

REVIEW

Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge

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Summary

Antibodies have long been recognized for their diagnostic and therapeutic potential. The rapidly increasing number of monoclonal antibodies approved for immunotherapy has paved the way to an even greater demand for these molecules. In order to satisfy this growing demand and to increase the production capacity, alternative systems based on antibody production in transgenic organisms are being actively explored. In this paper, we focus on transgenic plants as a promising system for the scale-up and processing of plant-made pharmaceuticals. In particular, we point out the advantages and limitations induced by glycosylation of plant-made antibodies for human therapy.

Introduction

For several decades, genetically modified organisms (GMOs) have been used to produce therapeutic molecules in a recombinant form. For a long time, the only GMOs used for such production were *Escherichia coli*, yeast and mammalian cells. Over the last 20 years, several proteins of pharmaceutical interest have also been produced in transgenic plants. These include blood and plasma proteins, such as human albumin (Sijmons *et al.*, 1990; Goddjin and Pen, 1995), aprotinin (Zhong *et al.*, 1999), enkephalin (Vandekerckhove *et al.*, 1989; Takamatsu *et al.*, 1990), haemoglobin (Dieryck *et al.*, 1995, 1997; Halling-Sorensen *et al.*, 1998), antigens produced for vaccination purposes (Richter *et al.*, 2000), growth factors, hormones, cytokines (Goddjin and Pen, 1995; Matsumoto *et al.*, 1995; James *et al.*, 2000), enzymes with therapeutic potential, conversion enzymes, angiotensin I (Hamamoto *et al.*, 1993), protein C (Cramer *et al.*, 1996), glucocerebrosidase (Cramer *et al.*, 1996; Steiner, 2000) and, finally, antibodies (Table 1, for illustration). Through this last example, we wish to illustrate the interest that molecular farming offers as an alternative to the current production systems of therapeutic molecules. We also wish to introduce the advantages and limitations associated with the glycosylation of plant-made pharmaceuticals (PMPs).

Plants as an alternative for the production of monoclonal antibodies

Antibody-based therapies (immunotherapies) have been highly developed over recent years. As early as 1998, 30% of the clinical trials concerning biotechnology-derived molecules involved antibodies (Hudson, 1998). This percentage has increased within the last 2 years, with almost 1000 therapeutic antibodies currently being tested for immunotherapies against various pathologies, such as immune system disorders, inflammatory diseases, cancers, disorders of the central nervous system and infectious diseases. More than 10 antibodies have already been clinically validated (Table 2), and more than 50 others may be administered clinically within the next 5 years. Immunotherapy often requires large quantities of antibodies. As an example, some treatments, such as the use of the Herceptin antibody (Herceptin) for breast cancer treatment, require the administration of 2–5 g of antibody per patient per year.

At the present time, most recombinant antibodies approved by the Food and Drug Administration (FDA) are produced in cultured Chinese hamster ovary (CHO) cells. This process is highly expensive and of limited capacity. Indeed, the investment needed to establish and operate a culture unit for mammalian cells is important (about 1 million \$US for the

Table 1 Recombinant antibodies produced in transgenic plants (see Figure 1 for illustration of the different antibody and antibody fragments presented in the second column)

Antigen	Type of antibodies	Indications	Transformed plant	Promoter	Targeting signal	Expression	Reference
Immunomodulations							
Cutinase	scFv	ND	<i>N. tabacum</i> cv Samsum		No SP	+	Schouten et al. (1996, 1997)
	scFv		<i>S. tuberosum</i>		No SP + KDEL	+ 0.2–1% of TSP	
Tobacco mosaic virus	IgG-2b	Reducing virus infectivity	<i>N. tabacum</i> cv petit Havana	35 S promoter +	MSP	15–45 µg/g wet weight	Voss et al. (1995) Fischer et al. (1999)
<i>A. thaliana</i> cyclin-dependent kinase	scFv	Immunomodulation in plant	Tobacco leaves	35 S promoter	No targeting signal	+ 1.2–1.8% of TSP	Eeckhout et al. (2000)
Potato starch branching enzyme A	Llama VHH antibody fragment	Inhibition of enzyme function	<i>S. tuberosum</i> (potato tubers)	35 S promoter	Chloroplast targeting peptide of granule-bound starch synthase	+ (0.03% of TSP)	Jobling et al. (2003)
Environment							
Plant pathogenic Mollicute (bacteria)	scFv 2A10	Plantibody controlled resistance to mycoplasma infection	<i>N. tabacum</i> cv PBD6	35 S promoter	Pectate lyase SP	+	Le Gall et al. (1998)
Urea herbicide paraquat	scAbs	Detection and removal of organic pesticides from contaminated water	<i>N. tabacum</i> cv Xanthi	35 S promoter	Plant PR1a SP	+ 0.006–0.028% of TSP	Longstaff et al. (1998)
Herbicide atrazine							
Antibody-based therapy (human and animal health)							
Human carcinoembryonic antigen	Mouse/human chimeric IgG1 antibody (cT84.66)	Antibody-mediated cancer therapy (colon cancer, breast cancer and tumour with epithelial origin)	<i>N. tabacum</i> cv petit Havana SR1 (transient expression)	35 S promoter	MSP	+ 1 mg/kg of FLW	Vaquero et al. (1999)
	scFv T84.66				MSP + KDEL MSP	+ 5 mg/kg of FLW	
	T84.66/G68 diabody		<i>N. tabacum</i> cv petit Havana SR1	35 S promoter	Plant codon optimized SP SP + KDEL	+ (1–5 mg/kg) + (4–12 mg/kg)	Vaquero et al. (2002)
	scFvT84.66				Maize ubiquitin promoter or 35S promoter	+ wheat and rice: 30–100 ng/g FLW + wheat: 50–900 ng/g of FLW rice: 1.5–29 µg/g of FLW	
Rabies virus protein	Monoclonal antibody (mAb SO57)	Rabies virus neutralization	<i>N. tabacum</i> cv Xanthi	35 S promoter	MSP	+ 3 µg/g of FLW (0.07% of TSP)	Ko et al. (2003)
					MSP + KDEL		

Human IgG monoclonal antibody	C5-1 IgG	Anti-human globulin reagent for phenotyping and cross-matching red blood cells of receivers and donors	<i>M. sativa</i>	35 S promoter	MSP	+	0.13–1.0% of TSP	Khoudi <i>et al.</i> (1999)
Streptococcal surface antigen SAI/II	Guy's 13 IgG IgA/G slgA/G	Tooth decay	<i>N. tabacum</i>	35 S promoter	MSP	+		Ma <i>et al.</i> (1994)
Colon cancer surface antigen	CO-17 A IgG	Antibody-mediated cancer therapy	<i>N. benthamiana</i>		MSP MSP + KDEL			Larrick <i>et al.</i> (2001) Ma <i>et al.</i> (1998) Ma <i>et al.</i> (1995) Verch <i>et al.</i> (1998)
HSV-2, protein from herpes simplex virus (HSV)	IgG, IgA, DigA or slgA IgG1 Fab and F(ab') ₂	immunoprotection against genital herpes and vaginal transmission of HSV	<i>O. sativa</i> <i>G. max</i>			+		Briggs <i>et al.</i> (2000) Zeitlin <i>et al.</i> (1998)
Zearalenone (mycotoxin)	scfv	Passive immunization of animals in their feed	<i>A. thaliana</i> (ecotype Columbia)	Inducible <i>Lac</i> promoter	No SP Plant PR1-b SP	– +		Yuan <i>et al.</i> (2000)
Human creatine kinase-MM	MAK33 IgG1	Cardiac disease, mitochondrial disorders, inflammatory myopathies, myasthenia, polymyositis, McArdle's disease	<i>A. thaliana</i>		<i>A. thaliana</i> 2S2 seed storage protein SP	+	0.02–0.4% of TSP of fresh leaf extract (10–12% of TSP of intercellular fluid)	De Wilde <i>et al.</i> (1996)
	Fab fragment	NMJ disorders, muscular dystrophy, ALS, hypo- and hyperthyroid disorders	<i>A. thaliana</i>	35 S promoter		+	1.3% of TSP	De Neve <i>et al.</i> (1993)
	MAK33 scFv	central core disease, acid maltase deficiency, myoglobinuria, rhabdomyolysis, motor neurone diseases, rheumatic diseases, and others that create elevated or reduced levels of creatine kinases	<i>N. tabacum</i> <i>N. tabacum</i> SR1	35S promoter	No SP	+	0.044% of TSP	Bruyns <i>et al.</i> (1996)
	MAK33 Fab fragment		<i>A. thaliana</i>	35 S promoter	<i>A. thaliana</i> 2S2 seed storage protein SP <i>A. thaliana</i> 2S2 seed storage protein SP SP + KDEL	+	6–25 ng scFv/mg TSP + 8–55 ng scFv/mg TSP + 6.53% of TSP (0.02% of TSP in seed) + 5.9% of TSP (0.015% of TSP in seed)	Peeters <i>et al.</i> (2001)
Tumour's surface Ig	38C13 scFv	B-cell lymphoma treatment	<i>N. benthamiana</i>	TMV coat protein promoter	Rice α -amylase SP	+	60 μ g/mL of intercellular medium	McCormick <i>et al.</i> (1999)
Hepatitis B virus surface antigen (HBsAg)	scFv	Immunoaffinity purification of recombinant HBsAg	<i>N. tabacum</i> cv petit Havana SR1	35 S promoter	No SP Sweet potato Sporamin SP Sporamin SP + PP Sporamin SP + KDEL	– + + + +	0.031% of TSP + 0.032% of TSP + 0.22% of TSP	Ramirez <i>et al.</i> (2002)
Zearalenone (mycotoxin)	scfv	Passive immunization of animals in their feed	<i>A. thaliana</i> (ecotype Columbia)	Inducible <i>Lac</i> promoter	No targeting signal Plant PR1-b SP	– +		Yuan <i>et al.</i> (2000)

ALS, amyotrophic lateral sclerosis; FLW, fresh leaf weight; KDEL, endoplasmic reticulum retention signal; MSP, murine signal peptide; NMJ, neuronuscular junction; PP, propeptide; SP, signal peptide; TSP, total soluble protein.

Table 2 Therapeutic antibodies clinically validated

Drug	Company	Indication	Molecule	Antigen	Year approved
ReoPro	Eli Lilly	Acute coronary syndromes	Abciximab Chimeric human murine Fab fragment	Integrine GPIIb/IIIa	1994
Orthoclone	Janssen	Acute kidney transplant rejection	Muromonab Murine monoclonal antibody	CD3	1986
Rituxan	Genentech	Non-Hodgkin's lymphoma	Rituximab Chimeric human/mouse monoclonal antibody	CD20	1997
MabThera		HER2+ metastatic breast cancer	Trastuzumab Humanized antibody	P185 (HER2/Neu)	1998
Herceptin					
Simulect	Novartis	Kidney transplant rejection	Basiliximab Chimeric human/mouse monoclonal IgG	Interleukin 2 receptor α -chain (CD25)	1997
Remicade	Centocor	Crohn's and rheumatoid arthritis	Infliximab Chimeric human/mouse monoclonal antibody	TNF α	1998/1999
Zenepax	Roche, PDL	Kidney transplant rejection	Daclizumab Humanized antibody	CD25 (IL-2R)	1997
Synagis	Medimmune	Prevention of RSV infection	Palivizumab Humanized antibody	F protein of syncytial respiratory virus	1998
Mylotarg/AHP	Celltech	Acute myeloid leukaemia	Gemtuzumab Humanized antibody that is likened to the toxin calicheamicin	CD33	2000
Campath	ILEX	Chronic lymphocytic leukaemia	Alemtuzumab Humanized antibody	CD52	2001

RSV, respiratory syncytial virus.

production of 1 kg of antibody per year) (Steiner, 2000). Currently, the capacity available for the production of monoclonal antibodies does not exceed 1000 kg per year. This means that almost all the current production capacity is used to provide sufficient amounts of the first 10 monoclonal antibodies clinically validated today (Table 2). Therefore, the pharmaceutical industry will need to invest a considerable amount of money to allow the commercialization of future monoclonal antibodies produced in culture units of CHO cells, which are currently undergoing clinical tests. Even in these conditions, and considering the delays needed for the construction of such equipment, it can be estimated that, in 2010, the production capacity would be, at best, 10 000 kg of antibodies with an estimated demand of 50 000 kg (Gura, 2002).

In this context, pharmaceutical companies are deeply interested in transgenic production systems which enable high production levels of therapeutic antibodies. Apart from cultured mammalian cells, only transgenic animals and transgenic plants are able to assemble, via disulphide bridges, the light and heavy chain constituents of an antibody (Figure 1). In particular, plant cells can reproduce the complexity of these proteins, as illustrated in 1989 by the first production of functional antibodies in plants (Hiatt *et al.*, 1989). Since this pioneering demonstration from Dr Hiatt's group, many antibodies, or fragments of antibodies, have been produced for therapeutic purposes in various plant systems, in particular antibodies directed against human immunoglobulins (Igs) (Khoudi *et al.*, 1999), against an antigen from *Streptococcus mutans* (Ma *et al.*, 1995; Smith and Glick, 2000), against a human creatine kinase (Conrad *et al.*, 1998) or against a tumoral antigen expressed in colon cancer (Vaquero *et al.*, 1999). Antibody fragments or diabodies have also been produced in plants, in particular against tumour-specific antigens (Stöger *et al.*, 2000; Vaquero *et al.*, 2002) (Figure 1).

Figure 1 Various antibodies and antibody fragments produced in plant systems. In a conventional immunoglobulin G (IgG), the constant heavy-chain (CH1, CH2, CH3) and the variable heavy-chain (VH) domains are shown in orange, and the constant light-chain (CL) and variable light-chain (VL) domains in green. In the recombinant single-chain Fv fragment (scFv), the variable domains are joined by a peptide linker. scFvs can be complexed into dimers (diabodies), trimers or tetramers (not illustrated). Diabodies have two functional antigen binding domains with similar or distinct antigen specificities. Some camelidae IgGs lack a light chain. Their antigen-binding domains consist only of the heavy-chain variable domain (VHH). The VHH antibody fragment is highly soluble and stable. A secretory IgA (slgA) is a dimeric IgA composed of 10 different polypeptides: four light chains, four heavy chains, a J chain that facilitates dimerization and a secretory component. The F(ab) fragment contains the light chain and the VH and CH1 domains of the heavy chain. F(ab')₂ is divalent and contains two F(ab) fragments linked by a disulphide bond.

Therefore, the use of transgenic plants could be a solution to the need for a scale-up in the production capacity of therapeutic antibodies. Indeed, even with relatively low expression levels of total soluble proteins (Hiatt *et al.*, 1989; Ma *et al.*, 1995) (Table 3), the production capacity of recombinant antibodies in transgenic plants is almost unlimited, as it depends only on the surface dedicated to the plant culture. Although dependent on the stability of the protein of interest and on the plant used to produce it (tobacco, corn, soybean or alfalfa), it is estimated that a plant 'bioreactor' will allow

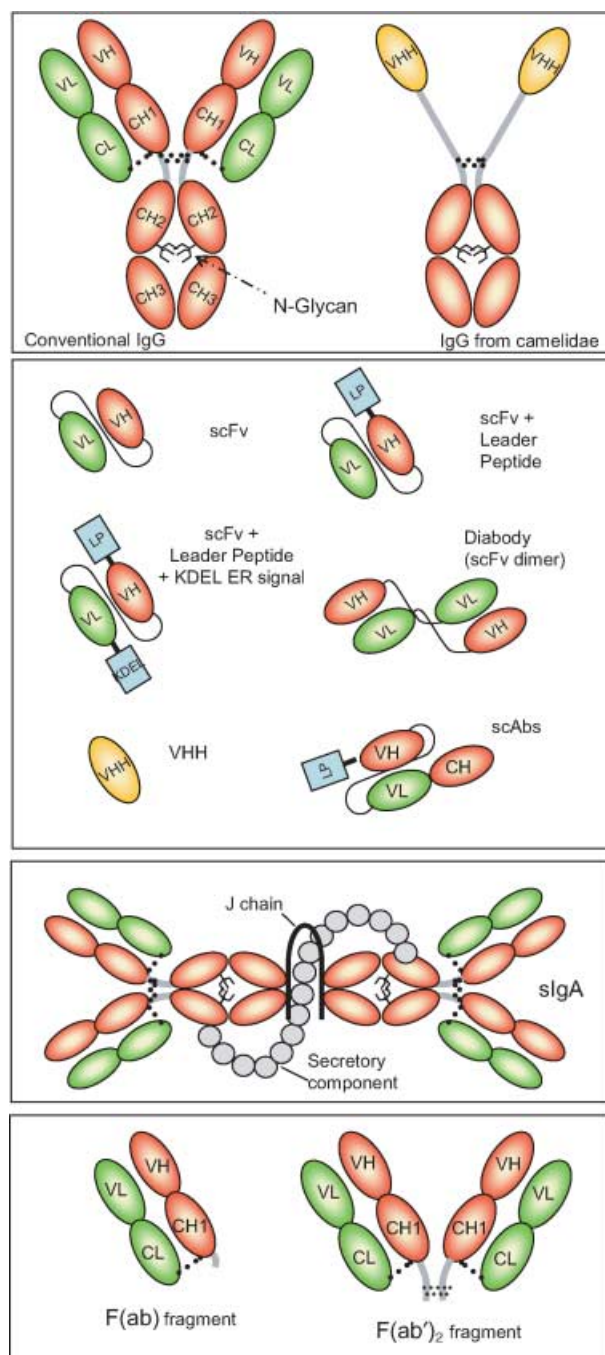


Table 3 Comparison of currently available recombinant protein production systems

	Bacteria	Yeasts	Mammalian cells	Insect cells	Transgenic mammals	Transgenic plants
Risk of pathogenicity	Yes	Unknown	Yes	Yes	Yes	Unknown
Production cost	Medium	Medium	High	High	High	Low-medium 10–50-fold lower than the same protein produced by <i>E. coli</i>
Time effort	Low	Medium	High	High	High	High
Scale-up cost	High	High	High	High	High	Low (unlimited biomass)
Production	Limited	Limited	Limited	Limited	Limited	Worldwide
Production yield (i.e. antibodies)	Medium 0.1–2 g/L	High > 0.25 g/L (scFv)	Medium to high 0.4–2 g/L	Medium to high 5–70 mg/L	High 4–14 g/L	High 500–1000 g/acre/cycle
Folding accuracy	Low	Medium	High	High	High	High
Glycosylation status						
N-glycosylation	No	Yes (unusual)	Yes	Yes	Yes	Yes
Site of glycosylation		Not conserved	Conserved	Conserved	Conserved	Conserved
Non-human sugar residues		Yes	yes	yes	Yes	Yes
Product homogeneity	Low	Medium	Medium	Low	Low	Medium to high
Protein complexity (i.e. antibody)	Fab	Fab	IgG, IgM	IgG	IgG	IgG, sIgA
Delivery vehicle	No	No	No	No	Yes	Possible
Storage	–20 °C	–20 °C	N ₂	N ₂	N ₂	Room temperature
Distribution	Feasible	Feasible	Difficult	Difficult	Difficult	Easy
Ethical concerns	Medium	Medium	Medium	Medium	High	Medium
Companies	GenWay Biotech Inc. Genentech ATG Laboratories Paragon Bioservices Biological Mimetics	Paragon Bioservices ApoLife, Inc.	GenWay Biotech Inc. Paragon Bioservices Cell Trends Acceptys, Inc. Lonza Biologics Micromet/Enzon Biological Mimetics Genentech	ATG Laboratories Paragon Bioservices Cell Trends Biological Mimetics	Nexia Biotechnologies GTC Biotherapeutics PPL Therapeutics Abgenix Pharming	Biolex Incorporated ProdiGene Greenovation Biotech GmbH SemBioSys Medicago Inc. Meristem Therapeutics Phytomedics, Inc. EpicYTE Pharmaceuticals

the production of recombinant proteins in the range of kilograms per hectare, regardless of the plant material considered (Austin *et al.*, 1994; Khoudi *et al.*, 1999). Another advantage of antibody production in transgenic plants, compared with classical production in culture units of mammalian cells, is that it would cost 500 times less (Table 3). Considering the expense and difficulty of producing antibodies by conventional means, it seems that production in crop plants is one of the most promising options for large-scale antibody production.

Only transgenic mammals could present an antibody production capacity equivalent to that estimated for transgenic plants. The efficiency of this production system has been well documented, as about 100 recombinant proteins have already been expressed in the milk of transgenic animals (Houdebine, 2002). However, this system also presents some limitations as the production of some recombinant proteins, such as therapeutic antibodies, might disturb the metabolism of the transgenic animal. Moreover, although PMPs cannot be contaminated by human pathogens, doubts still exist about the contamination of recombinant proteins, expressed in the milk of transgenic mammals, by human-transmissible pathogens.

Many plant systems are available for the production of recombinant antibodies

Several plant species have been successfully used to produce biologically active antibodies. In tobacco, alfalfa and some other species, expression is performed in leaves, whereas, in potato, corn, rapeseed, safflower, soybean, wheat or rice, the production and accumulation of recombinant antibodies occur in the tubers or in the seeds. Both production strategies present their advantages and drawbacks. Leaves present an active and complex metabolism which offers many possibilities, but they also contain a substantial protease activity which limits the accumulation of some proteins. Seeds present the advantage of offering a lower water content, and therefore represent a more stable accumulation site. In addition to the organ-specific storage of PMP(s), the increasing amount of information on protein targeting in a plant cell also allows for the storage of a protein of interest in various subcellular compartments. Up to now, most recombinant antibodies produced in plants have been secreted in the apoplast based on the efficiency of a signal peptide to target a foreign protein for secretion (Table 3). However, recent results have shown that the H/KDEL-mediated retention in the endoplasmic reticulum (ER) could present several advantages, including a strong increase in the stability of the recombinant protein (Wandelt *et al.*, 1992; Pueyo *et al.*, 1995; Tabe *et al.*, 1995; Conrad and Fiedler, 1998; Pagny *et al.*, 2003b) and

prevention of the addition of complex (i.e. immunogenic) N-glycans on plant-made antibodies (Finnern *et al.*, in press). Targeted expression of PMP(s) in the chloroplast also offers many advantages. For instance, by combining high expression levels and low proteolytic activity, a protein of interest expressed in this organelle could represent up to 20% of total chloroplast proteins (Maliga, 2003). This organelle looks particularly well suited to the production of very simple antigen binding molecules, such as VHH from camelidae IgG (Figure 1) (Jobling *et al.*, 2003), but, surprisingly, tobacco chloroplasts are also capable to properly fold complex proteins with disulphide bridges, such as human somatotropin (Staub *et al.*, 2000) and full-size antibodies (Daniell *et al.*, 2001).

A better knowledge of the signals and mechanisms responsible for protein targeting to the protein storage vacuole will help further investigations on the advantages and limitations of PMP(s) storage in this compartment (Paris and Neuhaus, 2002).

Plant-specific purification strategies

In all heterologous production systems, the recombinant protein will have to be extracted and purified from endogenous proteins of the producing organism. Protein purification is known to be a key stage in the preparation of a biopharmaceutical and it could represent more than 80% of its production cost. Antibody purification from plant protein extracts is generally achieved using existing technologies, such as affinity chromatography methods based on specific interactions with antigens, *Staphylococcus aureus* Protein A or *Streptococcus* sp. Protein G. The targeted expression of PMP(s) into specific subcellular compartments also offers plant-specific strategies to simplify the first steps of purification. For example, in oleaginous plants, the recombinant protein can be fused to a protein (oleosin) anchored in the membrane of lipid bodies, organelles that accumulate in the seed during maturation (Figure 2A). By combining this fusion with an expression targeted to the seed, researchers have established a simple expression/purification system in which the recombinant protein is recovered with the lipids after a centrifugation step. The recombinant protein, cleaved by targeted proteolysis, is then extracted from the lipid fraction by a second phase partitioning. This strategy has been used in rapeseed for the production of hirudin, an anticoagulant from leach salivary glands (Parmenter *et al.*, 1995; Boothe *et al.*, 1997). Recently, it has been adapted to the production of recombinant antibodies in seeds of another oilseed plant, safflower (Seon *et al.*, 2002). As shown in Figure 2(B), the transgenic plant synthesizes and accumulates two recombinant

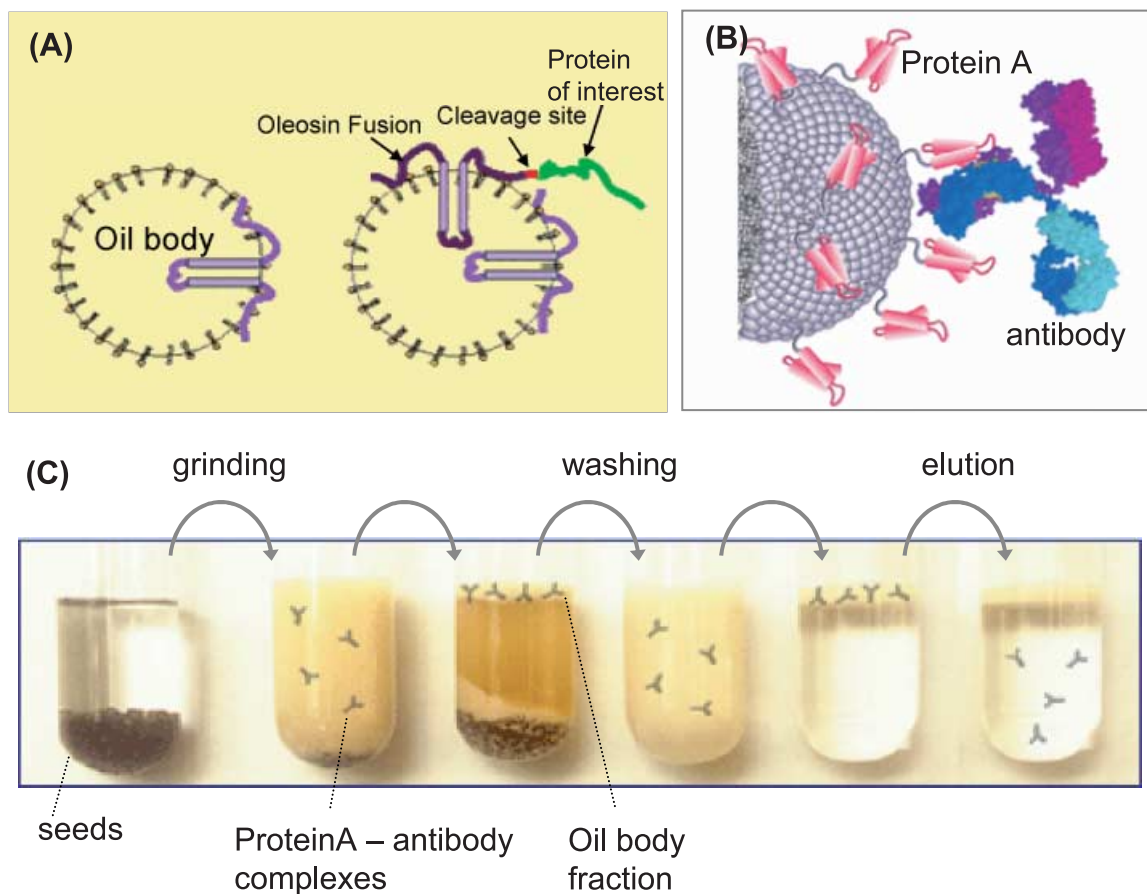


Figure 2 Strategy for the purification of a recombinant antibody by targeting to lipid bodies. Panel A. Anchoring of the oleosin molecule in the membrane of a native oil body (left). Recombinant lipid body presenting in its membrane an oleosin molecule fused to a protein of interest (right). Panel B. After grinding the seeds, the recombinant antibodies form complexes with the protein A molecules fused to oleosin and expressed on the surface of the recombinant lipid bodies. Panel C. Purification by centrifugation of antibodies from safflower seeds where the two recombinant proteins are stored: antibody and protein A fused to oleosin. (Adapted from Seon et al., 2002 and <http://www.sembiosys.ca/stratosome.html>)

proteins in two distinct cellular compartments of the seed: an antibody and a protein presenting a high affinity for this antibody, protein A fused to oleosin. The protein A-fused oleosin and the antibody form complexes when the seed is ground. These complexes are easily isolated, and purified with the lipid phase from the extract. Eventually, the antibodies are released after dissociation of the complexes by an acidic treatment (Figure 2C). Similar approaches, yielding simplified purification and a reduced proteolysis of the recombinant protein, have been developed using the advantages of protein expression in transgenic chloroplasts. As previously described for Bt toxin (De Cosa et al., 2001), due to its high expression level, human serum albumin (HSA) aggregates and forms inclusion bodies in tobacco chloroplasts. Inclusion bodies are easily purified from soluble cellular proteins, and HSA recovered from inclusion bodies is solubilized and *in vitro* refolded (Daniell and Dhingra, 2002).

Conformity of recombinant antibodies produced in plant systems

Plant cells are able to reproduce the complexity of human proteins. The homologies shared by the protein biosynthesis and maturation machinery in animal and plant cells are well illustrated through the ability of plants to produce various types of recombinant antibodies, such as IgGs or secretory IgAs (sIgAs) (Table 1). Antibodies are complex molecules. Immunoglobulins of the IgG class are tetramers consisting of two identical polypeptides of 450 amino acids (heavy chains) and two identical polypeptides of 250 amino acids (light chains). These four IgG-constituent polypeptides are linked together by several disulphide bridges. The complexity of an sIgA is even greater, as these immunoglobulins are composed of two IgA molecules linked together by two polypeptides (Figure 1). The assembly of an sIgA requires the action of

two different cell types in mammals. sIgA, IgGs and IgAs have been produced with success, in a biologically active form, in transgenic plants. This illustrates the ability presented by the plant cell machinery to assemble mammalian proteins, even when the latter are extremely complex. However, further work is still needed to explain why secretory IgA/IgGs, expected to be secreted in the tobacco apoplast, have been found in different subcellular compartments, such as the vacuole and the ER (Frigerio *et al.*, 2000). This heterogeneous distribution of a recombinant antibody in a plant cell could result from either improper folding or targeting. Unfortunately, it could induce a large structural heterogeneity in the PMP due to different proteolytic maturations occurring on the proteins in the ER, vacuole and apoplast. The N-glycan maturation is also reflective of the localization of a glycoprotein in the secretory pathway (Lerouge *et al.*, 1998), and the strong heterogeneity observed in the glycosylation of IgGs produced in tobacco could also be explained through this heterogeneous distribution (Cabanes-Macheteau *et al.*, 1999; Bakker *et al.*, 2001). In contrast, an IgG produced in alfalfa presents a very homogeneous N-glycan structure, which could be explained by a higher efficiency of the secretion and/or folding machinery in this plant expression system (Bardor *et al.*, 2003b).

Antibodies produced in transgenic plants differ from mammalian antibodies: immunogenic glyco-epitopes

The correct folding and assembly of plant-made antibody molecules within the ER, through interactions with a number of chaperones, processing and glycosylation enzymes, illustrate that co- and post-translational protein maturation events are similar in plants and mammals. However, as illustrated in Figure 3, antibodies produced in mammalian cells differ in their N-glycosylation from those produced in a plant system. This is illustrated with the monoclonal antibody Guy's 13, which is specific for an adhesin from *Streptococcus mutans*, the major pathogen contributing to the development of dental caries. When produced in mammalian cells, this IgG1 is glycosylated on its two N-glycosylation sites with oligosaccharidic structures (N-glycans) presenting an $\alpha(1,6)$ -fucose residue and about 10% terminal sialic acid. When produced in transgenic tobacco cells, the Guy's 13 antibody is also glycosylated on both N-glycosylation sites. However, the N-glycans borne by this antibody are of the high-mannose type (common structures to plants and mammals) and complex type, with typical characteristics for plants, such as $\beta(1,2)$ -linked xylose and $\alpha(1,3)$ -linked fucose (Cabanes-Macheteau

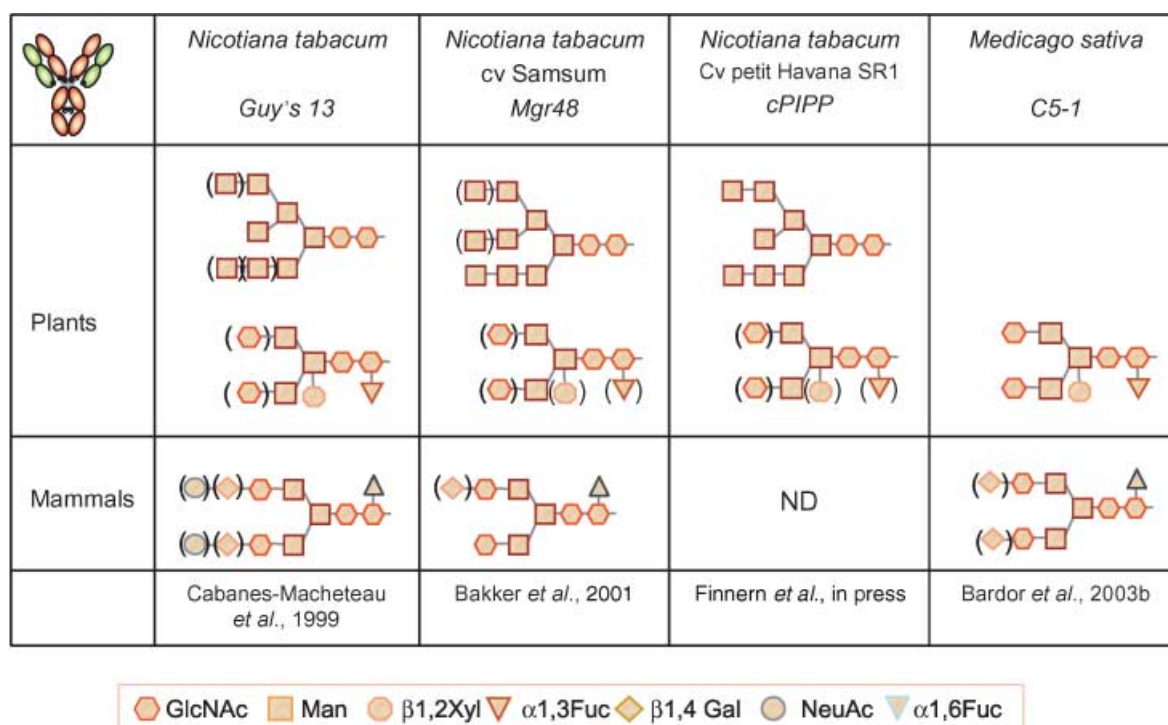


Figure 3 Structure of glycans N-linked to immunoglobulin G (IgG) molecules expressed in hybridoma and transgenic plants.

et al., 1999). Similar glycan heterogeneity and structural characteristics have been described for another monoclonal antibody (Mg48) produced in tobacco (Bakker *et al.*, 2001). In contrast with the IgG expressed in tobacco plants, the N-glycosylation of an alfalfa-derived antibody (C5-1) is restricted to a predominant mature oligosaccharide chain having a core $\alpha(1,3)$ -fucose, a bisecting $\beta(1,2)$ -xylose and two terminal GlcNAc residues. Although the homogeneity of glycosylation could differ from one plant expression system to the other, all plant species used so far to produce PMPs have the capacity to associate $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose residues on to complex N-glycans (Cabanes-Macheteau *et al.*, 1999; Bakker *et al.*, 2001; Bardor *et al.*, 2003b; Samyn-Petit *et al.*, 2003). These residues are constituents of glyco-epitopes, known to be important IgE binding carbohydrate determinants of plant allergens (Aalberse *et al.*, 1981; Faye and Chrispeels, 1988; Van Ree and Aalberse, 1995; Garcia-Casado *et al.*, 1996; Van Ree *et al.*, 2000). More importantly, it has recently been found that a plant N-glycan containing these glyco-epitopes not only shows IgE binding, but also causes mediator release by human basophils, when at least two of these N-glycans are present on the same protein (Westphal *et al.*, 2003). We and others have also reported that the immunization of goats (Kurosaka *et al.*, 1991) or rabbits (Faye *et al.*, 1993) with plant glycoproteins elicits the production of antibodies specific for these glyco-epitopes containing $\beta(1,2)$ -xylose or $\alpha(1,3)$ -fucose. More recently, *in vivo* experiments using BALB/c mice have shown that plant-made antibodies produced in tobacco do not elicit immunological response against their N-linked glycans of plant origin (Chargelegue *et al.*, 2000). Altogether, these data obtained in laboratory mammals raise the question of the immunogenicity of these glyco-epitopes in the context of a human therapy based upon PMPs. We recently addressed this question by reinvestigating their immunogenicity in rodents. We found that, in C57BL/6 mice and rats, but not using BALB/c mice, immunization with a model glycoprotein, horseradish peroxidase, elicited the production of antibodies specific for $\alpha(1,3)$ -fucose- and $\beta(1,2)$ -xylose-containing glyco-epitopes. Furthermore, we demonstrated that about 50% of non-allergic human blood donors contain in their sera antibodies specific for the $\beta(1,2)$ -xylose glyco-epitopes, whereas 25% have antibodies against the $\alpha(1,3)$ -fucose glyco-epitope (Bardor *et al.*, 2003a). These antibodies probably result from sensitization to environmental antigens. Although the immunological significance of anti- $\alpha(1,3)$ -fucose and anti- $\beta(1,2)$ -xylose antibodies is too speculative at the moment, the presence of such antibodies may, at least, induce a rapid immune clearance of glycosylated PMPs from

the circulation, which may greatly compromise their effectiveness as *in vivo* therapeutic agents. In addition to this accelerated clearance, the clinical effects resulting from the immune response caused by the administration of plant-derived glycoproteins in allergic or non-allergic patients are also questionable. As a consequence, for safety concerns related to the use of plant-derived therapeutic proteins, further experiments should be carried out in an appropriate animal model, as well as in human volunteers, by administering a therapeutic glycoprotein produced in a plant and analysing the immune responses to the plant glyco-epitopes in allergic and non-allergic populations.

Interestingly, even when antibodies are produced in mammalian expression systems, they also contain non-human sugar residues, such as the N-glycosylneuraminic acid (Neu5Gc) form of sialic acid (antibodies produced in CHO cells and in milk) or terminal $\alpha(1,3)$ -galactose (antibodies produced in murine cells). It has been shown that antibodies containing these sugar residues can also provoke undesirable side-effects, including an immune response in humans. Several examples of the genetic manipulation of CHO cells have been attempted to reduce the Neu5Gc level in recombinant glycoproteins (see Chenu *et al.*, 2003 for a recent paper). Similarly, the following part of this paper describes the current efforts carried out to prevent the association of immunogenic N-glycans to PMPs.

Towards the humanization of antibody glycosylation in plants

In plants, as in other eukaryotic cells, N-glycosylation starts in the ER, in a cotranslational way, by the addition of an oligosaccharide precursor ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) to specific asparagine residue constituents of the potential N-glycosylation-specific sequences, Asn-X-Ser/Thr, borne by the elongating polypeptide. Once transferred on to the nascent protein, and while the secreted glycoprotein is transported along the secretory pathway, the oligosaccharide undergoes several maturation reactions involving the removal or addition of residues in the ER and the Golgi apparatus (Figure 4). It is only in the late Golgi apparatus that plant and mammalian N-glycan maturation differs, which results in the absence of $\alpha(1,6)$ -linked fucose and sialic acids and the presence of bisecting $\beta(1,2)$ -xylose and core $\alpha(1,3)$ -fucose in the N-glycans of PMPs, as illustrated in Figure 5.

To be able to fully use the potential plants can offer for the production of recombinant antibodies, it becomes necessary to inhibit these plant-specific maturations in order to obtain 'humanized' non-immunogenic N-glycans. Numerous projects are currently in progress in order to prevent the addition of

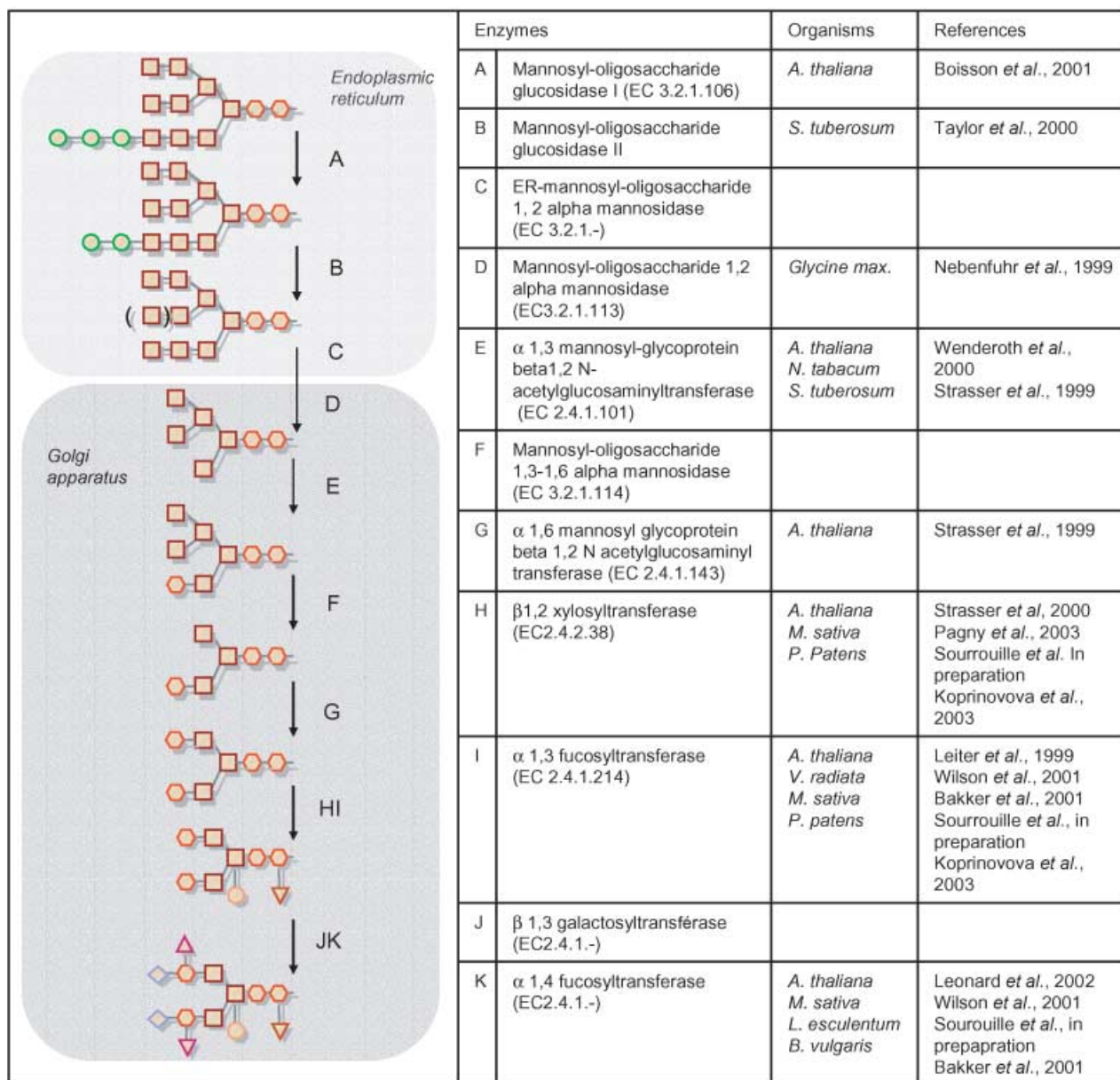


Figure 4 Processing of N-glycans in plants. N-glycosylation of plant proteins starts in the endoplasmic reticulum (ER) with the transfer of an oligosaccharide precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ on specific Asn residues of the nascent polypeptide chain. This precursor is then modified by glycosidases and glycosyltransferases mainly in the ER and the Golgi apparatus during the transport of the glycoprotein downstream of the secretory pathway. Glycosidases and glycosyltransferases responsible for plant N-glycan maturation are indicated from A to K in the left panel. Most of these plant N-glycan processing enzymes have recently been cloned from different plants as indicated in the right panel.

immunogenic glycans on to PMPs. One of the most drastic approaches is to prevent the addition of immunogenic N-glycans on plant-made antibodies by inactivating N-glycosylation sites through the mutation of Asn or Ser/Thr. In most cases, this strategy will not inactivate the antigen binding function of antibodies. However, many pharmaceuticals, including antibodies used for their effector functions, such as the triggering of the immune response (Wright and Morrison, 1994), require glycosylation for an increased *in vivo*

activity and stability. A recent observation has also shown that the addition of N-glycans to several recombinant protein or glycoprotein therapeutics increases their biological activity and half-life. This further illustrates a current tendency in glyco-engineering to increase, and not to reduce, the number of glycosylation sites on recombinant pharmaceuticals (Elliott *et al.*, 2003).

Promising results have been obtained in the production of plant-made glycosylated therapeutic proteins bearing

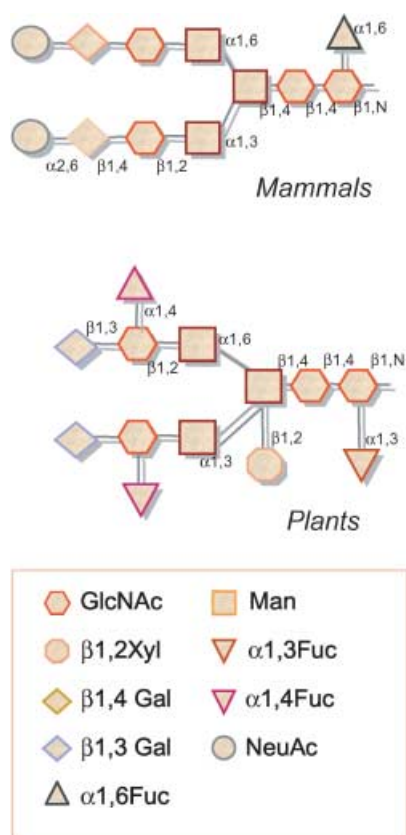


Figure 5 Plant and mammalian N-glycans have different structures. The first steps of N-glycan maturation are common to plants and mammals. Only the late steps differ, when glycosyltransferases specific for plants or for mammals act on the N-glycan. As illustrated here for typical plant and mammalian biantennary complex N-glycans, these differences in the processing machinery result in the absence of sialic acids in the terminal position of the antennae and the presence of a bisecting $\beta(1,2)$ -xylose and an $\alpha(1,3)$ -fucose residue instead of $\alpha(1,6)$ -fucose linked to the proximal N-acetylglucosamine of the N-glycans of plant-made pharmaceuticals (PMPs).

non-immunogenic N-glycans by storing antibodies within the ER, i.e. upstream of the compartment where plant-specific maturations take place. Other strategies, involving the inhibition of Golgi glycosyltransferases and the expression of mammalian glycosyltransferases within this compartment, are now under investigation. What has currently been achieved is detailed below.

Retention in the ER: control of glyco-epitope formation vs. PMP clearance

The structural analysis of plant ER-resident proteins has shown that natural reticuloplasmins bear high-mannose-type N-glycans (Navazio *et al.*, 1996, 1998; Pagny *et al.*, 2000). These oligosaccharide structures are common to plants and mammals, and therefore are probably not immunogenic. This observation

has suggested a strategy to prevent the association of immunogenic glycans to PMPs. This strategy consists in the storage of recombinant proteins within the ER, i.e. upstream of Golgi cisternae, where immunogenic glyco-epitopes are added to maturing plant N-glycans. It was first shown that the addition of H/KDEL sequences at the C-terminal end of a recombinant protein is sufficient for its retention in the plant ER (Gomord *et al.*, 1997, 1999 for a review). By this approach, we have shown that a model secretory protein (i.e. cell wall invertase) fused to an HDEL retrieval sequence was efficiently retained within the ER. A detailed structural analysis has shown that this cell wall invertase bearing the HDEL sequence (invertase-HDEL) displays mainly high-mannose-type glycans as well as a detectable amount of glycans containing the $\beta(1,2)$ -xylose epitope (Pagny *et al.*, 2000). This indicates that a small amount of this protein has first been transported across the secretory pathway down to the medial Golgi, where $\beta(1,2)$ -xylosyltransferase is located (Pagny *et al.*, 2003a), from which it is then retrieved and transported back to the ER.

Similar results have recently been obtained with a human monoclonal antibody containing KDEL sequences fused at the C-terminal end of both heavy chains, and expressed in tobacco plants (Ko *et al.*, 2003). As observed for invertase-HDEL, most glycans N-linked to this antibody were of the high-mannose type with six to nine mannose residues, but a fraction (about 10%) of these glycans contained the immunogenic $\beta(1,2)$ -xylose glyco-epitope (Figure 6). However, this antibody was not $\alpha(1,3)$ -fucosylated, a glycan modification occurring in the *trans*-Golgi side (Fitchette-Lainé *et al.*, 1994).

The retrieval efficiency is further increased when the ER retention sequence is fused to both heavy and light chains of the antibody. This was clearly illustrated with a chimeric mouse-human antibody expressed in tobacco and harbouring four KDEL retrieval signals on the fully assembled H2L2 form. As shown in Figure 6, this antibody presents exclusively high-mannose-type N-glycans with six to nine mannose residues (Finnern *et al.*, in press), indicating a very efficient recycling based on glycan maturation limited to enzymes located in the ER and *cis*-Golgi, such as α -mannosidase I (Nebenfuhr *et al.*, 1999).

Therefore, preventing the association of immunogenic N-glycans to PMPs through the fusion to ER retention signals is possible. However, when compared with their mammalian homologues, antibodies produced using this strategy present a very low stability after their injection in mice (Ko *et al.*, 2003). These antibodies with high-mannose N-glycans are probably rapidly degraded after binding to the mannose receptor and endocytosis by macrophages, as previously observed for

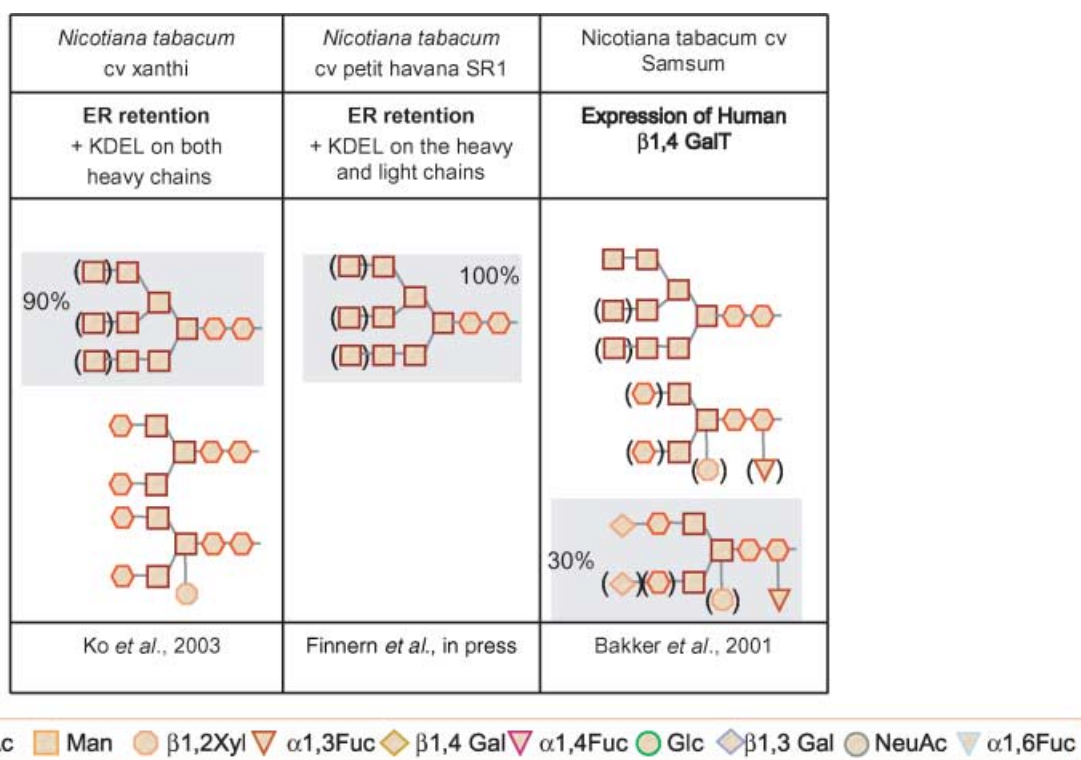


Figure 6 Some successful strategies towards plant N-glycan humanization. This figure illustrates glycosylation of plant-made antibodies produced with KDEL retrieval signals or in tobacco plants expressing a human β (1,4)-galactosyltransferase. An antibody fused with two (left panel) or four (middle panel) KDEL-ER retention signals is mostly or exclusively glycosylated with high mannose and probably non-immunogenic N-glycans. When an antibody is produced in tobacco plants expressing the human galactosyltransferase (right panel), 30% of its N-glycans show terminal N-acetylglucosamine sequences identical to those borne by this antibody when produced in hybridoma cells.

antibodies produced in Lec 1 mutant CHO cells (Wright and Morrison, 1998). In a similar clearance assay following intramuscular injection, a plant-made antibody with complex N-glycans (Bardor *et al.*, 2003b) has been shown to be as stable as its mammalian homologue in the bloodstream of mice (Khoudi *et al.*, 1999). This indicates that, in contrast with high-mannose N-glycans, matured plant N-glycans are not recognized by the mannose receptor. However, BALB/c mice used to study *in vivo* the stability of plant-made antibodies definitely provide the most inappropriate animal model to appraise both plant N-glycan immunogenicity and glycosylated PMPs' clearance in blood via carbohydrate-specific antibodies.

En route to the inactivation of plant glycosyltransferases

The analysis of an *Arabidopsis* mutant has shown that the inactivation of only one glycosyltransferase, N-acetylglucosaminyltransferase I (GnTI), is sufficient to block the biosynthesis of complex N-glycans (von Schaeuwen *et al.*, 1993). This glycosyltransferase has been cloned from several plants

(Figure 4). However, the expression of antisense GnTI in tobacco and potato plants has only shown a decrease in complex N-glycan biosynthesis in these plants (Wenderoth and von Schaeuwen, 2000).

Independent of its low efficiency in the prevention of immunogenic N-glycan formation, this pioneer study has stimulated the interest of several laboratories and molecular farming companies for plant glycosyltransferases. Many of these enzymes, in particular 'targets' for inactivation, such as β (1,2)-xylosyltransferase and α (1,3)-fucosyltransferase, have been cloned within the past 5 years in several plant expression systems (Figure 4). In the near future, the development of strategies allowing an efficient inhibition of these glycosyltransferases will prevent glyco-epitope formation on therapeutic proteins produced in higher plants. Recent results obtained in the moss *Physcomitrella patens* have paved the way to inactivation strategies in higher plants. N-linked glycosylation of mosses is highly similar to that in higher plants (Vi tor *et al.*, 2003), and its genes for α (1,3)-fucosyltransferases and β (1,2)-xylosyltransferase show 50% and 38% identity, respectively, with those already cloned from *A. thaliana*. *P. patens* is the only known plant system which shows a

high frequency of homologous recombination. This strategy has been used to knock out $\alpha(1,3)$ -fucosyltransferases and $\beta(1,2)$ -xylosyltransferase genes, permitting the disappearance of plant-specific glyco-epitopes without any effect on protein secretion in this moss (Koprinovova *et al.*, 2003).

As described for their mammalian counterparts, plant glycosyltransferases are type II membrane proteins. This prediction has been confirmed through the detailed characterization of $\beta(1,2)$ -xylosyltransferase from *A. thaliana*. The first 36 amino acids of this glycosyltransferase (i.e. cytosolic tail + transmembrane domain) are sufficient for its Golgi retention, but also contain information on sub-Golgi compartment targeting (Pagny *et al.*, 2003a). The analysis of several other glycosyltransferases is currently providing a panel of specific signals that are sufficient for protein targeting within the different Golgi subcompartments. These signals will help to target exogenous glycosyltransferases in the plant Golgi apparatus for an optimal efficiency in the engineering of the glycosylation pathway, as illustrated below. However, there will also be limits to N-glycosylation engineering in plants. This is illustrated by our recent results demonstrating the importance of the early steps of N-glycan maturation for the accumulation of seed storage proteins, the formation of protein bodies and cell differentiation during plant embryo development (Boisson *et al.*, 2001).

Expression of mammalian glycosyltransferases in the plant Golgi apparatus

Another attractive strategy to humanize plant N-glycans is to express mammalian glycosyltransferases in the plant, which would complete and/or compete with the endogenous machinery of N-glycan maturation in the plant Golgi apparatus. Based on these complementation strategies, we have hypothesized recently that the expression of human $\beta(1,4)$ -galactosyltransferase, in the Golgi of plant cells, could lead to a partial humanization of plant N-glycans and, possibly, compete with the addition of $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose. In agreement with this hypothesis, we have shown that the human $\beta(1,4)$ -galactosyltransferase, when expressed in plant cells, transfers galactose residues on to the terminal N-acetylglucosamine residues of plant N-glycans. Moreover, 30% of N-glycans carried on an antibody, produced in tobacco plants expressing this human galactosyltransferase, bear terminal N-acetylglucosamine sequences of the mammalian type. (Figure 6) (Bakker *et al.*, 2001). The efficiency of this strategy is further increased when a targeted expression of human $\beta(1,4)$ -galactosyltransferase is obtained after fusion of the catalytic domain of this glycosyltransferase with the

first 54 amino acids of *A. thaliana* $\beta(1,2)$ -xylosyltransferase (Bakker *et al.*, 2003).

However, as the N-glycans borne by the tobacco-derived antibodies are highly heterogeneous, the action of the human $\beta(1,4)$ -galactosyltransferase on this pool of glycans resulted in a highly complex mixture of N-glycans, some of them being partially humanized (Bakker *et al.*, 2001). These strategies developed to glycoengineer plant-made antibodies would be more efficient in plant systems such as alfalfa, where the N-glycosylation of antibodies is restricted to a predominant mature oligosaccharide chain harbouring terminal GlcNAc residues. These glycans present the perfect structure for *in vitro* or *in vivo* remodelling into a human-compatible N-glycosylation. As a proof of concept, we have shown that *in vitro* galactosylation using a $\beta(1,4)$ -galactosyltransferase of an antibody-made in alfalfa resulted in an efficient conversion of the plant N-glycan into oligosaccharides having homogeneously galactosylated antennae identical to those of the murine antibody (Bardor *et al.*, 2003b).

Concluding remarks

The promising results already obtained for the humanization of N-glycosylation will hopefully permit the creation of plant systems producing PMPs compatible with human therapy in the near future. Current efforts are concentrated on the association of complementation strategies with strategies allowing the inhibition of plant glyco-epitope biosynthesis. Our next goal, in the near future, is to produce plant-made antibodies presenting an N-glycosylation profile identical to that observed in mammals. In this respect, key steps will include the inactivation of plant $\beta(1,2)$ -xylosyltransferase and $\alpha(1,3)$ -fucosyltransferase using strategies that prove to be more efficient than the antisense oligonucleotide approach and improvement of the human $\beta(1,4)$ -galactosyltransferase efficiency. We are currently working at increasing the performance of this heterologous glycosyltransferase through a better control of its targeting in the Golgi cisternae.

Compared with the other plasma proteins, antibodies present a simple N-glycosylation, with partial terminal $\beta(1,4)$ -galactosylation and very few terminal sialic acids. However, sialic acids are very important for the clearance of many mammalian plasma proteins of pharmaceutical interest. The absence of such residues on these circulating proteins induces their fast elimination from the blood by interactions with galactose-specific receptors on the surface of hepatic cells. To our knowledge, sialic acids are absent from plant cells. Adapting the maturation machinery of plant N-glycans to obtain sialylated N-glycans in plants would require

the transfer of at least five heterologous genes encoding enzymes implicated in sialic acid biosynthesis, and transport within the Golgi. These enzymes should not only be expressed in a stable manner, but should also be active and correctly targeted in a plant cell.

Therefore, the production of humanized antibodies in plants pertains to today's technology, whereas the production of sialylated PMPs at large remains a much more ambitious objective for plant biotechnology.

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