



Biopharmaceutical production in plants: problems, solutions and opportunities

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There are major structural differences between plant and mammalian N-linked glycans, with those from plants being immunogenic in most laboratory mammals and eliciting glycan-specific IgE and IgG antibodies in humans, when delivered parenterally. However, because humans are constantly exposed to plant glycoproteins in the diet, glycosylated plant-made pharmaceuticals (PMPs) should be acceptable for topical and oral administration. To exploit fully the potential that plants offer for the production of therapeutic proteins for parenteral administration, it might be necessary to inhibit plant-specific post-translational modifications to obtain 'humanized' non-immunogenic N-glycans on PMPs. The benefits that could accrue are lower manufacturing costs, relative to mammalian cell culture, and a reduced risk of transmission of mammalian pathogens.

Plant and mammalian complex N-glycans differ in their structures

Antibodies produced in plants (plantibodies) bear both high-mannose (Man₅–Man₉ glycans) and biantennary complex type N-glycans, the proportions of each varying with plant species. The high-mannose-type N-glycans have the same structure in plant and mammalian glycoproteins. By contrast, complex-type N-glycans are structurally different in plants and mammals. For instance, in plants, the proximal N-acetylglucosamine of the core is substituted by an α 1,3 fucose in place of an α 1,6 fucose in mammals, and the β -mannose of the core is substituted by a bisecting β 1,2 xylose in plants, in place of a β 1,4 N-acetylglucosamine in mammals. In addition, β 1,3 galactose and fucose α 1,4-linked to the terminal N-acetylglucosamine of plant N-glycans form Lewis a oligosaccharide structures instead of β 1,4 galactose combined with sialic acids in mammals. The complex type N-glycans that are present on plantibodies produced in tobacco are highly heterogeneous with structures ranging from paucimannosidic to Lewis a type N-glycans (Figure 1) [1–4]. The glycoform profile of plantibodies can differ depending on the compartment of the secretory pathway in which they accumulate (e.g. ER, vacuole, apoplast) [4–7]. As illustrated in Figure 2, additional heterogeneity is observed for

plantibodies produced in tobacco because the glycoform profile differs between young leaves (30% high-mannose glycans) and older leaves (10% high-mannose glycans) [8].

Plant N-glycans are allergenic

The presence of IgE antibodies that are specific for plant N-glycans in the sera of allergic human patients is well documented [9–14]. Interestingly, repeated subcutaneous injections of a pollen homogenate in allergic patients during their desensitization result in the production of IgG4 antibodies that are specific for the glycan moieties of glycoallergens [15]. We have characterized the specificity of glycan-specific IgG4 in sera from allergic patients receiving grass pollen immunotherapy and demonstrated specificity for both the α 1,3 fucose and β 1,2 xylose glycoepitopes (Bardor *et al.*, unpublished results). Thus, humans allergic to plant glycoallergens produce both IgE and IgG4 that are specific for α 1,3 fucose and β 1,2 xylose glycoepitopes.

The clinical significance of carbohydrate-specific IgE remains uncertain. However, the major allergen of olive tree pollen, Ole e1 – a glycoprotein with N-glycan containing β 1,2 xylose and α 1,3 fucose glycoepitope [16] – was reported to elicit basophil histamine release [17].

Immunogenic potential of plant-derived therapeutic glycoproteins

Charlelegue *et al.* [18] reported that no glycan specific antibodies were produced in BALB/c mouse when exposed to a plantibody containing the α 1,3 fucose and β 1,2 xylose glycoepitopes [1]. However, BALB/c mice might be more tolerant than other species and responsiveness will be highly dependent on the dosing regimen. Indeed, in contrast with C57BL/6 mice, rats, rabbits and goats, BALB/c mice also do not produce anti-glycan antibodies following immunization with plant glycoproteins delivered in Freund's adjuvant, such as horseradish peroxidase (HRP) [3,19,20]. Antibodies induced to glycans N-linked to a purified plant glycoprotein are cross-reactive with glycoproteins from plants, insects or snails containing the same α 1,3 fucose and β 1,2 xylose glycoepitopes [20]. The specificity of these antibodies for plant N-glycans was further confirmed through their reactivity with the heavy chain of a plantibody containing α 1,3 fucose and β 1,2

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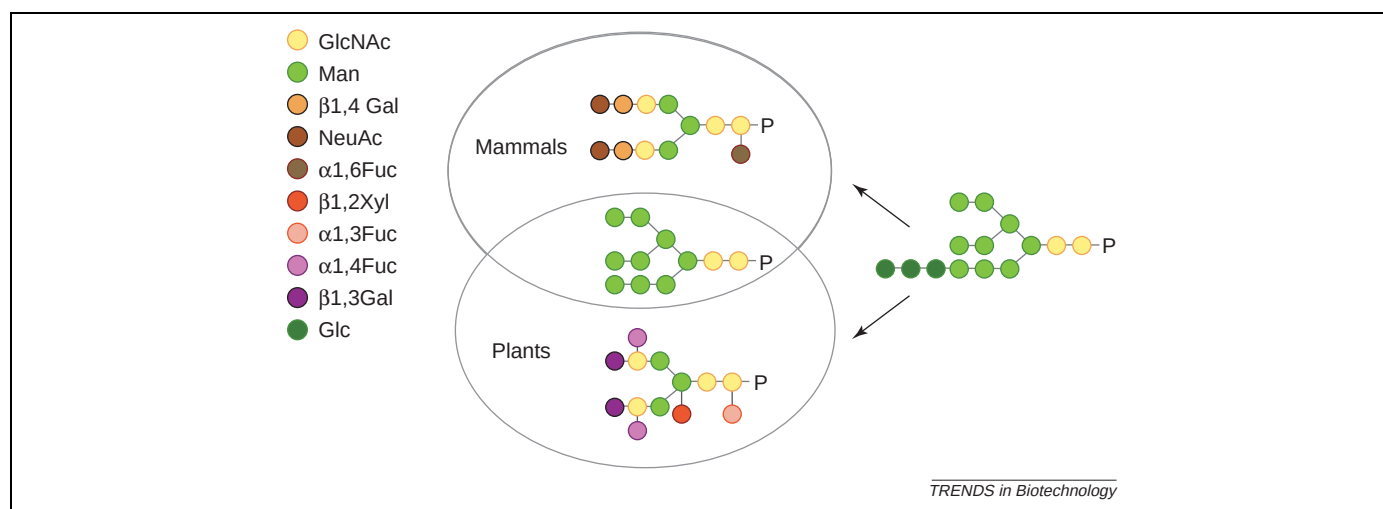


Figure 1. Plant and mammalian complex N-glycans differ in their structure. In plant and mammalian cells, two main classes of N-glycans are synthesized from the same oligosaccharide precursor added co-translationally to nascent glycoproteins. A first class of high-mannose-type N-glycans is made after a limited trimming of the precursor by glycosidases. These glycans have the same structure in plant and mammalian glycoproteins and contain two N-acetylglucosamine and 5–9 mannosyl residues. The second class of N-glycans, the complex type N-glycans, is made after extensive trimming of the precursor and addition of new sugar residues by glycosyltransferases, some of them being specific for the organism producing the glycoprotein. As a result, biantennary complex type N-glycans are structurally different in plants and mammals. For example, in plants the proximal N-acetylglucosamine of the core is substituted by an α 1,3 fucose (α 1,6 fucose in mammals) and the β mannose of the core is substituted by a bisecting β 1,2 xylose (a bisecting β 1,4 N-acetylglucosamine in mammals). In addition, β 1,3 galactose and fucose α 1,4 linked to the terminal N-acetylglucosamine of plant N-glycans form Lewis a oligosaccharide structures (instead of β 1,4 galactose combined with sialic acid in mammals [47,48]). Abbreviation: P, protein.

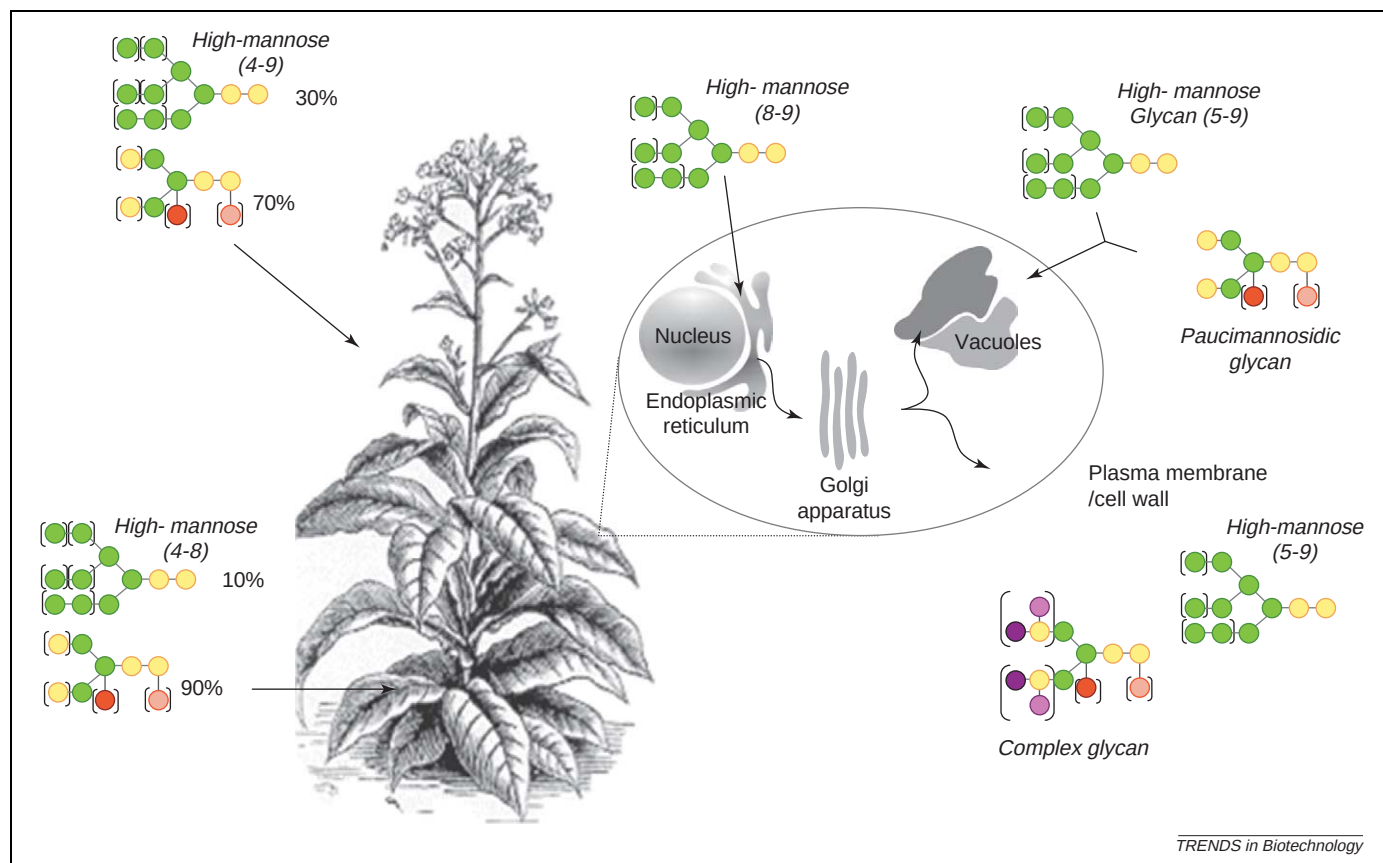


Figure 2. The glycoform profile of a plant-made pharmaceutical (PMP) will differ according to the plant in which it is synthesized and the organ and the subcellular compartment where it accumulates. When expressed in tobacco, plantibodies isolated from young leaves have a relatively high amount of high-mannose-type N-glycans compared with plantibodies from older leaves, which contain more terminal N-acetylglucosamine [8]. N-glycosylation also depends on the subcellular compartment where the glycoprotein is stored. For example, an endoplasmic reticulum (ER)-resident glycoprotein such as calreticulin or plantibodies fused with ER-retention signals have high-mannose-type N-glycans [4,5], whereas Lewis a antennae or terminal GlcNAc are detected on glycans N-linked to extracellular glycoproteins [6,7]. Finally, the paucimannosidic N-glycans are considered typical for vacuolar glycoproteins [47].

xylose glycoepitopes, whereas they do not react with the same heavy chain containing exclusively high-mannose N-glycans [4].

Although an abundant literature illustrates the immunogenicity of plant complex N-glycans in most laboratory mammals, the species-specific nature of the immune response and its dependence on the route and nature of the antigen presentation does not enable secure predictions of the immunogenicity of glycans N-linked to plant-made pharmaceuticals (PMPs) in man. Additional factors might influence the intrinsic immunogenicity of plant glycans associated with therapeutic proteins (see subsequent section).

Additional factors influencing potential immunogenicity

It is established that the IgG-Fc mediated effector activities of human antibodies are dependent on glycosylation and differ between individual glycoforms [21]. Therefore, the unique N-glycans added by plants could impact on both immunogenicity and functional activity. However, any such impact should to be assessed relative to the probability and consequences of immune responses directed at other portions of antibody molecules.

When the diversity of glycosylation-related effects on antibody functionality is considered [21], it becomes apparent that the immunogenic potential of a plantibody will be a highly complex function of inter-dependent attributes. Therefore, plant N-glycans could be immunogenic in their own right, or when co-presented in glycoprotein structures – but this has to be weighed against the expected immunogenicity of idiotypic and allotypic IgG motifs. In addition, plant complex N-glycans might modify significantly the pharmacokinetic behaviour of the plantibody – for example, by mediating more rapid clearance via liver mannose receptors [22] – and thereby reducing T-cell activation. This reduction of T-cell activation might be expected to not only reduce efficacy but also to abrogate the immunogenic risk.

'Humanized' therapeutic monoclonal antibodies are immunogenic

The licensed recombinant antibodies are either chimeric (mouse V and human constant regions), 'humanized' (mouse antigen binding sequences in a human background) or 'fully human' antibodies generated from a phage display library or transgenic mice; each has been seen to be immunogenic in a proportion of patients with the development of human anti-chimeric (HACA) or

human anti-human (HABA) responses, respectively (Table 1). Although in the phage display and transgenic mouse technologies antibodies are generated from human Ig genes, they represent products of 'forbidden clones' that would be eliminated in the intact human individual. The most 'human-like' glycosylation profiles for recombinant therapeutics are those produced in mammalian cells (e.g. CHO, NS0, Sp2/0). These cell lines yield some of the human glycoforms but not all; in addition, although a minor population of normal human IgG bears α 2,6 linked N-acetylneuraminic acid, CHO, NS0 and Sp2/0 cells add it in α 2,3 linkage. Of more concern is the ability of CHO, NS0 and Sp2/0 cells to add N-glycolylneuraminic acid as a terminal sugar [23]. Humans do not have a functional gene encoding the enzyme that generates N-glycolylneuraminic acid and this structure is known to be immunogenic. The addition of N-glycolylneuraminic can be anticipated for IgG produced in transgenic cows, sheep, goats and so on. Similarly, mammalian cell lines also have an ability to generate Gal- α 1,3 Gal structures [24]; humans and higher primates do not have a functional gene encoding the required transferase; however, constant environmental exposure to this structure results in most individuals having circulating IgG antibodies that are specific for this epitope. Thus, antibodies engineered to render them more 'human' and produced in the most 'human-like' expression systems are still regarded as altered self and have potential immunogenicity in human [25].

Risk assessment of the potential immunogenicity of plant-made pharmaceuticals

Regulatory authorities require that potential protein therapeutics are tested for immunogenicity in animal models; however, it is recognized that results obtained in mice, rabbits or primates might not be a relevant guide to immunogenicity in humans [26–29]. More sophisticated animal models that incorporate functional aspects of the adaptive human immune repertoire could become available for more routine application in future [30]. The development of validated bioanalytical methodologies to characterize the humoral immune response will also be required [31]. The specificity of an antibody response should be characterized as anti-idiotype, anti-isotypic or anti-plant glycan. The capacity to stimulate human T-cells *ex-vivo* should also be assessed, using T-lymphocytes and antigen-presenting cells from a broad cross-section of the human population [32]. Animal studies of the

Table 1. Most licensed recombinant antibodies are immunogenic in humans (adapted from Ref. [32])

Antibody	Indication	Type of antibody	% of patients with immune response
Remicade	Crohn's Disease and rheumatoid arthritis	Chimeric	10–60
Simulect	Transplant rejection	Chimeric	1.5
Rituxan	Lymphoma	Chimeric	1.1
Reopro	Cardiac	Chimeric Fab	6
Anti-A33	Cancer	Humanized	72
Anti-B72.3	Cancer	Humanized	75
Campath	Leukemia	Humanized	50
Zenapax	Transplant rejection	Humanized	8.4
Synagis	Respiratory syncytial virus infection	Humanized	1.1
Herceptin	Cancer	Humanized	0.1
Xolair	Allergy	Humanized	0
Humira	Crohn's Disease and rheumatoid arthritis	Fully-human	12

immunogenicity of plantibodies should also be evaluated in repeat-dose toxicology studies of suitable duration.

Evidence for immunogenicity might not exclude clinical application of PMPs but it would impact on the development process leading towards their therapeutic use. Initial clinical trials might be performed in an immunocompromised patient population, using a less immunogenic treatment route (intravenous rather than subcutaneous) with careful monitoring of immunogenicity before dose escalation; allergenicity screening tests before administration of PMPs could be mandatory [33]. Clinical studies of repeat-use products would be continued for a minimum of 12 months duration and, possibly, on re-administration following treatment breaks. Post-marketing commitment to monitoring in wider populations is a standard requirement for new biopharmaceutical products.

Strategies to reduce the potential immunogenicity of N-glycans in plant-made pharmaceuticals

Although wild-type plants might be inappropriate for the production of recombinant glycoproteins requiring parenteral administration, they could be acceptable for products requiring topical or oral administration because humans are constantly exposed to plant proteins in their diet without adverse reactions. Currently, the heavily glycosylated protein gastric lipase, produced in corn (maize), is in Phase II clinical trials for the treatment of patients suffering from exocrine pancreatic insufficiency (<http://www.meristem-therapeutics.com/GB/intro.htm>). The predominant antibody that is present in external secretions (e.g. breast milk) is of the IgA class; therefore, there

appear to be opportunities for the production of IgA plantibody therapeutics for topical or oral administration (<http://www.planetbiotechnology.com/technology.html>). With systemic applications in view, considerable progress has been reported for the generation of 'knockout' and 'knock-in' plants engineered to eliminate the addition of unwanted sugar residues and/or to 'humanize' PMP glycosylation (for review, see Ref. [34]). Possibly the most advanced system is the moss *Physcomitrella patens* because of the ease with which 'knockout' and 'knock-in' of genes can be accomplished by homologous recombination in this expression system [35]. Thus, *P. patens* has been engineered to produce a strain that does not add β 1,2 xylose or α 1,3 fucose but produces PMPs bearing a core heptasaccharide identical to that of human IgG, ([35]; see also <http://www.greenovation.com/>). The availability of glycosylation mutants has also enabled the production of glycoproteins that are devoid of plant-specific glycoepitopes in the model higher plant *Arabidopsis thaliana* [36] (Figure 3).

Most human glycoproteins are synthesized with oligosaccharides that are 'capped' by the addition of sialic acid to a penultimate galactose residue. The absence of terminal sialic acid and/or galactose residues might be expected to increase the clearance rate of glycosylated PMPs. This is why knock-in engineering has focused on the addition of terminal β 1,4 galactose and sialic acid residues to humanize PMP glycosylation. Tobacco plants expressing the human β 1,4 galactosyltransferase were shown to produce antibody with 30% of N-glycans bearing terminal N-acetylglucosamine sequences, identical to those associated to the N-glycans of an antibody produced

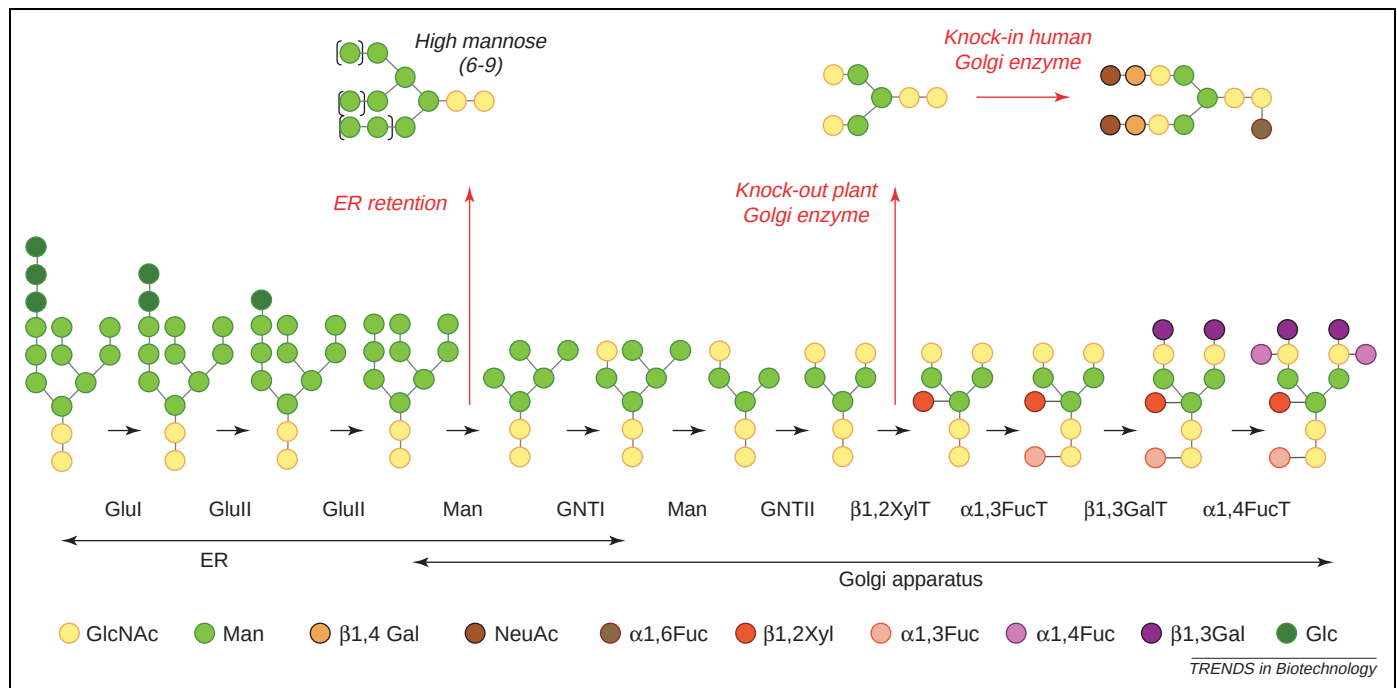


Figure 3. Current strategies to reduce the immunogenicity and to humanize plant complex N-glycans. It is already possible to produce plant-made pharmaceuticals (PMPs) without immunogenic complex N-glycans using either retention in the endoplasmic reticulum (ER) [4] or knockout of β 1,2 xylosyltransferase and α 1,3 fucosyltransferase [35]. Knock-in strategies toward humanization of plant N-glycans have also been successful with the addition of β 1,4 galactose residues to plantibodies produced in tobacco [2]. Engineering plants to add sialic acid is more demanding because plants are unable to synthesize sialic acid [48] and, consequently, getting sialylated PMPs will require the simultaneous expression of several heterologous genes. Abbreviations: Glu I, Glucosylase I; Glu II, glucosylase II; Man, mannosidase; GNT I, N-acetylglucosaminyltransferase I; GNT II, N-acetylglucosaminyltransferase II; β 1,2XylT, β 1,2 xylosyltransferase; α 1,3FucT, α 1,3 fucosyltransferase; β 1,3GalT, β 1,3 Galactosyltransferase; α 1,4FucT, α 1,4 fucosyltransferase.

in mammalian cells [2]. However, because the N-glycans carried by the tobacco-derived antibodies are highly heterogeneous, the action of the human β 1,4 galactosyl-transferase on this pool of glycans resulted in a complex mixture of N-glycans, some of them being only partially humanized [2,37–39]. This strategy has proved to be more efficient in plant systems, such as alfalfa, where the N-glycosylation of antibodies is restricted to a mature oligosaccharide chain bearing terminal GlcNAc residues [40]; see also, <http://www2.medicago.com/en/>.

Engineering plants to add sialic acid is more demanding because it requires the transfer of at least five heterologous genes encoding enzymes implicated in sialic acid biosynthesis and transport within the Golgi. In addition to stable expression, these enzymes also have to be targeted correctly within the plant cell. Interestingly, the absence in plants of a pathway for the addition of sialic acid is not restrictive to plantibody production. Indeed, the IgG-Fc oligosaccharide of normal human IgGs does not bear sialic acid and human IgGs are catabolized through the unique neonatal receptor (FcRn), not the asialoglycoprotein receptor of the liver. However, the natural plant N-glycans might be expected to affect adversely the half-life of IgG plantibodies and to compromise IgG-Fc functional activity.

Proteins that reside in the lumen of the plant endoplasmic reticulum (ER) contain high-mannose type N-glycans with structures common to mammals [5,41,42]. Protein retention within the lumen of the ER of eukaryotic cells is based on the presence of the C-terminal retention sequences: KDEL or HDEL. Thus, the addition of H/KDEL sequences at the C-terminal end of a secretory protein is sufficient for retention in the plant ER [43,44].

Recently, antibodies containing KDEL sequences fused at the C-terminal end of their heavy chains or both at the heavy and light chains, were expressed in tobacco plants [4,22]. In the first case, most N-linked glycans added were of the high-mannose type with 6–9 mannose residues but a fraction (~10%) still contains the immunogenic β 1,2 xylose glyco-epitope. By contrast, the antibody expressed with KDEL retention signals on both the heavy and light chains contained exclusively non-immunogenic high-mannose type N-glycans [4] (Figure 4). However, the *in vivo* half-life of these antibodies in mice is reduced relative to mammalian antibodies or plantibodies bearing complex oligosaccharides; probably due to endocytosis and degradation following binding to mannose receptors, as previously observed for high-mannose N-glycan bearing antibodies produced in the Lec 1 mutant CHO cells [40,45,46].

Relative risk posed by plant N-glycans

Although there is evidence that glycoproteins containing plant N-glycans crossreact with pre-existing antibodies (IgE, IgG₄, IgG₁), the question of whether recombinant proteins bearing plant N-glycans are immunogenic in humans remains open. Nevertheless, it would be desirable to eliminate/minimize plant-specific post-translational modifications to obtain 'humanized' non-immunogenic N-glycans on PMPs. The reward for the effort required would be the reduced risk of transmission of mammalian pathogens and cost efficiencies that will enable wider patient access to these products.

Currently, our knowledge of the long-term clinical impact of host anti-product antibodies is still incomplete, as are our attempts to monitor immune responses to

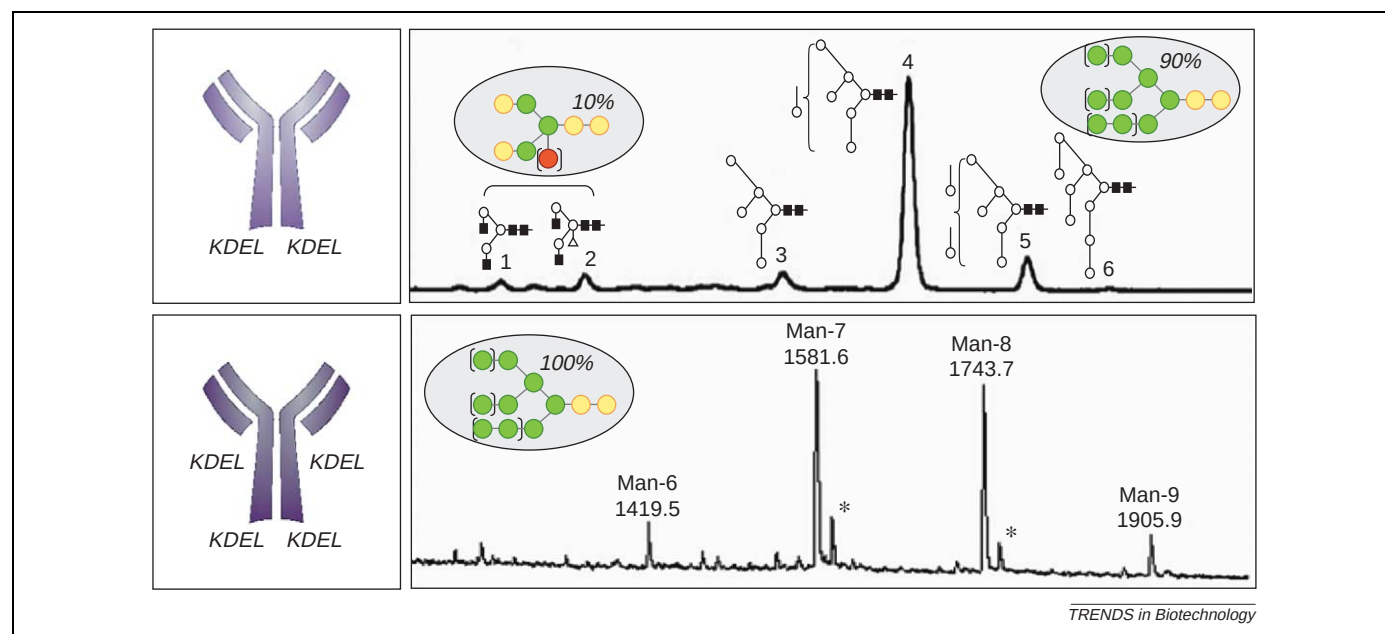


Figure 4. Retention in the endoplasmic reticulum (ER) prevents the biosynthesis of immunogenic N-glycans on plant-made pharmaceuticals (PMPs). As illustrated on the lower panels, after fusion of a KDEL, ER-retention signal, at the C-terminal end of both the heavy and light chains of a plantibody, it is possible to prevent the addition of immunogenic complex N-glycans completely [4]. By contrast, as shown in the upper panels, 10% of complex N-glycans are still observed on an antibody fused to KDEL only on the heavy chains [22]. Consequently, it appears that increasing the density of ER retention signals on a PMP could improve the efficiency of the retention/recycling machinery. However, it cannot be excluded here that differences observed in the glycosylation patterns are antibody-dependent. The upper and lower panels show an HPLC chromatogram and a MALDI-TOF mass spectrum, respectively, of N-glycans isolated from the KDEL-tagged antibody presented on the left of each panel. (Figure modified, with permission, from Refs [4] and [22]).

therapeutic proteins. Particular attention could be focused on detecting heightened responsiveness in certain clinical sub-populations, for example, HLA types. However, we anticipate many academic and industrial groups will continue to seek to exploit the many positive advantages of plant-expression systems to produce glycoprotein pharmaceuticals.

Acknowledgements

Work on glycobiology at the University of Rouen was supported by the Centre National de la Recherche Scientifique (CNRS) and by the 'Ministère de la Recherche'. We would like to thank present and former colleagues who contributed to the work described in this review and for their critical reading of the manuscript.

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