63$ oil objective lens. Red fluorescent protein was excited at 510-570nm and detected in the 571-638 nm range. The confocal microscope settings were kept constant throughout experiments. Image processing was done by using Zeiss Zen Blue edition software.

6.3.5 Fluorimetric analysis of culture medium.

Culture medium collected from HR cultures expressing RFP at different time points before and after induction, were centrifuged at 13000 x g and then analyzed using the VICTOR fluorimeter (PerkinElmer, Singapore) by using an excitation wavelength of 550 nm and an emission of 600 nm. Serial dilutions of

commercial RFP (Thermo Fisher Scientific, UK) starting from of 500 ng, were used as internal standard for quantification, while MS culture medium alone was used as negative control. Three different biological replicas were performed and each HR sample was assayed in triplicate and RFP amounts were interpolated in the linear portion of the standard curve.

6.3.6 Western blot analysis of HR clones.

To assay the presence of the 2B8-Fc Δ XTFT or RFP secreted in HR liquid cultures, the culture medium was collected at different time-points before and after induction and filtered using a MiraclothTM (Millipore). The filtered medium was then centrifuged at 13000 x g, 16 °C for 15 min, proteins were separated on 10-12% SDS-PAGE under non-reducing conditions essentially as previously described (chapter 4). 2B8-Fc Δ XTFT detection was performed with anti-human γ chain horseradish peroxidase conjugated (A8419 Sigma-Aldrich) diluted 1:5000 in 2% (w/v) non-fat milk in PBS. RFP detection was performed using an anti RFP antibody 6G6 (Chromotek, UK) diluted 1:3000 in 2% (w/v) non-fat milk in PBS and as secondary an anti-mouse HRP (KPL 4741802, UK) diluted 1:5000 in 2% (w/v) non-fat milk in PBS. Membrane was developed using ECL Plus Western blotting detection system (ECL Plus; GE Healthcare). The ImageQuantTM LAS 500 (GE Healthcare, Germany) was used for chemiluminescence signal detection. Sharp Mass VI (Euroclone, Italy) or Pink plus (GeneDireX, USA) were used as protein molecular weight markers.

6.3.7 ELISA assays.

Aliquots of HRs collected at different time-points before (T0) and after induction (T8, T10) were measured by quantitative ELISA as previously detailed (Chapter 5). Serial dilutions of purified 2B8-Fc were used as a positive internal standard for quantification, starting from a concentration of 500 ng/well while PBS 1X was used as negative control. Three different biological replicas were performed and each HR sample was assayed in triplicate and 2B8-Fc Δ XTFT values were interpolated in the linear portion of the standard curve.

6.3.8 Statistical analysis.

Statistical analysis was performed using Student's *t*-test on Graphpad Prism Software (version 6.01). P values < 0.05 were considered statistically significant.

6.4 Results.

6.4.1 Generation of HRs expressing RFP.

N. benthamiana leaf discs were infected with *A. rhizogenes* A4 strain carrying the RFP gene coding sequence fused to the secretory signal peptide SP from phaseolin (Hunter *et al.*, 2007) (Fig. 1). Infected leaf discs were cultured for about 20 days in MS plates (supplemented with 3% sucrose) and emerging hairy roots clonal lines expressing RFP were visualized and selected by using a mercury vapor lamp (Fig. 2A) that allowed a very fast and efficient preliminary screening. The best expressing clones were then analyzed by confocal microscopy (Fig. 2B) (510-570 nm/excitation and 565-645 nm/emission) that revealed fluorescence in the area included between cells, indicating localization of RFP in the apoplast (Fig. 2C). In addition, RFP was observed homogeneously in all the tissues, including hairy lateral roots (Fig. 2C). Among 86 positive clones, clone 17 showed the highest expression levels and was chosen for further analysis (Fig. 3).



Figure 1: Schematic representation of pBI-RFP expression vector. RFP encoding gene bearing a signal peptide sequence for secretion (*SP-rfp*) was cloned in the pBI-Ω plant expression vector. The gene was under the control of 35S cauliflower mosaic virus (CaMV-35S) constitutive promoter fused to the translation enhancer sequence of tobacco mosaic virus (Ω) and nopaline synthase terminator (Nos-ter) from *A. tumefaciens*. The nptII represents the neomycin phosphotransferase II gene that confers resistance to kanamycin. Nos-pro, nopaline synthase promoter from *A. tumefaciens*.

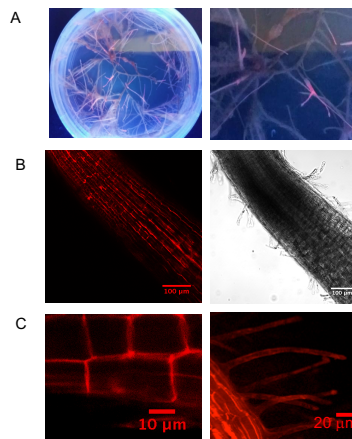


Figure 2: Example of Hairy root clone 17 expressing RFP visualized using a mercury vapor lamp (A, B). Typical confocal microscopy image of a root from clone 17 visualized at 10x magnification with (B left panel) and without red channel activation (B right panel). Detail of the root showing RFP apoplast accumulation at 40x magnification (C left panel). Detail of lateral root hairs showing high accumulation of RFP (C right panel) at 20x magnification.

6.4.2 Secretion of RFP in culture medium.

Hairy root cultures of clone 17 were established in liquid MS medium. Secretion of RFP was obtained as previously reported (chapter 4; Häkkinen *et al.*, 2014) by adding naphthaleneacetic acid (NAA) at a concentration of 19 mg/L, without addition of potassium nitrate (KNO_3) and polyvinylpyrrolidone (PVP). The auxin was added at day 20 and 22 days post-inoculation and flasks were left for other eight days (30 days after inoculum) prior collecting the last aliquot. Clarified culture medium samples, showing a visible red color gradient (Fig. 3A), were collected before (T0) and after induction (T2, T8, T10) and measured with a fluorimeter using excitation/emission wavelengths of 531/590 nm (Fig. 3B). Results showed increasing concentration of RFP during the time-course, with a peak 8 days after induction (T8). Accumulation of RFP in the medium was also analyzed by reducing 12% SDS-PAGE followed by Coomassie stain (Fig. 3C). A band at about 30 kDa corresponding to the intact RFP and additional degradation products of about 22 and 14 kDa were revealed. These results were confirmed by Western blot analysis (Fig. 3D), that showed the same band pattern observed in the Coomassie. Confocal microscopy analysis was also performed on the HR clone 17 before (T0) and after induction (T8) (Fig. 4A and 4B, respectively), keeping constant photomultiplier gain and offset settings throughout experiments. The results revealed that RFP signal in the apoplastic space of induced roots is typically lower than the one observed in non-induced hairy roots (T0).

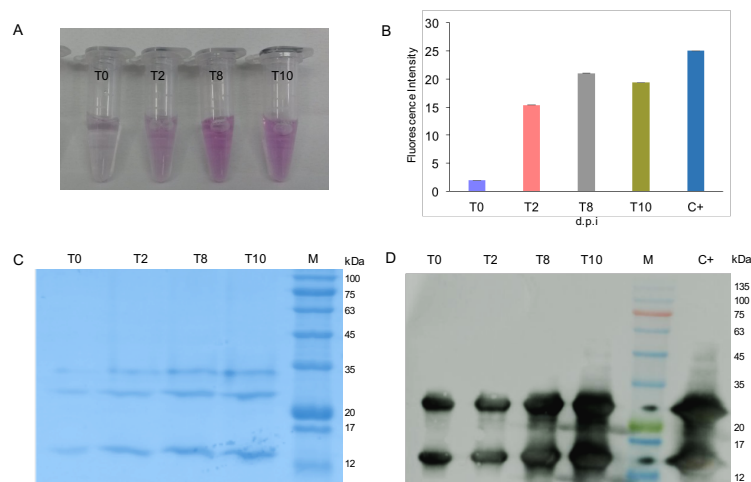


Figure 3: Analysis of RFP expression. (A) Liquid culture medium from HRs expressing RFP induced with NAA collected at different time-points, before (T0) and after induction (T2, T8, T10). (B) Fluorimetric analysis of culture medium samples (50µl) before (T0) and after induction with NAA 19 mg/L (T2, T8, T10). Three micrograms of commercial RFP were used as positive control (C+). (C) Coomassie stain performed by loading 5 µl of the medium samples collected before (T0) and after induction (T2, T8, T10). (D) Western blot analysis performed by loading 1 µl of the samples; as positive control, 100 ng of commercial RFP, were used. M: molecular weight marker. d.p.i.: days post induction.

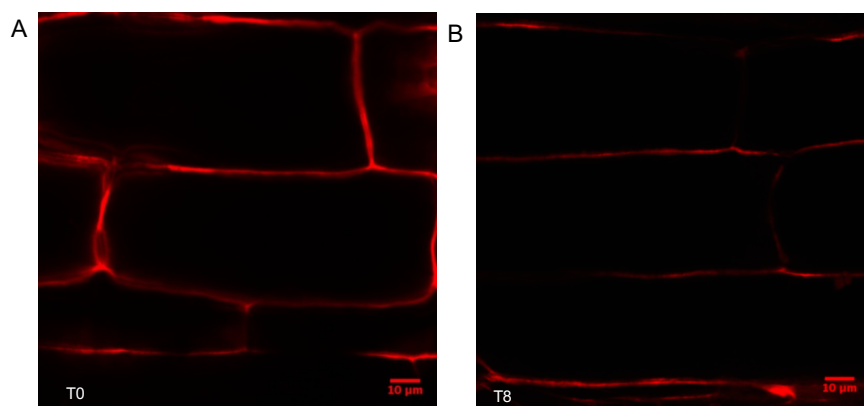


Figure 4: Typical confocal microscopy analysis performed on HRs of clone 17 expressing RFP before (T0) and after induction (T8). Magnification 63x: Images were acquired keeping constant photomultiplier gain and offset settings.

6.4.3 RFP secretion in the culture medium by using different auxins.

In order to increase the yield of heterologous protein in the culture medium, the secretion of RFP was induced by adding different auxins such as 1-Naphthalene acetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). To this aim, hairy root

cultures expressing RFP were induced at 20 and 22 (T2) days post-inoculation and flasks were left for other eight days (30 days after inoculum) prior collecting the last aliquot (T10). Samples were collected from culture medium before (T0) and after induction at all time points (T2, T8, T10). Culture medium samples (50 μ l) clarified by centrifugation were measured using a fluorimeter and quantification of RFP in terms of mg/L was performed using a calibration curve obtained with a commercial RFP. All cultures showed a peak of RFP accumulation ten days after induction (T10), except for IBA that displayed a higher amount eight days after induction (T8) (Fig. 5). The highest concentration achieved with of NAA was 235 ± 10 mg/L of RFP at the concentration of 1mg/L (Fig. 5A), while the induction performed with 1mg/L of 2,4-D led to the reported value of 228 ± 27 mg/L of RFP (Fig. 5B). Lower amounts of secreted RFP were also obtained using IAA and IBA (152 ± 11 mg/L and 118 ± 11 mg/L, respectively) (Fig. 5C-5D).

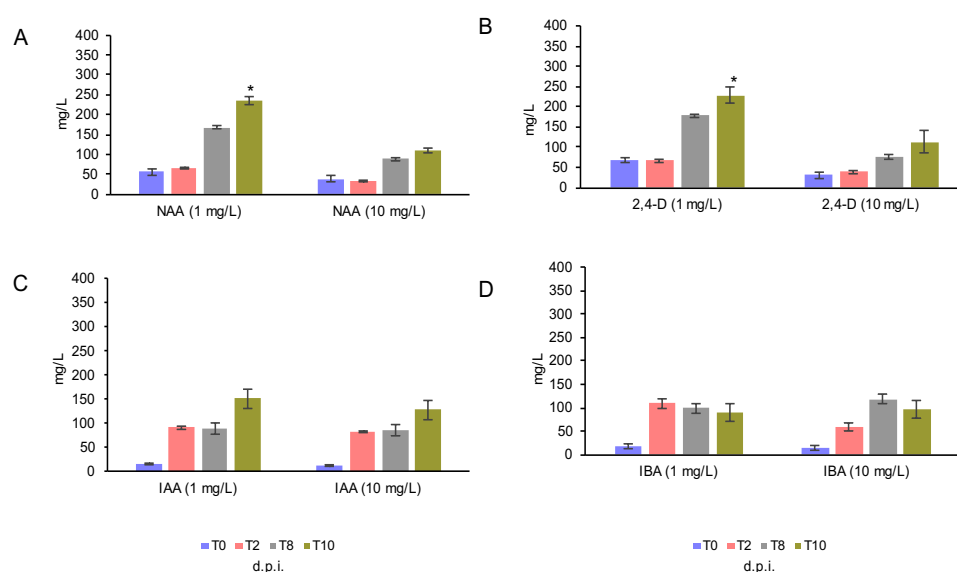


Figure 5: RFP secretion in the culture medium by using different auxins. Amount of RFP in the HR culture medium was quantified by fluorimetric analysis before (T0) and after induction (T2, T8, T10) with NAA (A), 2,4-D (B), IAA (C) and IBA(D) added at the concentration of 1 mg/L or 10 mg/L. Data analysis was performed from three independent experiments. Values represent mean SD. * $P < 0.05$ for NAA T10 (1mg/L) versus NAA (10 mg/L) and for 2,4-D T10 (1mg/L) versus 2,4-D (10mg/L). d.p.i.: days post induction.

6.4.4 Improving RFP secretion in the culture medium using NAA and 2,4-

D.

Among all the auxins tested NAA and 2,4-D showed the highest RFP accumulation levels in the culture medium with the lowest hormone concentration tested (1 mg/L). An additional analysis has been carried out with a specific range of concentrations in order to select the best conditions for high-yield RFP secretion. To this aim, HRs expressing RFP were induced with 0.1, 0.5, 1 and 5 mg/L of both NAA and 2,4-D and the culture medium collected at different time-points before (T0) and after (T2, T8, T10) induction was analyzed using the fluorimeter. The best yields were obtained by adding either 0.1 mg/L of 2,4-D or 0.5 mg/L of NAA, reaching levels of 334 ± 22 mg/L and $240 \text{ mg/L} \pm 11$ of RFP, respectively (Fig. 6). Also in this case the highest accumulation was obtained at day 10 post induction. A typical result of the induction with 2,4-D (0.1 mg/L) of a HR culture of 30 ml in a 150 ml flask is shown in Figure 7A. The high accumulation of RFP in the medium 10 days after induction (T10) and differences between induced (T10) and not induced (T0) HR cultures are clearly visible. Accumulation of RFP is even more evident illuminating flasks (Fig. 7B, T0, T10 upper panel) and the clarified culture supernatant (Fig. 7B, T10 lower panel) using a mercury vapor lamp. As negative control hairy root expressing the 2B8-Fc Δ XTFT antibody have been used (Fig. 7B, C-). Samples collected from HR cultures (clone 17) induced with 0.1 mg/L of NAA (Fig. 8A) or 2,4-D (Fig. 8B) were separated by reducing SDS-PAGE and gels were stained with Coomassie. Analysis revealed the presence of a major band at about 30 kDa corresponding to the intact RFP and minor bands of about 22 and 14 kDa, possibly corresponding to degradation products. As positive control (C+) a commercial RFP was loaded. Moreover, Western blot analysis performed on the 2,4-D induced samples using an anti-RFP antibody showed, as expected, the same band pattern observed in the Coomassie stained gel (Fig. 8C). Densitometric analysis of bands in the Coomassie stained gel revealed that RFP represented about 50% of total proteins in the culture medium.

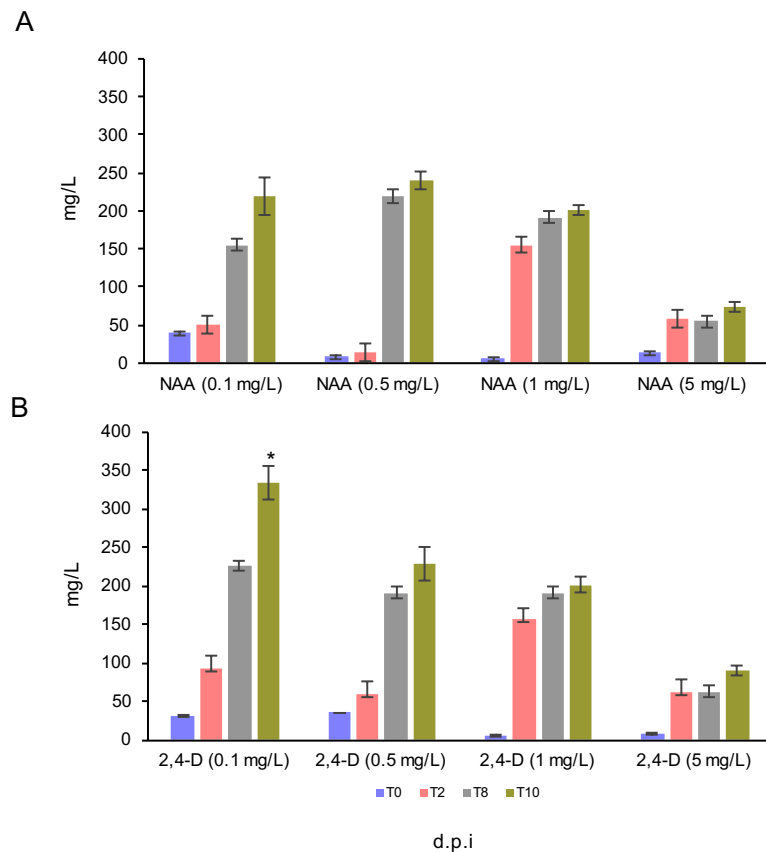


Figure 6: Accumulation of RFP in HR culture medium at different time-points before (T0) and after induction (T2, T8, T10) with different concentrations of NAA (A) or 2,4-D (B). Data analysis was performed from three independent experiments. Values represent mean SD. * $P < 0.05$ for 2,4-D T10 (0.1 mg/L) versus the other 2,4-D concentrations and versus all NAA concentrations. d.p.i.: days post induction.

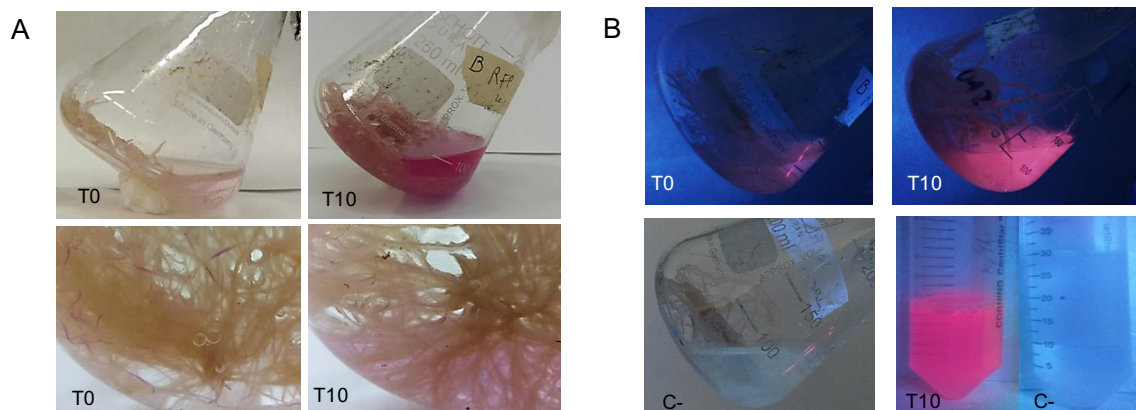


Figure 7: Hairy root culture clone17 expressing RFP (induced with 2,4-D 0.1 mg/L) was visualized under normal light (A) before (T0) and 10 days after induction (T10). In panel B the same culture illuminated with a mercury vapor lamp. Clarified culture supernatant collected in a

Falcon (T10) was also visualized with a mercury vapor lamp. As a negative control (C-), a HR culture expressing the 2B8-Fc Δ XTFT antibody was used.

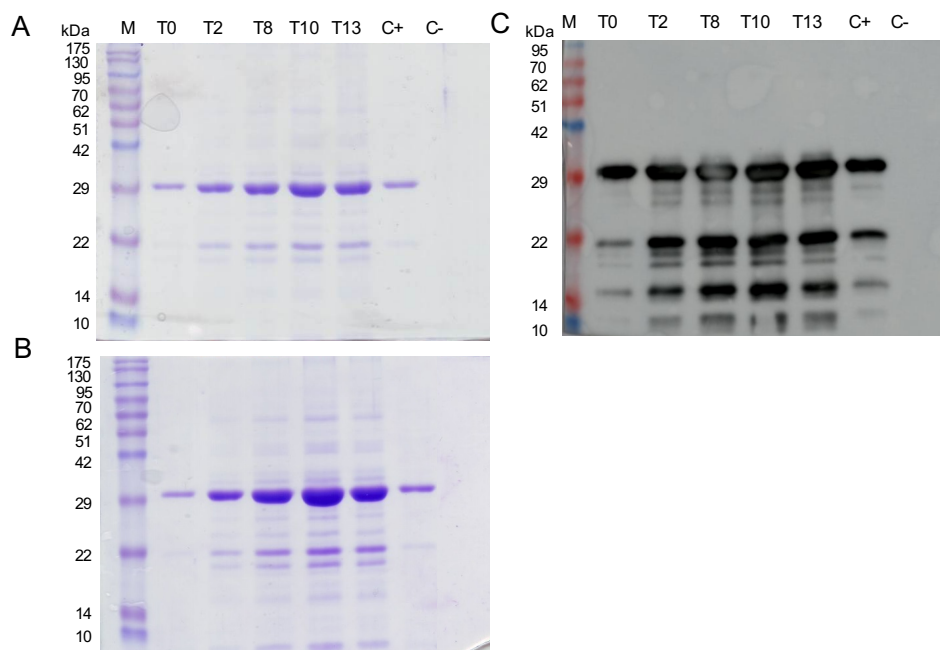


Figure 8: Accumulation of RFP in the culture medium. Coomassie stained SDS-PAGE analysis of HR culture medium samples expressing RFP induced with 0.1 mg/L of NAA (A) or 0.1 mg/l of 2,4-D (B). Samples (10 μ l) were collected before (T0) and after induction (T2, T8, T10, T13) with the hormones. Western blot analysis performed on the 2,4-D induced samples using an anti-RFP antibody (0.5 μ l of samples were loaded on gel). C+: Commercial RFP was used as positive control (200 ng); As a negative control, a HRs expressing the 2B8-Fc Δ XTFT antibody was used (C-). M: Molecular weight marker.

6.4.5 Secretion of the tumour-targeting 2B8-Fc Δ XTFT in HRs culture medium using NAA and 2,4-D.

In order to validate the HR induction protocol based on NAA and 2,4-D to trigger the secretion of a tumour-targeting protein of interest in the culture medium, 20-days old HRs expressing 2B8-Fc Δ XTFT were induced by adding the two hormones. Optimal concentrations of NAA and 2,4-D (0.1 mg/L) were used based on the previous results obtained with RFP. Culture medium aliquots were collected before (T0) and after induction (T8 and T10) and analyzed by non-reducing 12% SDS-PAGE and Western blot analysis (Fig. 9A). A major band at about 110 kDa corresponding to the intact dimeric antibody was visible in all samples after induction. Intensity of this band was stronger in the T10

samples. The induced samples showed also a degradation product at about 95 kDa and a very faint band at ~ 55 kDa that probably corresponds to the non-assembled monomeric 2B8-Fc Δ XTFT (Fig. 9A). To quantify the antibody, in the culture medium, collected samples were analyzed by ELISA, adding serial dilutions onto anti-HC antibody coated wells. A secondary anti-HC HRP-conjugated antibody was used for detection. As positive control (C+), serial dilutions of 2B8-Fc Δ XTFT purified from agro-infiltrated Δ XTFT *N. benthamiana* leaves was used. The amount of 2B8-Fc Δ XTFT in the medium was 16.3 ($\pm$ 2.4) mg/L in the case of HRs induced with 2,4-D and 8.35 ($\pm$ 1.02) mg/L with NAA. It must be noted that no significant differences in root fresh weight were detected between roots treated with NAA or 2,4-D in all the three biological replicates, nor between induced and non-induced HR cultures (data not shown).

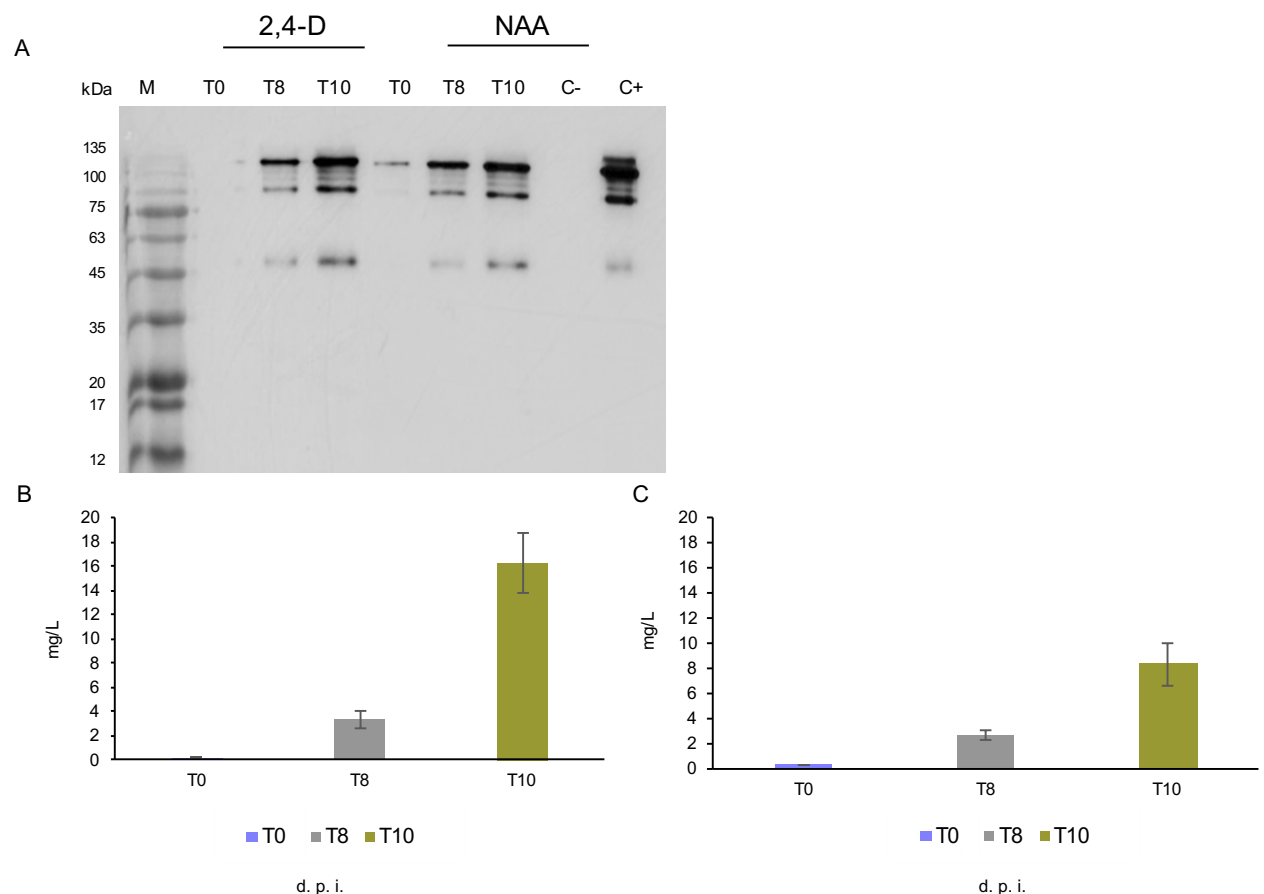


Figure 9: Accumulation of 2B8-Fc Δ XTFT in HR culture medium. (A) Western blot analysis using an anti-HC on HR cultures expressing 2B8-Fc Δ XTFT induced by adding of NAA or 2,4-D

(0.1mg/L) at different time-points before (T0) and after induction (T8 and T10). M: molecular weight marker; C+: 2B8-Fc Δ XTFT purified from agroinfiltrated *N. benthamiana* leaves. C-: a HRs expressing the RFP was used. Accumulation levels of 2B8-Fc Δ XTFT in the culture medium quantified by ELISA, at different time-points before (T0) and after induction (T8 and T10) with 2,4-D (B) and NAA (C). All the experiments were conducted in triplicate. Values represent mean SD. d.p.i.: days post induction.

6.5 Discussion.

Hairy roots are a valid expression system for heterologous protein production due to several advantages such as the possibility of working in safe contained conditions, fast biomass accumulation, lack of human pathogens, secretion of the recombinant product in the culture medium. On the other hand, one of the limitations of this system is the low yields of recombinant protein generally obtained compared to other systems such as *Agrobacterium* mediated transient expression platforms. Several strategies have been adopted in order to increase heterologous protein yield in the liquid culture medium as for example the use of PVP or gelatin for their protein stabilizing and colloidal properties, that has been shown to enhance intact antibody accumulation in culture medium by almost 3 fold, although their use could be a disadvantage in the purification downstream processing (Häkkinen *et al.*, 2014; Lee *et al.*, 2002). Another strategy contemplates the use of KNO_3 as source of nitrate for root growth. The addition of KNO_3 to the liquid culture was shown to increase the amount of the monoclonal antibody M12 recovered from the medium by potentially increasing the capacity of the protein synthesis machinery in the roots (Häkkinen *et al.*, 2014). Another work characterizing the secretion of the M12 antibody from roots of tobacco plants showed that the addition of nitrate enriched hydroponic medium increased antibody accumulation by more than 40 fold reaching levels of 45mg/L (Madeira *et al.*, 2016). The most effective strategy to specifically increase the secretion of the recombinant protein in the culture medium was shown to be the use of auxins. Hairy roots naturally produce auxins, but it was demonstrated that by adding auxin to the culture medium, the yield of recombinant protein increased consistently. In particular, it was demonstrated that auxins play a role in root development by increasing lateral and adventitious root formation that may provide increased surface area for the production and secretion of recombinant antibodies (Drake *et al.*, 2009; Häkkinen *et al.*, 2014). Another observation was made in the tobacco hydroponic culture medium induced with auxins identifying mRNAs encoding several cell wall remodelling genes, including a subtilisin-like protease and expansins that may attest a role of auxins in cell wall remodeling (Madeira *et al.*,

2016). In a recent work, it was possible to predict the optimal amount of KNO_3 , PVP and the auxin NAA necessary to boost the accumulation of the monoclonal antibody M12 in the spent medium using a mathematical model. The secretion capacity was tested in three individual root clones and the optimal induction medium was added with 14 g/L KNO_3 , 19 mg/L NAA, and 1.5 g/L PVP to obtain a maximum antibody yield of 5.9 mg/L (Häkkinen *et al.*, 2014). The previous examples show that due to the complexity of the system, it is very difficult to set up novel protocols to increase the secretion yield of recombinant proteins in HR culture medium. The variables to be taken into account are numerous and the detection system used to assay the yield of recombinant protein in the medium may be difficult and laborious. For this reason, we decided to simplify this task by devising a model system based on HR cultures expressing the RFP fused with an apoplast secretion sequence that could allow the rapid testing of different substances and their impact on recombinant protein secretion. Using this model system, we tested the role of different auxins in the induction of protein secretion in *N. benthamiana* HRs expressing secretory RFP. Characterization of these roots by confocal microscopy showed, as expected, that RFP was localized in the apoplastic space. Furthermore, we also assayed the capacity of these roots to secrete RFP in the medium after induction with NAA (19 mg/L) which is the most used auxin for HR induction as described in literature. We omitted on purpose the use of KNO_3 and PVP to evaluate solely the effect of the hormone. We were able to show by microscopy that two days after the addition of NAA, RFP fluorescence in the apoplast strongly decreased indicating an active secretion in the medium. Furthermore, fluorescence analysis of the medium confirmed the accumulation of the recombinant protein that reached a maximum peak 10 days after induction that was also confirmed by Western blot analysis. Next step was to compare the effect of different auxins on the secretion of RFP in the culture medium. To this aim (Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α -Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) were tested by adding two different concentrations in the medium 1 mg/L and 10 mg/L. The selection of this range of concentrations was chosen based on previous studies in literature (Häkkinen

et al., 2014; Madeira *et al.*, 2016). By simply measuring the fluorescence of the culture medium at different time points after the addition of auxins we showed that the highest accumulation of RFP was obtained using NAA and 2,4-D at the lowest concentration of 1g/L. Both IBA and IAA performed better at higher concentrations (10mg/L) but with very low accumulation levels of RFP. Additional tests using a different range of concentrations (0.1, 0.5, 1, 5 mg/L) of NAA and 2,4-D revealed that the highest accumulation of RFP was still obtained using the lowest concentration of 2,4-D (0.1 mg/L). This result is important since high concentrations of hormones in the culture medium could represent a limitation in the downstream purification processes of recombinant proteins from the culture medium. A Coomassie stained gel performed on collected culture medium samples at different time-points after induction showed a specific very abundant band corresponding to the monomeric RFP, with a maximum peak 10 days post induction and some minor bands possibly corresponding to the degraded protein. At this time point RFP reached a concentration of 334 mg/L, which represents the highest level ever reported for a secreted recombinant protein in HRs. Most interestingly analysis of the medium showed that RFP was highly represented accounting for a high percentage of all proteins secreted in the medium (~50% as evaluated by densitometric analysis of Coomassie stained gels). This result is extremely high compared to previous results which showed an accumulation of the M12 antibody in the hydroponic medium of tobacco plants to levels of ~25% of all secreted proteins while cyanovirin-N produced with the same system accounted for 13% of all proteins (Madeira *et al.*, 2016). Moreover, we showed that auxins have no influence on the overall accumulation of biomass after induction since no difference was found comparing fresh root weight of induced versus non induced HR cultures indicating a direct effect on heterologous protein secretion in the medium. To validate this new protocol on a recombinant protein of interest, the HR clone 63 expressing the tumour-targeting 2B8-Fc Δ XTFT recombinant antibody was induced either with NAA or 2,4-D (0.1mg/L) and accumulation of the protein in the medium was compared and quantified by ELISA. We showed that highest levels of accumulation were obtained with 2,4-D reaching levels of 16.3 mg/L

which were nearly two times higher compared to those obtained with NAA and to those previously reported in chapter 5 (7.76 mg/L). Furthermore, we showed that the protein profile of the antibody produced in hairy roots is very similar to that obtained in agroinfiltrated *N. benthamiana* leaves.

In conclusion, we established an HR root clone producing the RFP that was used as model system to optimize protocols for the secretion of heterologous proteins in the culture medium of hairy roots. We compared different auxins to induce protein secretion and found that the highest RFP yield of 334.24 mg/L was obtained by adding 0.1 mg/L of 2,4-D to the liquid culture medium. This accumulation level in the medium was the highest reported for heterologous proteins produced in HRs. The same protocol was tested on the tumour targeting 2B8-Fc Δ XTFT recombinant antibody showing very high accumulation levels (16.3 mg/L) even without the use of KNO₃ or the stabilizer PVP. Future efforts need to be focused on devising novel improved induction protocols to enhance recombinant protein expression and secretion, in order to transform HRs into a competitive production system for heterologous protein production.

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Chapter 7. Published work.

Research Article

Production of a tumor-targeting antibody with a human-compatible glycosylation profile in *N. benthamiana* hairy root cultures

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Hairy root (HR) cultures derived from *Agrobacterium rhizogenes* transformation of plant tissues are an advantageous biotechnological manufacturing platform due to the accumulation of recombinant proteins in an otherwise largely protein free culture medium. In this context, HRs descending from transgenic *Nicotiana tabacum* plants were successfully used for the production of several functional mAbs with plant-type glycans. Here, we expressed the tumor-targeting monoclonal antibody mAb H10 in HRs obtained either by infecting a transgenic *N. tabacum* line expressing H10 with *A. rhizogenes* or a glyco-engineered *N. benthamiana* line (Δ XTFT) with recombinant *A. rhizogenes* carrying mAb H10 heavy and light chain cDNAs. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields (2–3 mg/L). *N*-glycosylation profiles of antibodies purified from HR supernatant revealed the presence of plant-typical complex structures for *N. tabacum*-derived mAb H10 and of GnGn structures lacking xylose and fucose for the Δ XTFT-derived counterpart. Both antibody glyco-formats exhibited comparable antigen binding activities. Collectively, these data demonstrate that the co-infection of Δ XTFT *Nicotiana benthamiana* with recombinant *A. rhizogenes* is an efficient procedure for the generation of stable HR cultures expressing the tumor-targeting mAb H10 with a human-compatible glycosylation profile, thus representing an important step towards the exploitation of root cultures for the production of 'next generation' human therapeutic antibodies.

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Abbreviations: FW, fresh weight; GFP, Green Fluorescent Protein; GUS, β -glucuronidase; HC, heavy chain; HR, hairy root; LC, light chain; LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; MS, Murashige and Skoog medium; NAA, 1-naphthaleneacetic acid; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; TNC, tenascin C

1 Introduction

Expression of therapeutic proteins in in vitro plant systems under contained conditions represents an advantageous manufacturing strategy in terms of uniform cultivation conditions, product quality and downstream purification process [1]. Indeed, the first plant-derived human therapeutic protein, taliglucerase α for Gaucher's disease treatment, is currently produced in carrot cells [2]. Along with cell suspension cultures, hairy root (HR) cultures

* These authors contributed equally to this work.

represent a valid alternative thanks to their time efficiency in establishing transgenic HR lines, intrinsic genetic stability, fast biomass accumulation and manufacturing scalability [3]. HR cultures are normally generated by infecting transgenic plants expressing the recombinant protein with *Agrobacterium rhizogenes* or by transforming plant tissues with *A. rhizogenes* strains bearing a plant expression vector encoding the protein of interest [4]. These cultures lead to the formation of extensive secondary roots that can be cultivated under contained sterile conditions in hormone free media. Importantly, recombinant proteins can be secreted in the culture medium facilitating the downstream purification processes [5]. Hairy roots have been successfully used to express a wide range of complex recombinant proteins, such as enzymes [6], vaccine components [7], monoclonal antibodies (mAbs) [8] and anti-HIV microbicides [9]. Antibodies produced in *Nicotiana tabacum* HRs demonstrated that accumulation in the culture medium can be improved by the addition of hormones as well as protein stabilizing agents such as polyvinylpyrrolidone (PVP) [7, 9]. Recently, an interesting study showed that components such as 1-naphthaleneacetic acid (NAA) can rise yields of the tumor targeting human IgG1 M12 produced in *N. tabacum* HRs by even 30-fold (≈ 6 mg/L of culture medium) [11]. Despite recent improvements, antibody yields obtained in HRs do not meet those reported for other plant expression systems, such as transgenic plants and *Agrobacterium*-based transient expression systems. Infact, by agroinfiltration of *Nicotiana benthamiana* leaves using plant virus derived expression vectors, up to 500 mg/kg FW of the human tumor-specific mAb A5 were obtained [12].

The goal of the present work was to produce the tumor-targeting antibody mAb H10 in HR cultures derived from glyco-engineered Δ XTFT *N. benthamiana* with the aim of improving the quality of the final product. This human IgG1 (λ) derived from a selected single-chain variable fragment (scFv) is directed against the C domain of the large isoform of the tumor-associated antigen tenascin C (TNC). TNC has been associated with a variety of tumors including breast, squamous cells, lung and prostate carcinomas, melanoma and malignant glioma [12, 13]. Anti-TNC antibodies have been recently used with success for the therapy of patients with acute myeloid leukemia and metastatic breast cancer [14, 15].

An important feature for many therapeutically interesting mAbs is proper Fc-glycosylation. It is now well established that certain glycan structures may alter the function of antibodies [17]. A prominent example is the lack of core fucose, which confers increased in vitro and in vivo activities [12, 13]. Recently it was shown that a mAb produced in wild type *N. tabacum* HRs carries, as many other plant-produced glycoproteins, complex structures bearing plant typical β 1,2-xylose and α 1,3 fucose [11]. A series of studies demonstrated that plants are

particularly amenable to glycan engineering towards human-type structures as reviewed recently [20]. A major achievement was the elimination of plant-typical fucose and xylose residues using RNA interference, as in the case of duckweed (*Lemna minor*) and Δ XTFT *N. benthamiana* transgenic line [15, 16]. However, whether mAbs with modulated N-glycan profiles can be produced in HR cultures was not investigated so far.

In this study we have monitored different approaches for the generation of HRs producing human mAb H10. To this purpose, we used two plant binary expression vectors bearing heavy chain (HC) and light chain (LC) coding sequences under the control of the cauliflower mosaic virus promoter (35S) and the tobacco mosaic virus (TMV) translational enhancer (Ω) with the nopaline synthase terminator sequence at the 3' end. A signal peptide sequence deriving from an embryonic mouse immunoglobulin HC coding gene was used to direct the recombinant antibody to the secretory pathway [13]. Hairy roots were either obtained by infecting transgenic tobacco cv. Petite Havana SR1 plants expressing mAb H10 [13] or by the co-infection of a glyco-engineered Δ XTFT *N. benthamiana* line with transformed *A. rhizogenes* strains bearing the plant expression vectors encoding antibody HC and LC sequences. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields. Purified antibodies were characterized for glycosylation and functional activity in vitro showing the presence of plant-typical complex structures (i.e. GnGnXF) for *N. tabacum*-derived mAb H10 and of GnGn structures lacking xylose and fucose for the Δ XTFT-derived counterpart. Both antibody glyco-formats exhibited comparable antigen binding activities.

2 Materials and methods

2.1 Hairy roots generation

N. tabacum cv. Petite Havana SR1 HRs were prepared from a T4 generation transgenic plant line (line 7.6) expressing mAb H10 HC and LC which was previously obtained by Villani and colleagues [13]. Briefly, wild-type *A. rhizogenes* A4 was grown in LB medium containing 50 mg/L rifampicin to $OD_{600} = 0.5$, at 28°C and 160 rpm. Bacteria were pelleted by centrifugation at 3000 $\times g$ for 10 min and resuspended at $OD_{600} = 1$ in MS medium with 30 g/L sucrose and 100 μ M acetosyringone, pH 5.8 [23]. Leaves were cut in segments (size: 1 cm $\times$ 1 cm), wounded with a sterile needle (10 pricks per 1 cm²) and inoculated by immersion in the *A. rhizogenes* suspension for 30 min, swirling in the dark. Leaves were dried and transferred to culture plates containing MS agar medium without phytohormones in the dark, for two days. Leaves were rinsed with sterile water and transferred to fresh MS agar medium containing 30 g/L sucrose and 0.5 g/L cefotaxi-

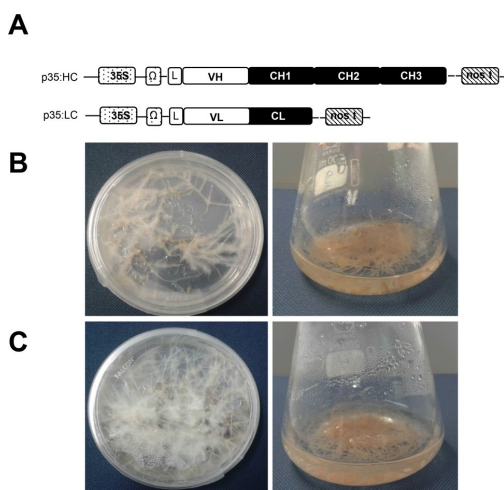


Figure 1. Generation of HRs expressing mAb H10. (A) Δ XTFT *N. benthamiana* HRs expressing mAb H10 were obtained by leaf disc co-infection with *Agrobacterium rhizogenes* strains bearing p35-HC and p35-LC plant expression vectors. Both HC and LC coding sequences were under the control of the cauliflower mosaic virus 35S promoter (35S) and the translation enhancer sequence of tobacco mosaic virus (Ω). L: signal peptide sequence derived from an embryonic mouse immunoglobulin HC coding gene. Solid and liquid medium HR cultures expressing the mAb H10 antibody; Δ XTFT *N. benthamiana* clone 15 (B) and *N. tabacum* clone 6 (C).

me. Leaves were transferred onto fresh plates every 15 days. Roots emerging from leaves (typically after 40 days) were excised and transferred to new plates. *A. rhizogenes* was eradicated by transferring roots every 15 days onto MS agar plates using decreasing cefotaxime concentrations (0.25, 0.125, 0.05 g/L) until no antibiotic was added. *A. rhizogenes* eradication was confirmed by PCR using *virC* specific primers (Supporting information, Fig. S1). Glyco-engineered Δ XTFT *N. benthamiana* plants [22] were used to obtain HRs expressing mAb H10. Leaf segments were infected with a co-culture of *A. rhizogenes* A4 strains transformed with the plant expression vectors p35S-HC or p35S-LC (Fig. 1A). Each strain was resuspended in inoculation medium to a final OD₆₀₀ of 1. Next steps were carried out using the same protocol described for *N. tabacum* HR generation with the exception that both solid and liquid MS culture media were supplemented with kanamycin (100 mg/L).

2.2 Selection of HRs expressing mAb H10 by Western blot analysis

The selection of HRs expressing mAb H10 was performed by Western blot analysis using anti-HC and LC antibodies. Root tissues (100 mg) were ground in liquid N₂ and homogenized in 100 μ L of phosphate-buffered saline

pH 7.2 (PBS) containing a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany). After centrifugation at 20 000 $\times$ g, 4°C for 30 min, the supernatant was recovered and analyzed by Western blotting. Samples were separated on 12 or 4–15% (Mini-PROTEAN® TGX™ Precast Gels, BIORAD, USA) SDS-PAGE acrylamide gels, and proteins were electrotransferred to a PVDF membrane (Millipore, Bedford, MA) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare, Freiburg, Germany) for 40 min at 0.4 mA/cm² and 30 V. The mAb H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and extracts from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative control. Membranes were incubated in PBS with 4% w/v non-fat milk, for at least 2 h. Protein detection was performed with anti-human γ chain (A8419 Sigma-Aldrich) and with anti-human λ chain secondary antibody (A5175; Sigma-Aldrich, Gillingham Dorset, UK) horseradish peroxidase conjugated diluted 1:5000 in 2% w/v non-fat milk in PBS. The membranes were washed three times with 0.1% Tween-20 in PBS, two times in PBS and developed using ECL Plus Western blotting detection system (ECL Plus; GE Healthcare). The ImageQuant™ LAS 500 (GE Healthcare) was used for chemoluminescence signal detection. Protein molecular mass markers were used in this analysis as reference (Color burst™, C1992 Sigma and Sharp Mass VI, Euroclone, Italy).

2.3 Establishment of HR culture in liquid medium and antibody secretion

The tobacco HRs were grown in 250 mL Erlenmeyer flasks using 40 mL of MS medium supplemented with 3% sucrose. Starting inoculum weight was typically $\approx$ 0.2 g of root FW and flasks, sealed with a cotton plug and aluminum foil, were orbitally shaken at 80 rpm ($\varnothing$ 19 mm), in the dark, at 26°C. Hairy root growth was analyzed by measuring roots FW at different time points before and after induction. Data shown represent the average values of three independent experiments $\pm$ standard deviation (SD). Measurement was performed by gently removing medium and weighing flask and roots; fresh root net weight was then obtained by subtracting flask weight. Measurements were performed in triplicate at day 8, 15, 20, 25, 28, 30 and 33 from the initial inoculum. For antibody production, HRs were grown for three to four weeks, as described before, and secretion was triggered by the addition of 10 mL of MS culture medium supplemented with 14 g/L of KNO₃, 19 mg/L of NAA and 1.5 g/L of PVP (Mw. 40 000). Three days after the first induction, flasks were again supplemented with 19 mg/L of NAA and 1.5 g/L of PVP, and left for five days before collecting the spent culture medium for antibody purification. An aliquot of 20 μ L of HR culture medium at different time points (before and after induction) was collected in order

to analyze the amount of secreted antibody by Western blot analysis (as described before). mAb H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and culture medium from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative controls. Densitometric quantification of bands was performed by ImageQuant TL 7.0 Image analysis software (GE Healthcare). Induced and non-induced HRs from both *N. benthamiana* and *N. tabacum* were observed by stereomicroscopy 33 days after inoculum. Analysis was performed using a macro-photo microscope WILD/Leica M420.

2.4 Antibody purification from roots and medium of HR cultures

Culture medium was separated from HRs by filtration using Miracloth™ (Millipore). Supernatant was centrifuged at $15\,000 \times g$ for 15 min, and filtered through 0.45 µm syringe filters (Millipore) ready for protein-A purification. Remaining roots (typically 4 to 11 g FW) were washed with PBS, ground in liquid nitrogen and homogenized using Ultra-Turrax homogenizer T25 equipped with a S25N-18G disperser (IKA, Staufen, German) in two volumes of 1x PBS. Extract was filtered using Miracloth™, centrifuged at $15\,000 \times g$ for 15 min, passed through 0.45 µm syringe filters and subjected to affinity chromatography. Antibody purification was performed by affinity chromatography essentially as previously described [24]. Briefly, the clarified root extract or culture medium was loaded onto a protein-A affinity column (1 mL HiTrap™ rProtein A FF; GE Healthcare) previously equilibrated with extraction buffer (1x PBS) at a flow rate of 1 mL/min. The column was washed with 10 mL of PBS (10 column volumes) and the antibody was eluted with 0.1 M citric acid (pH 3) and buffered with 1/5 volume of 1 M Tris-HCl, pH 8. Eluted fractions were analyzed by SDS-PAGE, followed by Coomassie staining. Reference molecular mass markers were used (Amersham, RPN5800). Antibody-containing fractions were dialyzed using slide-A-Lyzer™ Dialysis cassettes (Thermo Scientific) against 1x PBS. Antibody concentration was determined spectrophotometrically by measuring the absorbance at 280 nm [25].

2.5 ELISA assays

For the quantification of mAb H10 secreted in HR culture medium or in the root biomass, aliquots taken eight days after induction (33 days after inoculum) were analyzed by quantitative ELISA. Briefly, the purified antigen, human TNC (CC065; Merck Millipore), was coated directly onto Nunc-MaxiSorp® 96 well plates at a concentration of 2 µg/mL in PBS, and incubated at 4°C overnight. Plates were then blocked with 4% milk w/v in PBS, at 37°C, for 1 h. Hairy root culture media were centrifuged at $15\,000 \times g$ for 15 min, at 4°C, the supernatants were recovered and

serial dilutions were added to the wells (100 µL final volume) and incubated at 37°C, for 2 h. In the case of root biomass (for quantification of antibody in the roots), 0.2 g of roots were ground in liquid nitrogen in two volumes of 1x PBS, clarified by centrifugation $15\,000 \times g$ for 15 min, at 4°C, and serial dilutions were added to the wells and incubated at 37°C, for 2 h. After washing, the anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was added at 1:5000 dilution in 2% milk w/v PBS, and incubated at 37°C, for 1 h. As an internal standard for quantification, we used serial dilutions of purified mAb H10 positive control starting from a concentration of 3 µg/mL. Each HR sample was assayed in triplicate and mAb H10 concentrations were interpolated in the linear portion of the standard curve. For the functional characterization of the HR purified antibodies, the ELISA assay was performed essentially as described before. Purified antibodies in triplicate samples were added to TNC coated plates at serial dilutions starting from a concentration of 3 µg/mL. The anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was used as secondary antibody. After 30 min, enzymatic activity was measured at 405 nm on a microplate reader (TECAN-Sunrise, Groedig, Austria) using 2,2-azino-di-3-ethylbenz-thiazoline sulfonate as substrate (ABTS, KPL).

2.6 Glycan analysis

The *N*-glycosylation profile was determined by LC-ESI-MS as previously described by Stadlmann and colleagues [26]. In brief, purified IgG was separated by reducing SDS-PAGE, and bands corresponding to the HC and LC were excised from the Coomassie stained gel. Upon S-alkylation and tryptic or tryptic/GluC digestion, fragments were eluted from the gel with 50% acetonitrile and separated on a reversed-phase column (150 × 0.32 mm BioBasic-18, Thermo Scientific) using a gradient of 1–80% acetonitrile. Glycopeptides were analyzed with a Q-TOF Ultima Global mass spectrometer (Waters). Spectra were summed and deconvoluted for the identification of glycoforms. Glycans were annotated according to the proglycan nomenclature (www.proglycan.com).

3 Results

3.1 Hairy roots generation in *N. tabacum* and glyco-engineered ΔXTFT *N. benthamiana*

Twenty-three HR clones were obtained from *N. tabacum* cv. Petite Havana SR1 (T4 generation transgenic plant line 7.6) expressing mAb H10 HC and LC [13] by leaf disc infection with wild-type *A. rhizogenes* A4 strain. Twenty-five HR clones were also obtained from leaf discs of ΔXTFT *N. benthamiana* plants [22] infected with a co-culture of *A. rhizogenes* A4 strains transformed with the plant

expression vectors p35-HC and p35-LC [13] encoding mAb H10 HC and LC, respectively (Fig. 1A). Hairy root clones of both *N. tabacum* and Δ XTFT *N. benthamiana* were grown in plates with solid MS and using liquid MS in shake flasks (Fig. 1B and 1C).

3.2 Selected hairy root clones expressing the full-size mAb H10

All Δ XTFT *N. benthamiana* HR clones were screened for mAb expression by non-reducing Western blot analysis using an anti-HC antibody. Only five clones showed a band at 150 kDa indicating the presence of the full size mAb H10 (about 20% of the clones) and three clones (11, 15 and 16) exhibited the highest mAb H10 levels (Supporting information, Fig. S2). In the case of HRs derived from *N. tabacum* transgenic line, 14 clones were positive to antibody expression (about 60% of the clones), with clones 3, 4, and 6 showing the highest antibody levels (Supporting information, Fig. S2). Two clones were selected (clone 15 for *N. benthamiana* and clone 6 for *N. tabacum*) and analyzed; they were resolved on a gradient gel under reducing and non-reducing conditions and then subjected to Western blot analysis using both anti-HC and LC antibodies (Fig. 2). In the anti-HC blotting under non-reducing conditions, both *N. tabacum* and Δ XTFT *N. benthamiana* clones showed a similar band pattern, with the expected band migrating at high molecular mass ($\approx$ 150 kDa) corresponding to the assembled mAb. Additional lower bands were also observed: an intense band (at about 100 kDa) and very faint ones (at about 135, 75 and 50 kDa) that probably relate to antibody assembly intermediates (HC dimers/monomers) or putative degradation fragments. In the corresponding analysis under reducing conditions, a 50 kDa band associated with intact HC was visible as well as a faint band at about 25 kDa indicating specific degradation of the heavy chain (Fig. 2A). The anti-LC blot under non-reducing conditions (Fig. 2B) showed a 150 kDa band in both clones, together with additional lower faint bands at about 135, 100 and 75 kDa, which were already observed in the anti-HC blot. A very strong band at about 45 kDa was visible, which also occurred in the control. This may correspond to the homodimeric form of free LC. Under reducing conditions, the 25 kDa band corresponding to the LC was detected in both clones. It must be noted that both anti-HC and LC polyclonal antibodies seem to better recognize the antigens in their monomeric form, when they are separated under reducing conditions. This was already evidenced in previous works [13, 26].

3.3 Secretion of mAb H10 in the culture medium

Hairy root cultures of Δ XTFT *N. benthamiana* (clone 15) and *N. tabacum* (clone 6) in liquid MS medium were established. Secretion of mAb H10 in the culture medium

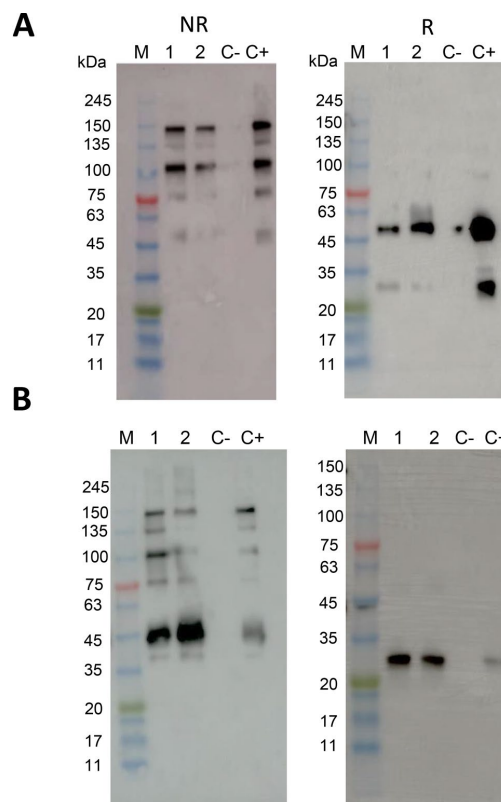


Figure 2. Western blot analysis of HR clones expressing mAb H10. *N. tabacum* clone 6 (1) and Δ XTFT *N. benthamiana* clone 15 (2) were analyzed using anti-HC (A) and anti-LC (B) antibodies under non-reducing (NR) and reducing (R) conditions. Extraction was performed on 0.1 g of root tissue for all samples and 20 μ L of each extract were loaded on 4–15% SDS-PAGE gel. C+, mAb H10 purified from agro-infiltrated *N. benthamiana* leaves. C-, HR clone expressing an unrelated protein (antimicrobial peptide); M, molecular mass markers.

was induced by the addition of KNO_3 as nitrogen source, the plant growth regulator NAA and PVP as protein stabilizing agent [10]. Growth curves of HR cultures were obtained by measuring root fresh weight (FW) at different time points before and after induction (Fig. 3A). Antibody secretion was induced at day 25 and 28 post-inoculation and flasks were left for another five days prior collecting the culture medium for antibody purification (33 days after inoculum). The two clones showed a significant difference in their growth, with Δ XTFT *N. benthamiana* (clone 15) reaching about 11 g FW/40 mL (275 g FW/L) 25 days post-inoculation, compared to about 4 g FW/40 mL (100 g FW/L) in *N. tabacum* (clone 6). Moreover, we recorded a decrease in growth for both clones after the

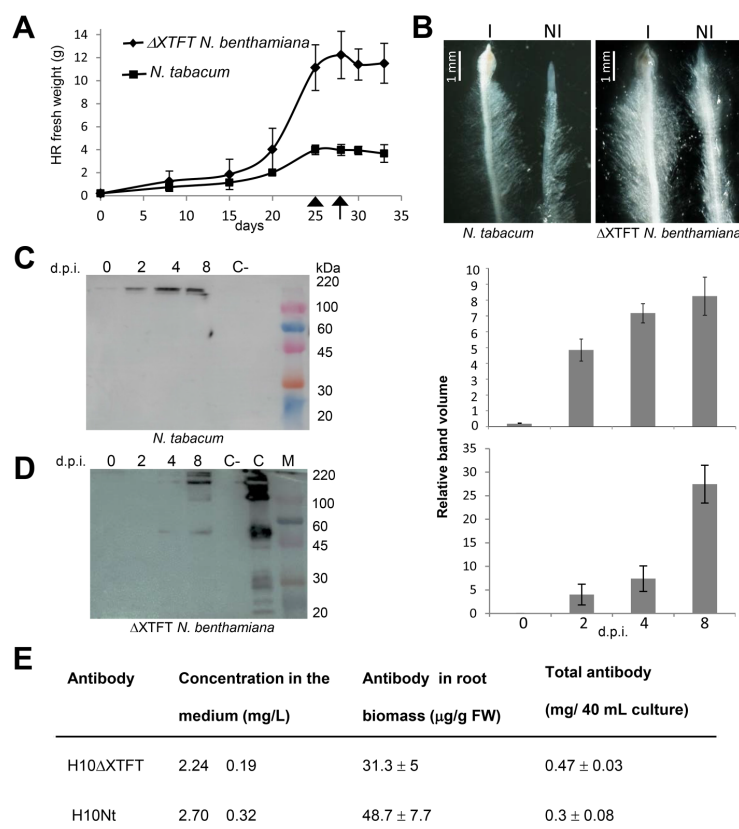


Figure 3. Analysis of mAb H10 accumulation in the biomass and liquid medium of HR cultures. **(A)** The growth rate of ΔXTFT *N. benthamiana* clone 15 and *N. tabacum* clone 6 was measured as root FW at different time points before and after the induction of mAb H10 secretion in the culture medium. The secretion of mAb H10 was triggered by the addition of KNO₃, NAA and PVP (days 25 and 33 after inoculum indicated by arrows). **(B)** A typical microscopy analysis showing phenotypic differences between induced (I) and non-induced (NI) HRs in both *N. tabacum* and ΔXTFT *N. benthamiana*. Induced HRs show typical swollen root tips and enhanced root hair proliferation. Antibody secretion in culture medium of *N. tabacum* clone 6 **(C, left panel)** and ΔXTFT *N. benthamiana* clone 15 **(D, left panel)** was assayed by Western blot analysis at different time points before (day 0) and after induction of antibody secretion (day 2, 4 and 8). C+, H10 purified from agro-infiltrated *N. benthamiana* leaves. C-, culture medium from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein. Post-induction accumulation of mAb H10 in culture medium of *N. tabacum* **(C, right panel)** and ΔXTFT *N. benthamiana* HRs **(D, right panel)** was evaluated by densitometry analysis of band volumes (values are the mean ± SD of two independent experiments). **(E)** Quantitative TNC-binding ELISA was used to calculate antibody concentration in the HR culture medium and in root biomass; values shown in the panel are the mean ± SD obtained from three independent HR cultures. Total antibody: secreted antibody + antibody in root biomass.

start of induction at day 25 (Fig. 3A). Microscopy observations at day 33 showed a general enhanced fragility of roots with swollen tips and root hair proliferation, compared to non-induced ones (Fig. 3B).

The accumulation of mAb H10 in culture medium was assayed by Western blot analysis using an anti-HC (γ chain) antibody. Samples were collected at different time points before (day 0) and after induction of antibody secretion (days 2, 4 and 8), and separated by non-reducing SDS-PAGE (Fig. 3C and 3D, left panels). Very faint

bands were observed at day 0 (before induction) at about 150 kDa, while stronger bands were detected in the following days. Maximum mAb H10 levels were typically reached at day 8 for both *N. tabacum* (Fig. 3C, right panel) and ΔXTFT *N. benthamiana* (Fig. 3D, right panel).

The amount of active antibody in HR culture medium was quantified by ELISA. HR supernatant was recovered at day 8 after induction and serial dilutions were added to the ELISA wells coated with human TNC. Results showed a similar concentration of functional antibody in the

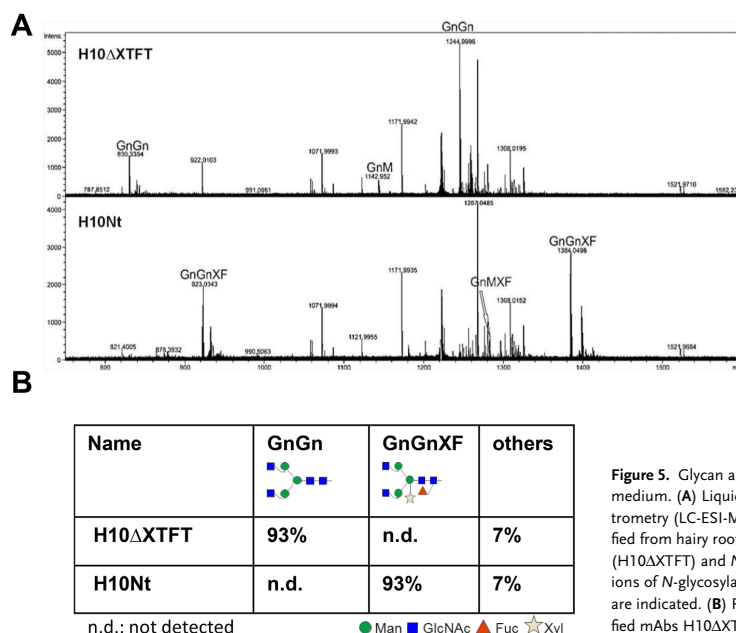


Figure 5. Glycan analysis of antibodies purified from HRs culture medium. (A) Liquid chromatography-electrospray mass spectrometry (LC-ESI-MS) of tryptic glyco-peptides of mAb H10 purified from hairy roots culture medium of Δ XTFT *N. benthamiana* (H10ΔXTFT) and *N. tabacum* (H10Nt). Doubly and triply charged ions of *N*-glycosylated variants of the expected tryptic fragments are indicated. (B) Relative amounts (%) of *N*-glycans in the purified mAbs H10ΔXTFT and H10Nt.

same pattern of bands observed for the HR derived antibodies. In the case of non-reducing SDS-PAGE, a band at about 150 kDa corresponding to the assembled antibody was present in both tobacco HR purifications. Additional faint (at about 100 kDa) and stronger bands (at about 40 kDa; indicated by a black triangle in Fig. 4A) demonstrated the presence of degradation products/assembly intermediates. The observed pattern of bands was similar to that of mAb H10 control from agro-infiltrated leaves (Fig. 4A).

The antibody was also purified from the root biomass of the induced HRs. Clarified root extracts were passed through protein-A column and eluted fractions were separated by SDS-PAGE under reducing conditions (Supporting information, Fig. S3). Average yields from three purifications were 16.2 ± 1.7 μ g/g FW for mAb H10Nt from *N. tabacum* HR and 12.4 ± 1.5 μ g/g FW for mAb H10ΔXTFT from Δ XTFT *N. benthamiana* HR (Fig. 4C). Band pattern was identical to that observed for antibodies purified from culture medium.

The integrity of both antibody versions was estimated by their ability to bind the antigen human TNC. ELISA plates were coated with recombinant C domain of human TNC (Fig. 4B), and incubated with purified antibodies in serial dilutions. Both antibodies recognized the antigen in a concentration dependent manner, thus demonstrating functional activity in a similar range. Glycan analyses of H10Nt-Fc by LC-ESI-MS revealed the presence of a single

glycan species, i.e. plant-typical GnGnXF structures (Fig. 5). In contrast, H10ΔXTFT carried GnGn structures lacking xylose and core α 1,3-fucose (Fig. 5).

4 Discussion

4.1 Hairy roots generation using a multiple gene co-transformation strategy

In literature, there are only a few examples of the successful expression of monoclonal antibodies in HRs. All were developed from stable *N. tabacum* transgenic plants [7, 10]. In this study, we evaluated the production of a tumor targeting mAb in tobacco HRs by using two different approaches. Hairy roots were either generated 'classically' by *A. rhizogenes* infection of a transgenic *N. tabacum* line expressing the antibody or by co-infection of glyco-engineered Δ XTFT *N. benthamiana* with a mix of recombinant *A. rhizogenes* strains bearing the antibody *hc* and *lc* genes. We showed that the percentage of clones expressing detectable amounts of recombinant protein was higher in *N. tabacum* (60% of the clones were positive in Western blot analysis) than in co-transformed Δ XTFT *N. benthamiana* (20% of the clones were positive). This result is merely indicative, due to the low number of HR clones screened, and does not allow for a thorough comparison of the two different approaches. It simply demon-

strates that co-infection of leaf discs with a mix of *A. rhizogenes* carrying antibody HC and LC coding sequences, although at a lower efficiency, can be used for the rapid establishment (approx. two months) of stable HR clones expressing functional mAbs. A previous work based on the same multiple gene co-transformation strategy, i.e. transgenes located in different plasmids and transformed in different batches of *A. rhizogenes*, was used to co-express GUS and GFP in *N. tabacum* hairy roots. In this case a co-transformation efficiency of 65% was obtained, while a higher value (82%) was obtained when the reporter genes were inserted in two different T-DNAs within the same binary vector [4].

4.2 Triggering antibody secretion from HR cultures

Comparison of the two best mAb expressors in liquid culture showed a faster growth (nearly three-fold) in the *N. benthamiana* clone, compared to the *N. tabacum* one. Starting from an inoculum size of ≈ 0.2 g in a 40 mL culture, the FW biomass value of Δ XTFT *N. benthamiana* HRs was 11 g (275 g/L) after 25 days, while in the case of *N. tabacum* it was lower (100 g/L), but comparable to that already described by other groups [11]. It has been previously demonstrated that transgene expression in HRs is strongly influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA), affecting not only protein expression but also hairy root growth and morphology [4, 28]. This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic HRs expressing monoclonal antibodies in different plant species [8, 11]. A major advantage of HRs is the possibility to secrete the recombinant proteins in the culture medium. Previous literature showed that the addition of protein stabilizing agents (PVP and gelatin) and KNO_3 in the medium improved antibody recovery [7, 9]. Moreover, some authors also highlighted the importance of the auxin NAA in increasing rhizosecretion of recombinant proteins [29]. An optimized protocol was recently developed leading to high yield accumulation of a mAb in *N. tabacum* HRs, which is based on KNO_3 and NAA to induce rhizosecretion and PVP as protein stabilizing agent [11]. We used a similar protocol to compare mAb H10 secretion in the two best expressing HR clones and the concentration of secreted antibody was similar in both *N. tabacum* (2.7 mg/L) and Δ XTFT *N. benthamiana* (2.24 mg/L) with levels comparable to those previously reported in literature [11]. Maximum antibody accumulation was typically achieved eight days post-induction (33 days after inoculation) and, most interestingly, antibody accumulation in the medium before induction was hardly detectable (Fig. 3C and 3D). Overall, our results showed that the amount of functional antibody secreted in the medium by *N. benthamiana* clone 15 represents about 20% of total antibody. This amount is slightly lower but comparable to that of *N. tabacum* clone 6 (35% of total antibody),

indicating that there is no major difference in trafficking ability of the two mAb H10 glyco-variants. Significant variations in the amount of secreted antibody in three different *N. tabacum* HR clones expressing mAb M12 were reported in a previous work with levels ranging from 25 to 57% of the total antibody yield [11]. Microscopy analysis of roots after induction revealed a peculiar phenotype with the formation of swollen root tips, root hair proliferation and elongation. This phenomenon was already described by Häkkinen and colleagues who observed the formation of lateral root primordia in the swollen root tip, associated with morphological changes in the inner cortex and pericycle cells [11]. A similar root morphology was also revealed in hydroponic tobacco cultures rhizosecreting a human monoclonal IgG after NAA induction [30].

4.3 Efficient antibody purification from HR biomass and culture medium

Antibody purification from culture medium was performed using a two-step protocol in which the medium was clarified by filtration/centrifugation and directly passed through a protein A column with no need for pH adjustment or DNase treatment. Purification yield of mAb H10 from liquid medium cultures of Δ XTFT *N. benthamiana* and *N. tabacum* HR clones were similar, in the range of 1.5 mg/L (Fig. 4C). It is interesting to note that the amount of antibody purified from the HR spent medium represents about 60% of the total secreted antibody while only 30% of the total antibody in the roots was recovered after protein A purification of root extracts. Purification yields from culture medium are slightly lower than those described by Häkkinen and colleagues which reported yields of 5.9 mg/L for mAb M12, reflecting possible lower antibody degradation and higher accumulation levels in the culture medium [11]. It is important to note that the performance of the protein-A affinity column used for antibody purification from the medium remained unaltered even after several purification cycles, thus indicating that the filtered HR culture medium can be directly used in the downstream processing without the need of further clarification steps. As expected, mAb H10 purification yields in HRs are sensibly lower compared to those obtained by using transient expression systems based on *N. benthamiana* agroinfiltration [27]; the latter yielded up to 75 mg/kg FW of the antibody. Nevertheless, HR cultures still represent an advantageous system in terms of manufacturing and downstream processing for the contained root growth in sterile conditions and the secretion of the recombinant protein in the culture medium.

4.4 Characterization of antibody degradation profiles in HRs

Degradation of recombinant proteins in the HR culture medium represents a major problem and previous studies

demonstrated that the addition of PVP or gelatin can enhance antibody stability [7, 9]. In particular, the addition of PVP had stabilizing effects on HR-secreted mAb M12 increasing accumulation by two times [11]. Previous studies reported that the susceptibility of recombinant antibodies to plant proteolysis is largely dependent on their primary sequence including the variable domains [21, 25]. Degradation profile of mAb H10 has been extensively studied in different plant species as well as production systems. This molecule was shown to be particularly susceptible to proteolytic cleavage in the proximity of the interdomain antibody sequences [33]. In particular, a previous study from our group demonstrated that mAb H10 purified from leaves of the transgenic tobacco line 7.6 was almost completely degraded leading to the formation of Fab fragments [13]. Reducing SDS-PAGE analysis of purified mAb H10 from Δ XTFT *N. benthamiana* and *N. tabacum* HRs, and from agro-infiltrated *N. benthamiana* leaves revealed a similar pattern with two major bands, corresponding to the HC and LC and a band at a lower molecular mass associated with HC degradation as a result of a proteolytic event close to the hinge region [13]. Identical patterns for the three purified antibodies were also observed in non-reducing conditions, with a major degradation product at about 40 kDa. In a previous work, a similar degradation product was described and characterized as a functional antibody Fab fragment deriving from specific cleavage in the antibody hinge region [32]. Identical band patterns for the two HR clones were also observed in Western blot analysis of root extracts under non-reducing conditions using an anti-LC antibody (Fig. 2). A strong signal at about 45 kDa was obtained suggestive for the formation of LC homodimers. The occurrence of LC dimers in *N. benthamiana* had been already described in a recent work in which dimerized monoclonal camelidae variable heavy chain domain antibodies (VHH) were obtained through the C-terminal fusion of a k light chain domain [34]. Non-reducing analysis using anti-HC antibody revealed the presence of an intense band at about 100 kDa and a fainter one at about 50 kDa possibly representing HC dimers and monomers, respectively. The presence of free LC and HC polypeptides in HR clones indicated that they are not synthesized in stoichiometric ratios. A marked difference was observed in the presence of free HC and LC homodimers between the two co-transformed Δ XTFT *N. benthamiana* hairy root clones 11 and 15 (Supporting information, Fig. S2). Since HC and LC coding genes were located in separate plasmids and transformed in different batches of *A. rhizogenes*, further used for hairy roots co-transformation, it is very likely that transgenes were inserted in these two clones in different regions of genomic DNA and possibly in different copy numbers, thus accounting for the differential accumulation of monomeric HC and LC. It is interesting to note that no significant differences in band patterns were observed between the two HR secreted

antibodies or mAb H10 produced in *N. benthamiana* leaves, indicating that equivalent proteolytic events take place in both species and production systems. A similar evidence was observed for analogous proteolytic activity of *A. thaliana* or *N. tabacum* thizosecretomes on a human IgG [35].

4.5 Glyco-modified antibodies with enhanced immunotherapeutic potential

Plant-derived mAbs differ from their mammalian counterpart in their Fc N-glycan composition. Non-human glycosylation may represent a limitation in their therapeutic application since plant typical complex glycan structures moieties may cause immunogenic reactions [35, 36] and may also interfere with Fc γ receptors binding [19, 37]. This has a negative impact on immunotherapeutic mAbs, since their effector functions, such as antibody dependent cell cytotoxicity (ADCC), rely on the interaction with Fc γ receptors [39]. Recent studies demonstrated that antibodies lacking plant specific xylose and fucose have enhanced binding to Fc γ receptors and improved biological activity [40]. With the aim of improving mAb H10 immunotherapeutic potential we have demonstrated here that it is possible to obtain HRs from Δ XTFT *N. benthamiana* producing mAbs with a human-compatible glycosylation pattern. Häkkinen and colleagues previously characterized the glycosylation profile of mAb M12 obtained in *N. tabacum* HRs, showing that the secreted antibody carries mainly GnGnXF plant typical complex-type glycans [11]. In this work, we have shown that mAb H10Nt has a similar profile to that observed for mAb M12 (GnGnXF), while mAb H10 Δ XTFT carries predominantly GnGn structures (>90%). To our knowledge, this is the first example of a functional glyco-modified mAb obtained from HRs lacking typical plant-type sugars, thus representing a step towards the exploitation of root cultures for the production of 'next generation' human therapeutic antibodies.

In conclusion, we have shown here that it is possible to rapidly establish HR cultures secreting full size mAbs with a targeted glycosylation profile by using a simple *A. rhizogenes* infection protocol of Δ XTFT *N. benthamiana* leaf segments. As reported in literature, maximum antibody secretory yields in HRs are in the range of few milligrams per liter of culture medium. To our view, this is the major limitation of this production system, especially if compared to the high-yield methods (hundreds of milligrams per kg of FW) based on transient expression systems using whole plants. For this reason, efforts have to be focused on devising novel expression vectors as well as cultivation and induction protocols to enhance recombinant protein production and secretion, making stably transformed HRs a competitive production system of glyco-modified antibodies for cancer immunotherapy.

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