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review

N-Glycosylation of Recombinant Therapeutic Glycoproteins in Plant Systems

Biljana Balen and Marijana Krsnik-Rasol*

Department of Molecular Biology, Faculty of Science, University of Zagreb, Horvatovac 102a, HR-10000 Zagreb, Croatia

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Summary

Transgenic plants have recently emerged as a promising system for low-cost and large-scale production of therapeutic proteins. Despite many advantages plants offer as hosts for expression of recombinant proteins, there are certain limitations caused by major structural differences between plant and mammalian *N*-linked glycans. Thus, efforts are being made in order to eliminate, or at least minimize, plant-specific *N*-glycosylation as well as to supplement the plant endogenous system with human glycosyltransferases to obtain »humanized« non-immunogenic *N*-glycans on plant-made pharmaceuticals aimed for human therapy.

Key words: *N*-glycosylation, transgenic plants, glycosyltransferases, sialic acid, recombinant protein, therapeutic glycoproteins

Introduction

The technology of recombinant DNA, which has rapidly developed in the last two decades, has allowed the expression of heterologous recombinant proteins in different host systems. The majority of the early work was directed to expression of recombinant therapeutic proteins in prokaryotic hosts, mainly in bacteria *Escherichia coli*, because of the low overall cost and short production timescale. Due to limitations of prokaryotic systems, such as low product quality and the lack of post-translational modifications, biotechnology has turned to eukaryotic hosts: yeast, insect and mammalian cell cultures as well as transgenic animals. These production systems have drawbacks in terms of cost, scalability, product safety and authenticity (Table 1) (*1*). Therefore, a simple and inexpensive system that allows large-scale production of safe recombinant proteins would be highly desirable. Studies performed recently have revealed the great potential of plants as an alternative system for expression of recombinant proteins due to many practical, economic and safety advantages in comparison with conventional systems (*2*,*3*).

Plants have a great potential as biofactories for the production of therapeutic proteins. Consequently, the number of recombinant proteins successfully produced in various plants, including mono- and dicotyledons, is rapidly increasing (*4–6*). Plant expression systems are much less likely to harbour human pathogens than mammalian expression systems. Therefore, plants are convenient hosts for production of therapeutic proteins such as vaccines and antibodies. Moreover, it is possible to direct the expression of the recombinant protein to specific parts of plant, such as fruits, seeds, leaves and tubers. Several examples have shown that plants allow the production of complex human proteins with appropriate biological features and activity (*7–10*).

Most of the therapeutic proteins are subjected to several posttranslational modifications, among which glycosylation is the most frequent one. The oligosaccharide chain can be either *N*- or *O*-linked. *N*-glycosylation occurs in the endoplasmic reticulum (ER) and the primary

*Corresponding author; Phone: ++385 1 46 06 268; Fax: ++385 1 46 06 286; E-mail: bbalen@zg.biol.pmf.hr

Table 1. Comparison of available hosts for production of recombinant therapeutic proteins

Host	Production cost	Production timescale	Product quality	Glycosylation	Risk of pathogenicity	Storage cost	Ethical concerns
Bacteria	Low	Short	Low	No	Medium	Moderate	Medium
Yeast	Medium	Medium	Medium	Yes (unusual)	Low	Moderate	Medium
Mammalian cells	High	Long	Very high	Yes	High	Expensive	Medium
Insect cells	High	Long	High	Yes (minor differences)	Medium	Expensive	Medium
Transgenic mammals	High	Very long	Very high	Yes	High	Expensive	High
Plant cell cultures	Medium	Medium	High	Yes (minor differences)	Low	Moderate	Medium
Transgenic plants	Low-medium	Long	High	Yes (minor differences)	Low	Low	Medium

oligosaccharide chain is further processed during its exit from the ER and passage through the Golgi apparatus (GA). The latter cell compartment is also a site of protein *O*-glycosylation. Carbohydrate binding to a polypeptide chain has a great impact on physicochemical properties of proteins such as its resistance to thermal denaturation, protection from proteolytic degradation and solubility. Glycosylation can change basic biological functions of a protein including imunogenicity, specific activity, and the ligand-receptor interaction. Although plants represent a promising system for production of recombinant proteins, small differences in protein *N*-glycosylation between plants and humans can pose problems for the production of fully functional therapeutic proteins.

Types of Plant *N*-glycans

All *N*-glycans share the common core structure, $Man_3GlcNAc_2$ (Fig. 1), constituted of *N*,*N*'-diacetyl chitobiose unit, a β-mannose residue attached to the chitobiose and two *α*-mannose residues linked to hydroxyl 3 and 6 of the β-mannose (*11*). Depending on the additional sugars that can be attached to the core structure, plant *N*-glycans can be classified into four groups: high--mannose type, complex type, hybrid type and paucimannosidic type *N*-glycans (*12*).

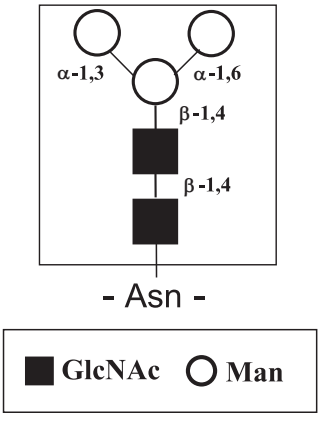

Fig. 1. Minimal core structure which is common for all *N*-glycans

High-mannose type *N*-glycans have 5–9 mannose residues attached to *N*,*N*'-diacetyl chitobiose unit. They are common in plants and mammals (Fig. 2). High--mannose *N*-glycans arise from the limited trimming of glucose and mannose residues from the oligosaccharide precursor. They were first identified in plants in soybean agglutinin (*13*). Afterwards, these *N*-glycans were found as glycan part of spinach and maize calreticulin (*12,14*) as well as linked to different vacuolar and extracellular glycoproteins (*15–18*).

Complex type *N*-glycans are formed after modifications of high-mannose type *N*-glycans in the GA. They are characterized by the α(1,3)-fucose residue attached to the proximal GlcNAc and/or β(1,2)-xylose linked to the β-mannose residue of the core. These are specificities of the plant glycoproteins since animal and human *N*-glycans have α(1,6)-Fuc attached to the proximal GlcNAc and do not contain any xylose residues (Fig. 2) (*19,20*). Complex type *N*-glycans also have one or two GlcNAc residues attached to α-mannose units of the core structure. More complex bi-antennary plant *N*-glycans, which contain additional side chains of α(1,4)-fucose and β(1,3)-galactose linked to the terminal GlcNAc units, have recently been identified (*21–24*). They have one or two terminal antennae containing an oligosaccharide sequence Galβ1-3(Fucα1–4)GlcNAc, named Lewis a (Le^a) after their mammalian counterparts.

Hybrid type *N*-glycans result from the processing of only α(1,3)-mannose branch of the intermediate Man_5-$GlcNAc_2$, which yields the oligosaccharide with α(1,3)--fucose and/or β(1,2)-xylose linked to the $GlcNAcMan_5$-$GlcNAc_2$ (*25*).

Paucimannosidic type *N*-glycans are plant specific *N*-linked glycans, which have not been detected in humans and animals (Fig. 2). They are modified oligosaccharides which contain only an α(1,3)-fucose linked to the proximal GlcNAc and/or a β(1,2)-xylose attached to the β-mannose residue of the intact, $Man_3GlcNAc_2$, or truncated core structure, $Man_2GlcNAc_2$. They have been found in many plant glycoproteins (*26–31*) and are considered as typical vacuolar type *N*-glycans.

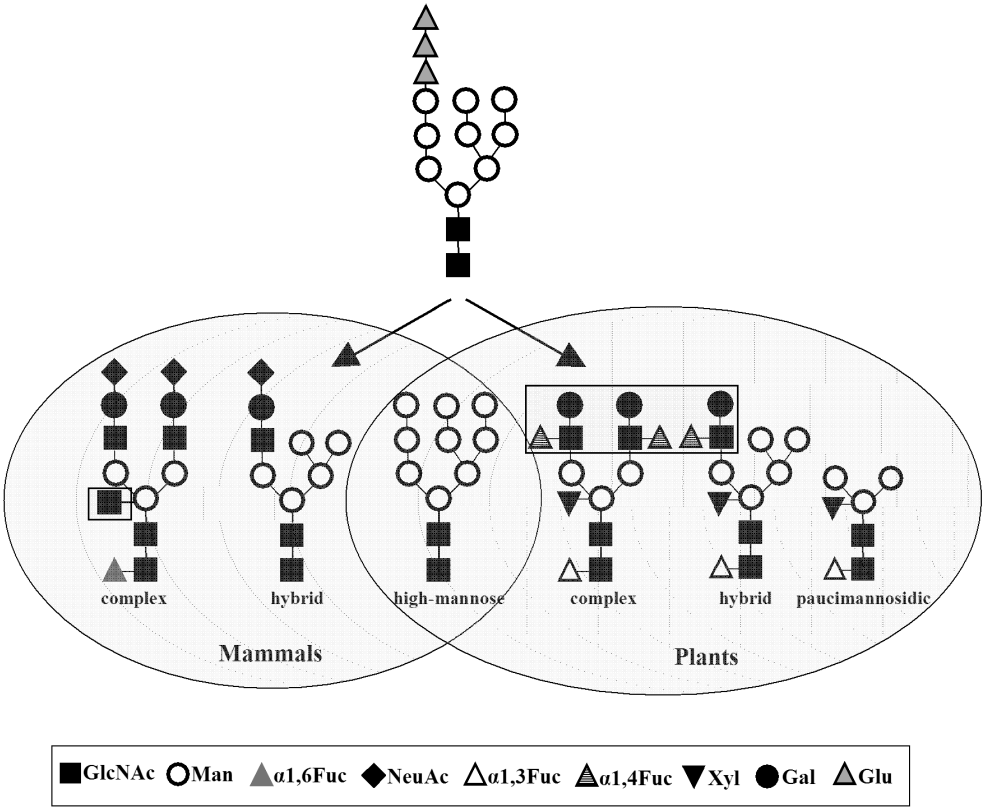

Fig. 2. Differences of *N*-glycan structures between plants and mammals. The high-mannose type *N*-glycans have the same structure in plant and mammalian glycoproteins. The complex and hybrid type *N*-glycans are structurally different, for example, in plant the proximal GlcNAc of the core is substituted by an α(1,3)-Fuc [α(1,6)-Fuc in mammals] and the β mannose of the core is substituted by a bisecting β(1,2)-Xyl [a bisecting β(1,4)-GlcNAc in mammals]. In addition, β(1,3)-Gal and α(1,4)-Fuc linked to the terminal GlcNAc of plant *N*-glycans form Lewis a oligosaccharide structures [instead of β(1,4)-Gal combined with α(2,3)-NeuAc or α(2,6)-NeuAc in mammals]. Paucimannosidic type *N*-glycans, which contain only α(1,3)-Fuc and/or β(1,2)-Xyl linked to the core, are typical glycans of plant vacuole and they have not been detected in mammals. Structures indicated in the rectangle (a bisecting β(1,4)-GlcNAc in mammals and Lewis a structures in plants) are optional

Biosynthesis of Plant *N*-glycans

N-linked oligosaccharides of plant glycoproteins are covalently linked to the asparagine residue (Asn) within the common consensus polypeptide sequence Asn-Xaa--Ser/Thr (where Xaa is any amino acid other than proline and aspartic acid), as they are in animal glycoproteins. Biosynthesis of plant *N*-glycans begins in the endoplasmic reticulum (ER) by the cotranslational transfer of a precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, from the dolichol lipid carrier to specific asparagine residues on the nascent polypeptide chain. Processing of this oligosaccharide into high-mannose, complex, hybrid or paucimannosidic type *N*-glycans occurs during the secretory pathway when the glycoprotein moves from the ER to its final destination.

Biosynthesis of high mannose N-glycans in ER

Three terminal glucose residues are trimmed from the oligosaccharide precursor by the glucosidases I and II in the ER (Fig. 3) (*32*,*33*). ER mannosidase specifically removes a single mannose residue in order to obtain $Man_8GlcNAc_2$ structure. This part of the biosynthetic pathway is identical in all eukaryotic cells and the final results are the high-mannose type *N*-glycans which can be further modified in the GA.

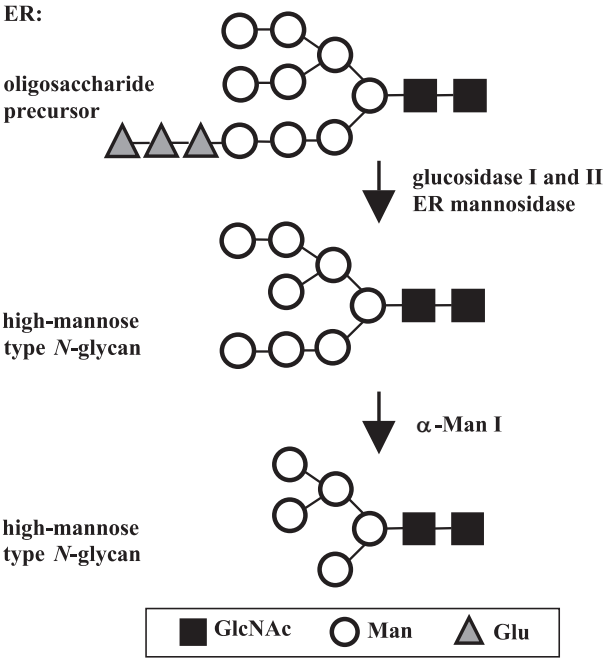

Fig. 3. Biosynthesis of plant *N*-glycans starts in ER. A first class of high-mannose type *N*-glycans is made after a limited trimming of the oligosaccharide precursor catalysed by glycosidases

Maturation of high-mannose N-glycans in GA

As illustrated in Fig. 4. high-mannose plant *N*-glycans can be modified into complex and hybrid *N*-glycans during their transport from *cis*-, through medial, to *trans*-Golgi cisternae. First, α-mannosidase I (α-Man) trims one to four α(1,2)-mannose residues and converts $Man_{8-9}GlcNAc_2$ to $Man_5GlcNAc_2$ (*34*,*35*). Biosynthesis of complex *N*-glycans begins with the addition of the first GlcNAc residue to the α(1,3)-mannose branch of the $Man_5GlcNAc_2$ high-mannose oligosaccharide. This step is catalysed by *N*-acetylglucosaminyltransferase I (GNT I) (*36*–*38*). Two additional mannoses are subsequently removed from the oligosaccharide by the α-mannosidase II (α-Man II) (*39*), then *N*-acetylglucosaminyltransferase II (GNT II) transfers the second GlcNAc to the α(1,6)-mannose branch (*36*,*40*). In the case of hybrid type *N*-glycans, the modifications occur only at α(1,3)-mannose branch, while the α(1,6)-mannose branch keeps its mannose residues. At this stage, α(1,3)-fucosylation and β(1,2)-xylosylation of the core structure may occur to yield plant-specific *N*-linked glycans. The study of the substrate specificity of α(1,3)-fucosyltransferase (α(1,3)--FucT) and β(1,2)-xylosyltransferase (β(1,2)-XylT) has revealed that the presence of at least one terminal GlcNAc is a prerequisite for transfer of the α(1,3)-fucose and β(1,2)--xylose (*36*,*37*,*41*). The identification of plant *N*-glycans containing either α(1,3)-fucose or β(1,2)-xylose proved that this two transfers are not correlated and are completely independent events (*29*,*30*,*42*,*43*).

The complex type *N*-glycans can be further processed by the addition of galactose and fucose moieties on terminal GlcNAc residue(s) by β(1,3)-galactosyltransferase (β(1,3)-GalT) and α(1,4)-fucosyltransferase (α(1,4)--FucT) in order to obtain one or two terminal antennae containing an oligosaccharide sequence Galβ1–3(Fucα1–4)-GlcNAc (Lewis a epitope). *N*-glycans, which are directed to the extracellular matrix, will not be further modified. Those *N*-glycans whose final destination is the cell vacuole will be processed during the transportation to and in the vacuole.

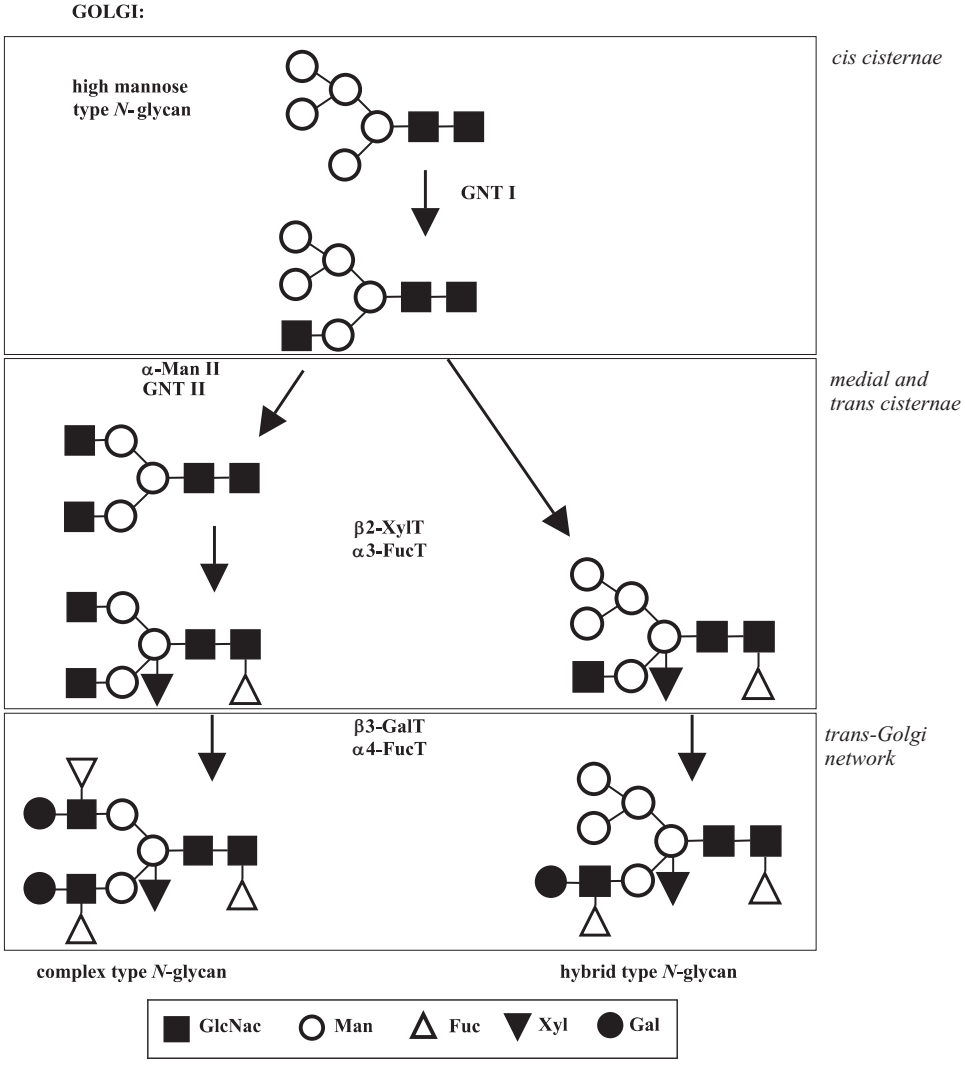

Fig. 4. Processing of plant high-mannose *N*-glycans into complex and hybrid *N*-glycans during the transport of glycoproteins from the *cis*- (mannose trimming and addition of the first GlcNAc), through the medial part (addition of the second GlcNAc and subsequently of the β(1,2)-Xyl), to the *trans*-Golgi cisternae (addition of the α(1,3)-Fuc) and *trans*-Golgi network (addition of galactose and fucose moieties on terminal GlcNAc residue) by different glycosyltransferases

Modifications of complex and hybrid type N-glycans in plant vacuole

Paucimannosidic *N*-glycans, which are specific for plant glycoproteins, are formed by processing of complex and hybrid *N*-glycans by different exoglycosidases in plant vacuole (Fig. 5). Most of the vacuolar glycoproteins described so far contain fucose and/or xylose, but they lack terminal GlcNAc. Since the presence of at least one terminal GlcNAc is the prerequisite for the transfer of fucose and xylose residues, this type of vacuolar *N*-glycans can result from post-Golgi modifications of complex *N*-glycans (*12*).

Expression of Recombinant Glycoproteins in Plant Systems

Since the late events of *N*-glycan modification in plant differ from those in mammals, recombinant proteins produced in transgenic plants will not be identical to mammalian glycoproteins. However, the folding and assembly of recombinant proteins as well as the transfer of an oligosaccharide precursor to *N*-glycosylation sites can be correctly accomplished in transgenic plant systems (*44*).

Expression of recombinant plant glycoproteins in plant systems

The first attempts of the recombinant glycoprotein expression in plants involved expression of plant glycoproteins in different plant systems. Phytohaemaglutinin (PHA) is a vacuolar storage glycoprotein of the common bean *Phaseolus vulgaris*. It consists of two isolectin subunits: PHA-E and PHA-L. A cDNA encoding PHA-L was introduced in different plant systems (*44–46*). The analysis of oligosaccharides from bean and recombinant PHA revealed that the *N*-linked glycans of this model glycoprotein were correctly processed in all tested plant expression systems. Furthermore, recombinant PHA was found to be uniformly *N*-glycosylated in different plant organs. The absence of organ specificity in *N*-glycosylation may prevent the production of heterogeneously *N*-glycosylated recombinant proteins after purification from whole transgenic plants. These experiments have shown that plants can express correctly assembled and *N*-glycosylated recombinant glycoproteins. In the recent study Drakakaki *et al.* (*47*) investigated *Aspergillus niger* phytase as a model glycoprotein to compare the intracellular fate of a recombinant protein in the leaves and seeds of rice. The recombinant protein was efficiently secreted from leaf cells, while within endosperm cells it was retained in endoplasmic reticulum-derived prolamin bodies and protein storage vacuoles. These results indicate that the intracellular deposition and modification of a recombinant protein is tissue dependent.

Expression of recombinant mammalian glycoproteins in plant systems

The next step was an expression of mammalian glycoproteins in plant systems. Immunoglobulins are good model glycoproteins for the evaluation of the potential

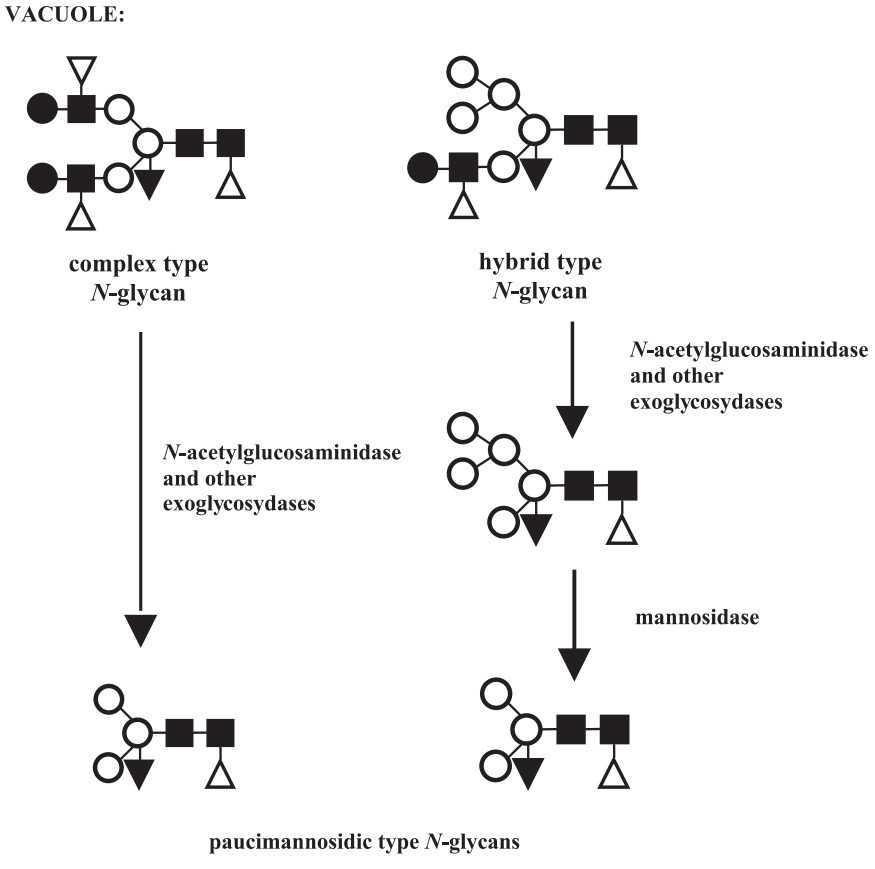

Fig. 5. Modifications of complex and hybrid *N*-glycans into paucimannosidic glycans by different exoglycosidases in plant vacuole

of an expression system for the production of therapeutic glycoproteins (*12*). It has been shown that some of their properties depend on glycosylation.

The monoclonal antibody (MAb) Guy's 13 is a mouse IgG1 class antibody, which recognizes a cell-surface protein of *Streptococcus mutants*, the bacterium that causes dental carries in humans. A full-length MAb Guy's 13 was expressed in tobacco (*48*). This plant MAb was found to be functional, considering recognition and binding. Cabanes-Macheteau *et al.* (*49*) revealed differences in the *N*-glycosylation of the MAb Guy's 13 produced in mouse and in transgenic tobacco plants. *N*-glycosylation analysis of the mouse antibody has shown that this IgG1 is *N*-glycosylated on both sites by biantennary *N*-glycans having α(1,6)-fucose and about 10 % of terminal sialic acid residues. The plant MAb Guy's 13 was also found to bear *N*-glycans on both sites of the heavy chains. High mannose and plant typical complex type *N*-glycans were identified. The plant antibody was found to be properly folded and functional (*48*) showing that plants can produce biologically active molecules.

Human lactoferrin is an 80 kDa glycoprotein which contains three potential *N*-glycosylation sites. The first two sites have complex *N*-glycans, while the third site is usually not glycosylated. Since this glycoprotein has very important anti-inflammatory, antibacterial, antifungal and antiviral activity, it is used as a supplement for infant foods. Comparative analysis of *N*-glycosylation of human lactoferrin (hLf) and lactoferrin produced in maize (mLf) and in tobacco (tLf) has revealed differences in natural and recombinant glycoproteins (6). *N*-glycosylation analysis of the hLf has shown that this glycoprotein is *N*-glycosylated at two sites by biantennary complex *N*-glycans that are more or less fucosylated and sialylated. Recombinant mLf and tLf were found to have mostly paucimannosidic *N*-glycans substituted by a bisecting β(1,2)-xylose and α(1,3)-fucose linked to the proximal GlcNAc on both *N*-glycosylation sites. This indicates that the first steps of *N*-glycosylation are similar in plants and humans and that the observed differences only arise from the specificity of the Golgi plant glycosyltransferases and from post-Golgi degradations of the matured plant *N*-glycans.

Although the homogeneity of glycosylation could differ from one plant expression system to another, all plant species used so far to produce recombinant therapeutic proteins have the capacity to associate α(1,3)-fucose and β(1,2)-xylose residues with the complex *N*-glycans (*6,49–51*). These plant specific glyco-epitopes are not new to humans who are exposed daily to such antigens in edible plant material. However, if people experience prolonged exposure to large quantities of these highly immunogenic plant *N*-glycans, sensitization to plant antigens may occur (*52*). To be able to exploit the potential a plant can offer for the production of recombinant proteins, it is desirable to inhibit these plant-specific modifications in maturation of complex *N*-glycans in order to obtain human-like *N*-glycan structures.

»Humanization« of Plant *N*-glycosylation

It is still not clear if recombinant proteins bearing plant *N*-glycans are really immunogenic. Nevertheless, it would be desirable to eliminate, or at least minimize, the plant-specific *N*-glycosylation to obtain »humanized« non-immunogenic *N*-glycans on therapeutic glycoproteins. On the other hand, none of the transgenic host systems currently available for the production of recombinant mammalian glycoproteins will produce these glycoproteins with the glycans identical to the ones produced by mammals (*1,52,53*). A good knowledge of the protein *N*-glycosylation machinery in plants and the availability of mutant plants allow scientists to define strategies to produce recombinant proteins with more mammalian-like *N*-glycan structures.

Strategies for reduction of α(1,3)-fucose and β(1,2)-xylose

Different strategies to prevent the formation of highly immunogenic plant *N*-glycans on recombinant proteins have been explored (*53*). One drastic approach is to prevent the immunogenic *N*-glycans to attach to the recombinant protein by modification of the *N*-glycosylation sites. However, this strategy does not work for heterologous proteins, which require *N*-glycosylation for *in vivo* stability and biological activity. Gomord *et al.* (*53*) have reported that the addition of *N*-glycans to several therapeutic recombinant proteins improves their biological activity and half-life. This illustrates a current tendency in glyco-engineering towards increasing instead of reducing the number of glycosylation sites on recombinant pharmaceuticals (*54*).

Recombinant proteins may be retained in the endoplasmic reticulum, which is upstream from the Golgi stacks where plant-specific modifications take place. Such recombinant proteins possess only high-mannose type *N*-glycans which are common to plants and mammals, and therefore are probably not immunogenic (*55–59*). However, it seems that high-mannose oligosaccaharides can affect chemicobiological properties of produced therapeutic glycoconjugates. Ko *et al.* (*59*) have shown that antibodies, which contain only high-mannose *N*-glycans, were less stable in comparison with their human homologues after injection into mice.

The third and the most promising approach is the inhibition of Golgi glycoslytransferases. Numerous plant glycosyltransferases, which are responsible for maturation of *N*-glycans, have been cloned in the past years. This resulted in knockout mutants which blocked the synthesis of typical plant complex *N*-glycans. The first plant deficient in any glycosyltransferase was the *Arabidopsis thaliana cgl* mutant lacking GNT I. This mutant cannot produce complex type *N*-glycans and accumulates high-mannose structures $Man_5GlcNAc_2$ (*60*). In contrast to the mice, where deficiency of this glycosyltransferase was shown to be lethal (*61*), there was no effect on *cgl* plant development or morphology. Strasser *et al.* (*42*) have generated knockout plants of *A. thaliana* that lack potentially allergenic and immunogenic α(1,3)-fucose and β(1,2)-xylose without any adverse impact on plant growth and development. Knockout lines display less heterogeneity than wild-type plants, while the high proportion of complex *N*-glycans, carrying terminal GlcNAc residues on both α(1,3)- and α(1,6)-linked mannoses, is a good prerequisite for further modification of plant *N*-glycosylation in a human-like fashion. In an

attempt towards humanization, the genes for α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase were disrupted by homologous recombination in *Physcomitrella patens* (*62*). This moss is an excellent plant system for targeted gene knockouts because of its high rate of homologous recombination, which is unique among plants (*63*). Moreover, it has been shown that the *N*-glycosylation pathway in *P. patens* is similar to that in higher plants (*64,65*). Single Δ*fuc-t* and Δ*xyl-t* plants, as well as the double knockouts, lacked transcripts of the corresponding genes, but they did not differ from the wild-type moss in morphology, growth, development or their ability to secrete recombinant, human growth factor $VEGF_{121}$ into the culture medium. *N*-glycosylation analysis revealed the absence of α(1,3)-fucose and β(1,2)-xylose residues. The moss strains generated in this study open up the possibility of using *P. patens* as a safe production system for therapeutic protein with human-like glycosylation patterns.

How to overcome the lack of certain monosaccharides in plant N-glycans

It has been commonly accepted that plants do not perform mammalian-like post-translational modifications. Most human glycoproteins are synthesized with oligosaccharides that contain terminal sialic acid added to a penultimate galactose residue. The presence of sialic acids at the nonreducing terminal position of various glycoconjugates is physiologically important (*66,67*). Sialylation affects the biological activity and circulatory half-life of many therapeutically important glycoproteins (*68*). To preserve the bioactivity and to fulfill regulatory requirements, it is crucial to ensure the desired glycosylation of glycoconjugate drugs. Until very recently, it has been firmly believed that plants are not capable of synthesizing sialic acids and sialylated glycoproteins (*69,70*). This is why knock-in engineering has focused on the addition of terminal β(1,4)-galactose and sialic acid residues to humanize glycosylation of therapeutic proteins.

The expression of mammal glycosyltransferases in plants is a very attractive approach for humanization of plant *N*-glycans with the aim to supplement plant endogenous system for Golgi maturation of *N*-glycans. So far, the successful expression of human β(1,4)-galactosyltransferase (β(1,4)-GalT) has been obtained in tobacco cells (*50,71*). These plants, expressing the human β(1,4)-GalT, were shown to produce antibody with 30 % of *N*-glycans bearing terminal *N*-acetyllactosamine sequences identical to those associated with the *N*-glycans of an antibody produced in mammalian cells (*50*). Early galactosylation, in *cis*-Golgi cisternae, is very convenient since it prevents transfer of α(1,3)-fucose and β(1,2)-xylose, which take place in medial and *trans*-Golgi. Recombinant glycoproteins obtained in this experiment are not immunogenic, and except from being devoid of sialic acid, they are very similar to human glycoproteins. However, as the *N*-glycans carried by the tobacco-derived antibodies are highly heterogeneous, the action of the human β(1,4)-GalT on this pool of glycans resulted in a complex mixture of *N*-glycans among which some were only partially humanized (*50,72,73*). This strategy was more successful in transgenic alfalfa plants, where *in vitro* galactosylation was performed; the *N*-glycosylation of produced antibodies was restricted to a mature oligosaccharide chain bearing terminal GlcNAc residues (*51*).

Engineering of plants with the potential of glycoprotein sialylation would be demanding. It seems now that this will not be necessary since recent results suggest the presence of a genetic and enzymatic basis for sialylation in plants. Shah *et al.* (*67*) found sialylated glycoconjugates in suspension-cultured cells of *A. thaliana* using biotinylated SNA-I and MAA lectins. The results were confirmed by reversed-phase C18 chromatography. On the contrary, the results obtained from *A. thaliana* and *N. tabacum* protein extracts by Seveno *et al.* (*74*) do not support the presence of detectable sialic acid in plants. According to their study the presence of 3-deoxy-D-manno-2-octulosonic acid (KDO), an α-ketoacid assumed to be solely found in the cell wall, could lead to misinterpretation of results under conditions that are otherwise specific for sialic acids in mammalian protein extracts. In their opinion, the lack of digestion by sialidase and the detection of signals in the *cgl* mutant indicate that, even if they do exist, sialic acid-related compounds are not associated with protein *N*-glycans. Nevertheless, the presence of sialic acids in different tissues of cactus *Mammillaria gracillis* grown *in vitro* (*75*) (shoot, callus, hyperhydric regenerant and tumour) was indicated by application of both biotin- and digoxigenin-labeled SNA-I and MAA lectins, revealing the terminal sialic acid-α(2,6)-galactose and sialic acid-α(2,3)-galactose structures, respectively (*76*). Since the use of lectins is very critical and can lead to unspecific signals, as it was shown by Seveno *et al.* (*74*), the high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of monosaccharide mixtures, obtained after total hydrolysis of detached *N*-glycans, was performed. This analytical method, which clearly resolves KDO and sialic acid residues (*77*), has demonstrated that *N*-acetylneuraminic acid is a constituent of the oligosaccharide chain in 42-kDa glycoproteins from the hyperhydric regenerant and tumour tissue. Additionally, by quadrupole time-of-flight electrospray mass spectrometry and an accurate mass determination the structures of the three disialylated species were postulated: $Hex_4HexNAc_4NeuAc_2$, $Hex_6HexNAc_4NeuAc_2$ and $Hex_6HexNAc_5NeuAc_2$ (Fig. 6). Since such structures have not been found in plant proteins so far, the presented glycan patterns require further detailed analysis by sequencing. However, these new data clearly indicate the presence of sialylated glycoconjugates in plants although the amounts of sialic acids are much smaller than in mammalian tissues (*78*). Thus, certain genetic manipulation might be required to enable plants to synthesize quantitatively significant amounts of sialylated glycoproteins. The pathways of sialylation in plants should be clarified in order to enhance the sialylation up to the desired level and to direct the enzyme activity to the protein of interest. Therefore, *in vitro* addition of sialic acid would be a more realistic option in the short run. Nevertheless, this discovery opens a new field of study in plants that involves the synthesis, localization, structural variations and function of sialic acids and shows that specific metabolic engineer-

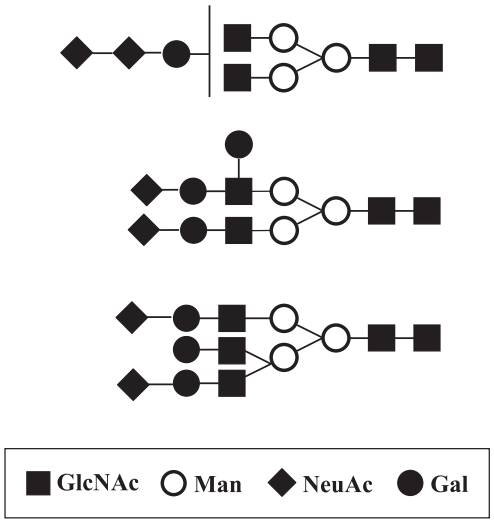

Fig. 6. Predictions of disialylated complex oligosaccharides found in the 42-kDa glycoprotein from the *Mammillaria gracillis* hyperhydric regenerant and tumour tissue. These structures were postulated by quadrupole time-of-flight electrospray mass spectrometry and accurate mass determination

ing of endogenous plant pathways could be an effective way to enhance the value of plants as production systems for mammalian proteins.

Conclusion

Results obtained so far indicate that plants, with small modifications of their glycosylation system, could become suitable hosts for massive production of therapeutic glycoconjugates compatible with human therapy in the near future. Differences in *N*-glycosylation between plants and mammals pose a certain limitation for production of some recombinant therapeutic glycoproteins at the moment. Overcoming these difficulties it soon might be possible to produce any therapeutic protein on the large-scale, which would fulfill pharmaceutical industry demands.

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