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N-Glycosylation of Plant-produced Recombinant Proteins

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Abstract: Plants are gaining increasingly acceptance as a production platform for recombinant proteins. One reason for this is their ability to carry out posttranslational protein modifications in a similar if not identical way as mammalian cells. The capability of plants to carry out human-like complex glycosylation is well known. Moreover, the targeted manipulation of the plant *N*-glycosylation pathway allows the production of proteins carrying largely homogeneous, human-type oligosaccharides. These outstanding results have placed plants in a favourable position compared to other eukaryotic expression systems. This review provides a comprehensive summary of the *N*-glycosylation of plant-produced recombinant proteins, the possible impact of plant-specific *N*-glycans on the human immune system, and recent advances in engineering the plant *N*-glycosylation pathway towards the synthesis of (complex) human-type glycan structures, highlighting challenges and achievements in the application of these powerful technologies.

Keywords: Plants, recombinant proteins, glycosylation, glyco-engineering.

1. INTRODUCTION

N-glycosylation, the enzymatic attachment of sugar moieties to a specific asparagine residue within the *N*-glycosylation recognition sequence of a protein, is a major posttranslational modification in eukaryotes. In humans, more than 50% of proteins are estimated to be *N*-glycosylated [1] and *N*-glycans can strongly influence the *in vivo* functionality of the protein (see below). *N*-glycosylation is a non-template driven reaction which normally results in the synthesis of a heterogeneous collection of different carbohydrate structures on an otherwise homogeneous protein backbone (microheterogeneity). Due to the large human glycome this microheterogeneity may comprise several thousand glycoforms [2], the exact number remaining elusive. Reflecting this diversity manifold functions have been attributed to the carbohydrate moiety of a protein, including folding, stability, conformation, solubility, quality control, half-life determination and oligomerization.

Serum proteins are particularly well known for their high glycan-microheterogeneity and it has been shown that different physiological conditions, e.g. disease, pregnancy or ageing strongly affect the *N*-glycosylation profiles of immunoglobulin G (IgG), an abundant serum protein. This indicates that some of the variable glycan residues might fine-tune antibody activity [3, 4]. In fact, dramatically altered effector functions were reported for an IgG without core α1,6-fucose, a residue normally present on human serum IgG [5]. Moreover, the presence/absence of sialic acid may reverse the function of IgG i.e. from pro- to anti-inflammatory [6].

Although the consequences of *N*-glycans are often well documented, the mechanisms behind these effects are in many cases unknown. This is unfortunate as *N*-glycosylated proteins play an ever more important role in the biotech industry [7] and patients would benefit from optimally glycosylated drugs. Thus, proper *N*-glycosylation is now regarded as a crucial factor by the biopharmaceutical industry and by regulatory authorities alike, leading to an increased number of industrial and academic laboratories trying to decipher the effects of *N*-glycans on proteins and uncover the underlying mechanisms.

Currently, the most used expression system for therapeutic glycoproteins are mammalian cell lines, most commonly Chinese hamster ovary cells (CHO). Mammalian cells provide the advantage of producing recombinant proteins with *N*-glycans very closely resembling those produced in the human body, however, differences exist. CHO cells for example lack the ability to sialylate proteins in the human-typical α2,6-position and add sialic acid in α2,3-linkage instead. While this difference does not seem to affect biological activity of recombinant human EPO, it might have an impact on other therapeutic molecules, like intravenous immunoglobulins (IVIG), whose activity seems to be dependent on the linkage of sialic acid [8]. Moreover, mammalian cell lines typically produce a heterogeneous mixture of glycoforms and control over the glycosylation is difficult. Thus, some human glycan structures are not produced (or not at the desired level) and in many cases the production of single glycoforms is not possible.

Another relevant issue is the presence of Galα1,3-Gal epitopes, a glycan not present in humans but produced by some mammalian cell lines e.g. SP2/0. Notably, about 1 % of the IgG in human serum is directed against this epitope (in particular to α1,3-galactose) [9]. Indeed, this structure present on the therapeutic mAb cetuximab, induced a hypersensitivity reaction in patients treated with this antibody [10]. Another difference between human and CHO cells is the absence of bisecting GlcNAc residues in the latter. The impact of this glycan formation is well documented for many proteins [reviewed in 11]. Finally, batch-to-batch reproducibility in terms of glycosylation is a challenge for protein production in CHO cells. Typically a heterogeneous mixture of glycoforms is produced and control over this heterogeneity is very difficult.

In summary, mammalian cell lines, despite being the most used production platform, suffer from several drawbacks including the difficulty to produce single glycoforms, low batch-to-batch glycosylation reproducibility, attachment of non-human glycoepitopes and absence of some human-type *N*-glycans. To overcome these drawbacks mammalian (and other) expression hosts are being engineered to allow production of tailor-made glycoproteins. Main aims are the removal of non-human and immunogenic epitopes, introduc-

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tion of human-type glycosylation reactions and reduction of the microheterogeneity to allow production of single glycoforms. However, the large glycome and the resulting high glycan-microheterogeneity hamper the targeted manipulation of the *N*-glycosylation pathway in many organisms [reviewed in e.g. 12]. Due to their rather small repertoire of glycosylation reactions plants carry out complex *N*-glycosylation at a striking homogeneity, which makes them especially amenable to glycoengineering. Indeed, over the past years many research groups have concentrated their efforts on modulating plant *N*-glycosylation to enable the production of recombinant proteins with human-like structures in plants.

In this manuscript we review the current status of the *N*-glycosylation of plant-produced recombinant proteins, we summarize different strategies for the production of targeted *N*-glycans, we give an overview of the impact of plant specific *N*-glycans on therapeutic applications and finally we highlight possible future developments.

2. GENERAL REMARKS ON PLANT MOLECULAR FARMING

Biopharmaceuticals are the fastest growing class of novel medicines and we are witnessing an accelerated development of protein-based drugs. Many of these novel proteins cannot be produced satisfactorily by established cell-based production platforms. Plants, on the other hand, offer a viable alternative technology with the potential to meet industry's standards. Recent success stories in expression levels, production speed and manufacturing scale-up have placed this expression system in an encouraging position. For example, the generation of mAbs and vaccines in gram levels within two weeks after obtaining the coding sequences provides a time frame that is unmatched by other established expression systems [13-16]. It is noteworthy that the U.S. governmental agency Defense Advanced Research Projects Agency (DARPA) awarded grants worth over $100 million for research into plant-based expression systems, recognizing it as the technology of choice for rapid large-scale manufacturing processes of vaccines and antibodies. Due to this financial support, large manufacturing facilities are being built, e.g. Kentucky Bioprocessing, GCon, Medicago Inc. Fraunhofer USA, with a size of 10,000-30,000 square meters each. Substantial progress has also been made in biosafety, regulatory compliance and public engagement [17], illustrating that therapeutic application of this technology is gaining acceptance by the regulatory authorities. At the time of writing at least three plant-derived recombinant proteins had been approved for human healthcare: the mAb CaroRx used for treatment of dental caries, human intrinsic factor used as a dietary supplement for the treatment of vitamin B-12 deficiency [18], and human glucocerebrosidase used for enzyme replacement therapy (see below). All three are glycoproteins.

3. THE PLANT *N*-GLYCOSYLATION PATHWAY

N-glycosylation is one of the major post-translational modifications of proteins in multicellular organisms. Thus, despite the high heterogeneity of the final glycosylation status of a protein, the corresponding biosynthetic pathways are largely conserved between kingdoms at a molecular level. In all eukaryotes, processing of *N*-linked glycans is initiated in the ER, where the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ (Man9) is converted to $Man_8GlcNAc_2$ (Man8) (Fig. **1**). In higher eukaryotes processing of Man8 in *cis* and *medial* Golgi compartments leads to the formation of the so-called complex *N*-glycans. Importantly, the *N*-glycan processing steps are virtually identical in plants and mammals up to the formation of the vital intermediate $GlcNAc_2Man_3GlcNAc_2$ (GnGn) (Fig. **1**). In mammals, GnGn oligosaccharides provide the substrate for extensive elongation/modification processes to give rise to the final diversification of *N*-glycosylation. In plants, modifications of these oligosaccharides are more limited and the GnGn structures are normally decorated with β1,2-xylose and core α1,3-fucose residues ($GnGnXF^3$, Fig. **1**). Although core fucosylation is observed in mammals as well, the fucose residues are α1,3-linked in plants as opposed to α1,6-linkage in mammals. In some cases plant cells are able to further elongate the $GnGnXF^3$ by attaching β1,3-galatose and α1,4-fucose residues to form Lewis-a epitopes (Le^a) [19, 20]. A plant peculiarity, common with insect cells, is the formation of paucimannosidic structures ($MMXF^3$). This truncated oligosaccharide formation results from the removal of terminal GlcNAc residues from $GnGnXF^3$ by the action of endogenous hexosaminidases [see below; 21, 22] (Fig. **1**).

In summary, although there are differences in the final structure of *N*-glycans in mammals and plants, they share a remarkably high degree of homology during processing along the secretory pathway.

4. GLYCOSYLATION OF RECOMBINANT PROTEINS PRODUCED IN NON GLYCAN-ENGINEERED PLANTS

Secreted Proteins

In contrast to the *N*-glycan profile of mammalian cell-derived recombinant proteins where a mixture of *N*-glycans is present, the plant produced counterparts exhibit generally a largely homogeneous glycosylation profile with a single dominant *N*-glycan species. Secreted heterologous proteins produced in plants typically carry one of two major types of *N*-glycans: complex-type or paucimannosidic oligosaccharides. How the final glycosylation profile of a particular protein actually looks like is *a priory* not predictable. Two factors seem to influence the process: (i) the route along the secretory pathway and the final destination/accu-mulation of the heterologous proteins, and (ii) the intrinsic character of the (recombinant) protein itself. Currently, the mechanisms and effects of both factors are poorly understood. For example, while recombinant mAbs, human transferrin and EPO that are targeted to the apoplast mainly carry complex *N*-glycans [23, 24], another secreted recombinant protein, follicle-stimulating hormone, carries virtually exclusively paucimannosidic structures [25], assumed typical for vacuolar proteins. The fact that this oligosaccharide formation carries core β1,2-xylose and α1,3-fucose residues indicates the proteins are processed through the Golgi, where the corresponding enzymes are active (XylT, α1,3-FucT, Fig. **1**). Currently it is not entirely known where paucimannosidic structures on secreted proteins are formed, (a) along the secretory pathway upon sorting after the *trans*-Golgi into a vacuolar compartment; or (b) in the apoplast by the action of hexosaminidases located in this compartment [22, 26].

In addition to the two major glycoforms ($GnGnXF^3$, $MMXF^3$), different levels of oligo-mannosidic glycans and glycans carrying Le^a motifs can be found on secreted recombinant plant-derived proteins. Interestingly, recombinant human EPO (rhEPO) produced in moss cells and *Nicotiana benthamiana* carries high amounts of Le^a motifs [27, 28]. This over-proportional synthesis of Le^a epitopes is a surprise, they are absent on native hEPO and rhEPO derived from mammalian cells. Notably, all plant derived complex *N*-glycan formations carry β1,2-xylose and core α1,3-fucose residues, structures which are not present in mammals.

Targeting to ER

A commonly used approach to express recombinant proteins in plants is their retention in the endoplasmic reticulum ER by addition of C-terminal retention/retrieval signals (H/KDEL). As a consequence proteins carry mainly oligo-mannosidic *N*-glycans (Man7, Man8, Man9) [for recent reviews see 12, 29, 30] and are largely devoid of plant-specific, immunogenic xylose and fucose (Fig. **1**). Additionally, some studies have reported enhanced accumulation of KDEL-tagged proteins in the ER [e.g. 31]. Thus, this strategy has been widely used for the expression of recombinant mAbs [among others 32-39]. However, therapeutic applications of such protein variants may be restricted to special cases since oligo-mannosidic structures are atypical on mammalian proteins and thus potentially

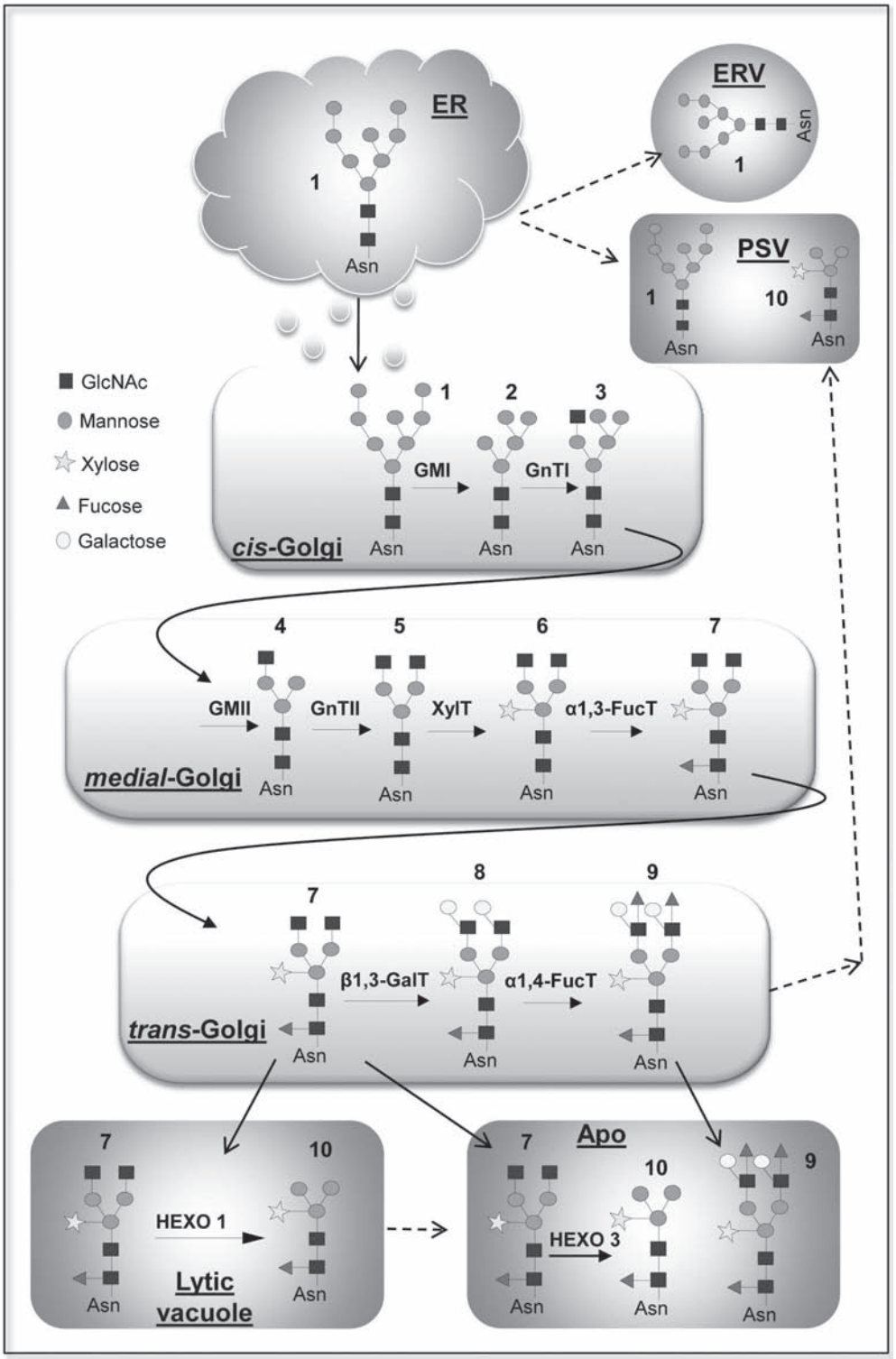

Fig (1). Protein *N*-Glycosylation pathway in plants. Sequential distribution of *N*-glycan processing enzymes across the Golgi apparatus, separated according to their action into early- and medial-acting (GMI, GnTI; GMII, GnTII, XylT and α1,3-FucT) and late-acting enzymes (β1,3-GalT and, α1,4-FucT). The majority of secreted proteins carry complex $GnGnXF^3$ structures. However significant amounts of paucimannosidic structures are present. Note: the majority of vacuolar glycoproteins carry paucimannosidic structures (i.e. $MMXF^3$). The formation of paucimannosidic structures in the vacuoles and in the apoplast is not fully understood. Adapted from [29].

ER: endoplasmic reticulum; **ERV**: ER-derived vesicles; **PSV**: protein storage vacuole; **Apo**: apoplast.

1: Man8; **2:** Man5; **3:** Man5Gn; **4:** MGn; **5:** GnGn; **6:** GnGnX; **7:** $GnGnXF^3$; **8:** $A^3A^3XF^3$ (β1,3-galactosylated structures; Le^a precursor); **9:** $(FA)(FA)XF^3$ (Le^a structures); **10**: $MMXF^3$ (paucimannosidic structure).

GMI: Golgi-α-mannosidase I; **GnTI**: *N*-acetylglucosaminyltransferase I; **GMII:** Golgi-α-mannosidase II; **GnTII:** *N*-acetylglucosaminyltransferase II; **XylT**: β1,2-xylosyltransferase; **α1,3-FucT**: core α1,3-fucosyltransferase; **α1,4-FucT**: α1,4-fucosyltransferase; **β1,3-GalT**: β1,3-galactosyltransferase.. **HEXO1**, **HEXO**3: β-*N*-acetylhexosaminidase 1 and 3.

immunogenic. In addition, macrophages quickly eliminate oligo-mannosidic glycoforms from the bloodstream via mannose receptors on their surface [40]. Indeed, the half-life of a plant-produced anti-rabies antibody bearing oligo-mannosidic *N*-glycans was clearly reduced when compared to the same antibody carrying mainly complex-type *N*-glycans [37].

High-level expression of recombinant KDEL-tagged proteins seems to generate *de novo* special, ER-derived compartments, similar to those frequently found in monocot seeds or induced by fusion to specific protein tags [33, 41, 42; reviewed in 43, 44]. Several studies have reported incomplete ER retention of KDEL-tagged recombinant proteins, a feature most often found in seeds [34, 41, 42, 45-47]. Consequently, *N*-glycosylation patterns of such proteins might differ from expectations. A peculiarity described in maize seeds is the presence of single-GlcNAc residues on recombinant mAbs irrespective of the subcellular localisation [33, 48, 49], a phenomenon not understood so far.

Targeting to Vacuoles

Plants seem to possess functionally distinct vacuolar compartments that can exist site by site in the same cell: lytic vacuoles and storage vacuoles [50]. Whereas storage vacuoles are mainly found in storage tissue (seeds, tubers, etc), lytic vacuoles are normally present in all tissues and share some of their basic properties with the lysosomes of animal cells. The mechanisms which direct soluble proteins from the secretory pathway to the different vacuolar compartments in plant cells are not fully understood. Different targeting signals, different sorting mechanisms and different routes are present and partially overlap [51-57]. Unexpected deposition in storage vacuoles has been reported for some proteins containing KDEL-fusions in seeds [34, 45-48], as well as for other recombinant proteins [48, 58-60]. Vacuolar targeting sequences, which direct (heterologous) proteins to vacuoles have been characterized [51, 61-63] and found use in the production of several recombinant proteins, among them the therapeutic human enzyme prGCD [see below; 64, 65, 66]. Paucimannosidic protein *N*-glycosylation has been regarded as most typical for vacuoles [67]; however, oligosaccharide analysis of proteins deposited in the vacuole revealed the possibility for oligomannosidic and complex-type *N*-glycosylation as well. Interestingly, paucimannosidic *N*-glycans are needed for *in vivo* efficacy of a special class of human enzymes used in enzyme replacement therapies, including glucocerebrosidase (GCD), which is used to treat Gaucher disease. Indeed, upon fusing GCD to a C-terminal vacuolar targeting signal, a recombinant enzyme that carries this truncated oligosaccharide was produced using carrot cells as expression platform [64]. This enzyme exhibited enhanced *in vivo* efficacy compared with the currently available drug Cerezyme® produced in CHO cells [64, 68]. The carrot-produced enzyme, also known as Elelyso™, has been approved by the FDA in March 2012. Notably, although Elelyso™ carries plant-specific xylose and fucose, no obvious adverse side effects that could be attributed to these *N*-glycan residues were reported during clinical trials. This work has been a significant milestone for the parenteral administration of plant-produced glycoproteins and at the time of writing it is the only recombinant plant-derived human therapeutic protein approved and on the market.

5. IMMUNOLOGICAL RELEVANCE OF PLANT-SPECIFIC CARBOHYDRATES

The possible adverse immune reactions of the "non-human" β1,2-xylose and core α1,3-fucose *N*-glycan epitopes on plant produced proteins have been a matter of debate. IgE antibodies that bind to these epitopes have been found in the serum of patients allergic to pollen and insect venoms [69]. *In vitro* assays using purified glycoprotein allergens from plants [70, 71] show that these *N*-glycans contribute to histamine release from basophil cells when incubated with sera of these patients. In contrast, skin prick tests suggest poor biological activity of these carbohydrate-specific IgE antibodies [72]. It was concluded that the carbohydrate-specific IgE antibodies are of limited clinical relevance since the observed biological reactions require high concentrations of glyco-allergens and these reactions were only observed with sera from a selected group of allergic patients [73]. Also IgGs binding to plant fucose and xylose epitopes have been found in sera, also from non-allergic people, albeit at low levels [74]. It is not likely that this is caused by dietary exposure of plant proteins, as this normally leads to tolerance. As in the case of IgE in allergic patients, appearance of these antibodies is most likely caused by pollen or venom exposure. Furthermore, rabbits were shown to raise carbohydrate specific IgG antibodies after parenteral immunization with plant produced antibodies carrying the fucose and xylose epitopes [75, 76]. However, immunization was in the presence of complete Freund's adjuvant, which is not likely to be used in humans. Mice do not mount such carbohydrate specific immune responses, suggesting that immunogenicity of these epitopes might be species specific [77]. It was shown that topical application of glycoproteins from plants does not cause adverse reactions on humans [78, 79]. It should also be noted that intravenous application of Elelyso™, the carrot-produced human glucocerebrosidase which does carry plant specific xylose and fucose, did not display obvious adverse effects during human clinical trials. Nevertheless, for both regulatory and safety issues, the presence of plant specific fucose and xylose residues has to be considered.

6. PLANT *N*-GLYCO-ENGINEERING

Elimination of Plant-specific *N*-glycan Residues

A major concern when using plant-produced recombinant glycoproteins in therapeutic applications is the presence of plant specific xylose and core α1,3-fucose residues (Fig. **2**, glycoform **1b**). Such glycan residues are not present in humans and are thus unwanted on proteins intended for therapeutic use. The elimination/disruption of the genes that are responsible for the synthesis of these glycan-epitopes, i.e. β1,2-xylosyltransferase and core α1,3-fucosyltransferase (XylT, α1,3-FucT) provides an elegant method to solve this issue. The feasibility of this strategy was proven by the generation of *Arabidopsis thaliana* knock-out plants lacking XylT and α1,3-FucT. Those plants were viable without any obvious phenotype under standard growth conditions but produced proteins carrying complex *N*-glycans lacking xylose and fucose (Fig. **2**, glycoform **2**) [42, 80, 81]. These results were a major breakthrough because they (i) demonstrated the plasticity of plants to tolerate the manipulation of the *N*-glycosylation pathway without an obvious adverse phenotype; (ii) generated the central acceptor template (i.e. GnGn structures) for a large number of modifications; and (iii) enabled a significant increase in glycan homogeneity, which may have substantial implications for downstream processing and meets regulatory requirements for therapeutic proteins. Subsequently, elimination/RNAi mediated knock-down of XylT and α1,3-FucT was performed in several other plant species potentially well suited for the production of human proteins, including the aquatic plant *Lemna minor* [82], the moss *Physcomitrella patens* [83], *Nicotiana benthamiana* (ΔXTFT) [23], *Medicago sativa* [84] and rice cells [85]. *N*-glycosylation profiles of mAbs (and other proteins) produced in some of the glyco-engineered hosts contained GnGn as a single dominant structure accounting for 90 % of the *N*-glycan structures [23, 86]. In some cases GnGn was the only carbohydrate species detected [82]. Biological activity assays of such glyco-engineered mAbs revealed unaffected antigen binding and CDC activity, but significantly enhanced ADCC potency compared with mAbs produced in wild type plants and CHO cells [82, 87]. Also, a plant-produced, fucose-free mAb against Ebola virus exhibited superior *in vivo* potency in immune protection assays [86].

Le^a (Fig. **2**, glycoform **1a**) is a *N*-glycan structure normally infrequently present on plant proteins; however it was detected at surprisingly high levels on some plant-produced recombinant proteins. This is the case for recombinant human EPO (rhEPO) produced in tobacco and moss [27, 28]. Even though Le^a structures can be synthesized by humans their abundance on human glycoproteins is low. Elimination of the responsible glycosyltransferases (α1,4-fucosyltransferase and β1,3-galactosyltransferase) by disruption of the corresponding genes was recently reported for moss [88]. As a consequence a rhEPO with largely homogeneous GnGn glycosylation lacking Le^a oligosaccharides was produced.

Overall results obtained by the *in planta* elimination of specific *N*-glycan residues have placed plants in a particularly favourable position for the production of the next generation mAbs and "biobetters". Thus, biopharmaceutical companies like Biolex therapeutics (*lemna*), Greenovation Biotech GmbH (moss) or Protalix Biotherapeutics (carrot cells) have prominently posted their plant-derived products with optimized *N*-glycosylation profiles in their company portfolio. Clinical trials with such potential "biobetters" are underway [89] and the outcome will hopefully confirm their therapeutic value in the near future.

Mammalian-type Core Fucosylation

N-glycans on human glycoproteins are typically decorated with core α1,6-fucose residues, a carbohydrate not synthesized in plants. In recent years, this special type of glycosylation has elicited a lot of interest because it modulates IgG (mAb-) Fc activity [reviewed in 4] and several studies have been published dealing with the modulation of this *N*-glycan residue in mammalian cells. Despite remarkable success it is to date impossible to use mammalian cells for the production of mAbs with identical *N*-glycosylation profiles differing only in the presence/absence of core α1,6-fucose [reviewed in 12]. Thus, apart from the presence/absence of fucose other minor differences in the glycosylation profiles might contribute at least to some extent to altered mAb activities.

Glycoengineered plants such as *N. benthamiana* ΔXTFT that allow the generation of mAbs with virtually a single GnGn *N*-glycoform provide an ideal host to address this issue. Indeed upon overexpression of the human α1,6-FucT (FUT8) in ΔXTFT, it was possible to produce human-type fucosylation (Fig. **2**, glycoform **3**), finally allowing the generation of mAbs with and without core α1,6-fucose while maintaining an otherwise identical *N*-glycosylation pattern [24, 87]. In the course of these studies Fc-glycosylation on the antiviral activity of the broadly neutralizing HIV mAb 2G12 was investigated. In contrast to the CHO-derived 2G12, which carries a mixture of 6 different glycoforms, the carbohydrate structures of 2G12 produced in different glycoengineered plants differed only in one or two glycan residues (Fig. **2**, glycoforms **1b**, **2**, **3** and **7**), thus allowing precise investigation of the impact of single glycan moieties on mAb activity [87]. These results demonstrate that presence/absence of fucose, irrespective of its α1,3- or α1,6-linkage, strongly influences downstream activities, like binding to Fc-γ-receptor III. In addition fucose-free 2G12 glycoforms mediated higher antiviral activity against various lentiviruses. This study was the first to investigate the *in vitro* and *in vivo* impact of particular glycoforms in the antiviral activity of mAbs, and pointed to the importance of optimal *N*-glycosylation for immunotherapeutic reagents.

Extension of Complex *N*-glycans by GlcNAc Residues

Bisected and branched (tri- and tetra-antennary) complex *N*-glycans are commonly synthesized in humans. These multi-antennary structures are involved in various biological functions [90] and are substrates for further galactosylation and multi-sialylation. They are generated by the transfer of terminal GlcNAc residues to the GnGn-core structure through the action of *N*-acetylglucosaminyltransferases (GnTIII-V Fig. **2**, glycoforms **4**-**6**). Like mammals, plants possess highly active GnTs that catalyse the transfer of β1,2-GlcNAc residues to the core α1,3- and α1,6-mannosyl residues (GnTI and GnTII), producing GnGn structures (Fig. **1**). However, activity of other GnTs has never been detected in plants and thus plant *N*-glycans must lack the respective carbohydrate formations.

Attempts to produce bisected *N*-glycans in plants (Fig. **2**, glycoform **4**) first relied on the overexpression of mammalian β1,4-*N*-acetylglucosaminyltransferase III (GnTIII), which indeed resulted in the formation of bisected carbohydrates, accompanied with unusual structures [91-95]. In the course of these studies it became evident that the correct sub-Golgi targeting of heterologous glycosylation enzymes is of the outmost importance in the formation of *N*-glycan structures. The use of chimeric GnTIII containing targeting sequences that direct it to the *trans*-Golgi compartment allowed the production of proteins carrying largely fully processed bisected structures, instead of unusual hybrid structures when the non-engineered version of the enzyme was expressed [24, 28].

To obtain tri- and tetra-antennary complex *N*-glycans in plants (Fig. **2**, glycoforms **5** and **6**) the respective mammalian enzymes GnTIV and V were overexpressed [96]. As already seen for GnTIII unusual incompletely processed structures were synthesized when the full length native protein was expressed. Thus, the catalytic domains of GnTIV and V were fused to different Golgi-targeting sequences to allow correct subcellular targeting. In this case it turned out that upon targeting the enzymes to a medial Golgi compartment the generation of tri- and tetra-antennary structures is most efficient [28,96]. Multi-antennary complex *N*-glycans provide an ideal starting point for further elongation of plant *N*-glycans.

β1,4-Galactosylation

β1,4-Galactosylation is a widespread modification of complex *N*-glycans in mammals. Although the precise impact of this residue on protein function is not known, two aspects have raised attention: (i) the degree to which the resulting glycoform can vary between different physiological conditions, which points to an active role of the *N*-glycan residue in modulating IgG activity *in vivo* [reviewed in 3, 4]; and (ii) the requirement of β1,4-galactosylation as an acceptor substrate for protein sialylation, the final and most complex type of human glycosylation.

Plant *N*-glycans do not carry this terminal elongation because they lack the corresponding enzyme, β1,4-galactosyltransferase (GalT). Several groups have reported the overexpression of the human enzyme in plants, with different degrees of success [97-101]. Although β1,4-galactosylated structures were formed in plants, the presence of incompletely processed structures and the reduction of xylose and fucose indicated that GalT and the endogenous enzymes compete for the same acceptor substrate. Advances in the generation of complex bigalactosylated structures were achieved by targeting GalT to a late Golgi compartment. mAbs coexpressed with such a hybrid enzyme exhibited a single dominant Fc-*N*-glycan species, namely a bigalactosylated structure (Fig. **2**, glycoform **7**) [102]. Conflicting reports about the role of terminal galactose residues in modulating IgG activity have been published [4]. Glyco-engineered plants as described by Strasser *et al*. [102] provide a suitable platform for producing bigalactosylated antibodies which can be used to further investigate the importance of this abundant IgG glycoform. Another important aspect is that these plants synthesize the appropriate acceptor substrate for the final glycosylation step in human, terminal sialylation.

Another human *N*-glycan structure is Lewis-X epitopes (Le^x; Fig. **2**, glycoform **10**), generated by the action of β1,4-GalT and α1,3-fucosyltransferase IXa. It is well known that Le^x-containing structures induce antigen-specific immune responses [103]. Indeed, by the overexpression of the two mammalian enzymes this oligosaccharide was synthesized in tobacco [104]. Thus, plants

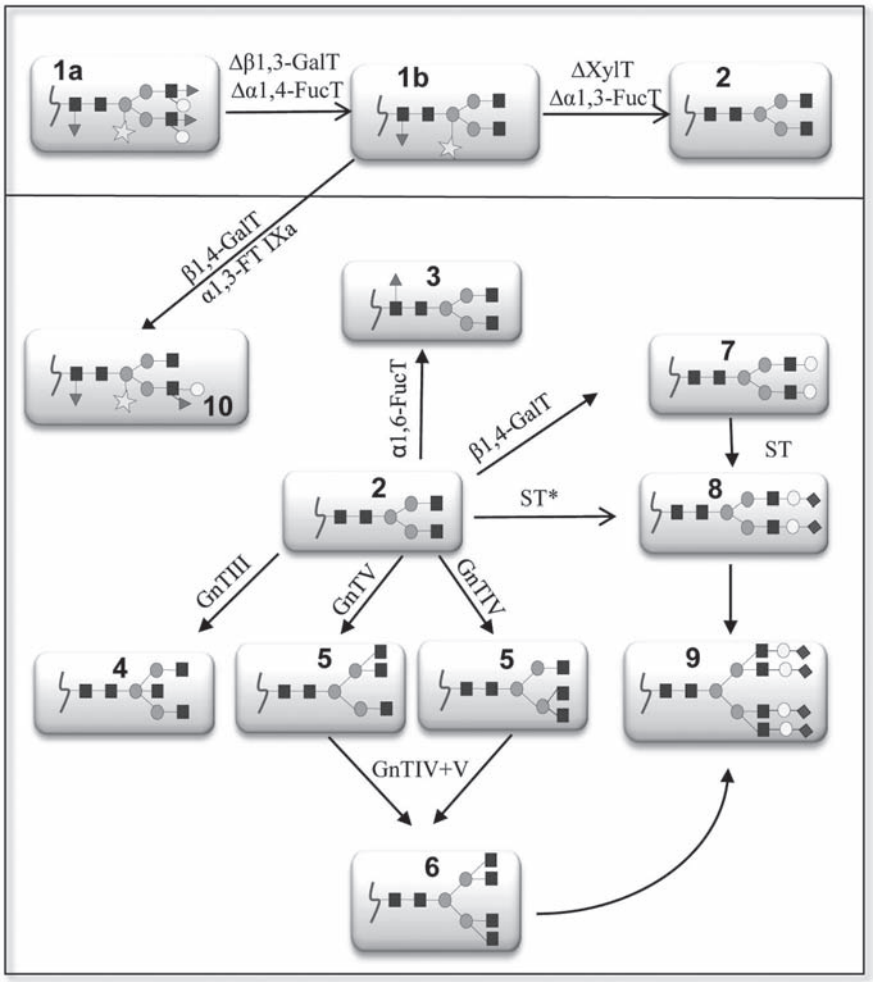

Fig. (2). Schematic illustration of plant engineered glycoforms. Reactions and enzymes to generate certain *N*-glycan structures are indicated. Numbers 1 to 10 refer to the different glycoforms generated on plant-derived proteins either naturally formed or engineered.
1a: (FA)(FA)XF^3 (Le^a structures); **1b**: GnGnXF^3 or through targeted glyco-engineering **2-10; 2**: GnGn; **3**: GnGnF^6; **4**: GnGn(bi) (bisected structures); **5**: [GnGn]Gn or Gn[GnGn] (triantennary structures); **6**: [GnGn][GnGn] (tetraantennary structures); **7**: AA (β1,4-galactosylated structures); **8**: NaNa (α2,6-sialylation); **9**: [NaNa][NaNa] (multiantennary α2,6-sialylation); **10:** Le^X containing *N*-glycans.
XylT: β1,2-xylosyltransferase; **α1,3-FucT**: plant core α1,3-fucosyltransferase; **α1,6-FucT:** core α1,6-fucosyltransferase (FUT8); **GnTIII:** β1,4-mannosyl-β1,4-*N*-acetylglucosaminyltransferase; **GnTIV:** α1,3-mannosyl-β1,4-*N*-acetyl-glucosaminyltransferase; **GnTV:** α1,6-mannosyl-β1,6-*N*-acetyl-glucosaminyltransferase; **β1,4-GalT:** β1,4-galactosyltransferase; **ST:** α2,6-sialyltransferase.

producing Le^x epitopes can be explored as a prospective host for production of vaccines with enhanced immunogenicity.

Terminal Sialylation

Terminal sialylation is the final and most complex step of human *N*-glycosylation. Many drugs require sialylated oligosaccharides for optimal therapeutic potency. Until recently, manufacturing has been restricted to mammalian cell-based systems that are able to perform this important posttranslational modification.

Convincing evidence suggests that plants do not sialylate glycoproteins [105, 106]; notwithstanding that genes homologous to mammalian CMP-sialic acid transporters and sialyltransferases have been detected in some plants [107, 108]. These findings are interesting since plants do not synthesize the donor substrate CMP-Neu5Ac or the acceptor substrate Galβ1,4-GlcNAc. Protein sialylation is particularly difficult to accomplish in plants, even in the presence of β1,4-galactosylated structures, because plants lack some further essential prerequisites: (i) the biosynthetic capability to produce the sugar nucleotide precursor CMP-sialic acid, specifically CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac); (ii) a transporter that delivers CMP-sialic acid into the Golgi in sufficient amounts; and (iii) a sialyltransferase to transfer sialic acid from CMP-Neu5Ac to terminal galactose on the nascent glycoprotein. The enzymes involved in protein sialylation and their substrates must work in a highly coordinated fashion at different stages of the pathway. Consequently, organelle-specific targeting of several components is required for proper protein sialylation. Initial attempts to introduce Neu5Ac residues into plant *N*-glycans involved the expression of some of these proteins in plants [109-111].

Recently, Castilho *et al*. [112] introduced six proteins from the mammalian sialylation pathway into plants, permitting the biosynthesis of sialic acid, its activation, transport into the Golgi, and finally its transfer onto terminal galactose. Namely, the mouse UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), the human *N*-acetylneuraminic acid phosphate synthase (NANS), human CMP-*N*-acetylneuraminic acid synthase (CMAS), mouse CMP-sialic acid transporter (CST), human β1,4-galactosyltransferase and the rat α2,6-sialyltransferase (ST). Coexpressed target proteins were decorated with structures sialylated up to 80 % [112,113]. This is remarkable considering the requirement

for coordinated overexpression of the mammalian proteins which are transiently delivered to the same cell and act at various different stages and in different subcellular compartments (i.e. cytoplasm, nucleus, Golgi). These results are thus a milestone in plant glyco-engineering because they show the enormous plasticity of plants which allows them to tolerate mammalian glycosylation and the large extent of conservation between the biosynthetic pathways of mammals and plants. Moreover, this reconstruction of an entire mammalian biosynthetic pathway in plants may serve as a model for engineering other complex traits into plants, which could open new possibilities in plant breeding and biotechnology.

Plant-produced EPO containing tri- and tetra-antennary *N*-glycans [28, 114] has paved the way for the generation of multi-antennary sialylated therapeutic proteins in plants (Fig. **2**, glyco-form **9**), a major prerequisite for full biological efficacy of many drugs [115]. Notably, sialylated multi-antennary structures are amongst the most complex structures found in mammals and the controlled generation of such structures in plants provides another major step towards the versatile use of plants as an expression system for proteins with highly complex glycosylation patterns.

7. FUTURE PERSPECTIVES

Plants are amazingly amenable to glyco-engineering. Many glyco-traits have already been introduced in plants and very often this has resulted in high glycoform homogeneity of the co-expressed target proteins. This offers several opportunities, both with respect to basic research as well as for applications.

Perspectives for Fundamental Research

It is clear that *N*-glycans play crucial roles in protein-protein interactions, and through that, in processes such as cell-to-cell communication, signal transduction and infection. This became apparent with the recognition of the *N*-glycan nature of the ABO blood group types [116]. More recently, it has been shown that even minor modifications can completely reverse a molecule's function, e.g. the addition of a single sialic acid molecule converts IgG from a pro-inflammatory into an anti-inflammatory molecule [6]. However, the role of different *N*-glycan structures on proteins participating in many biological processes is still unknown. Progress in this research is to a large extent impeded by the lack of availability of defined and homogeneous glycoforms of the proteins under study. Panels of glyco-engineered plants that can produce different glyco-variants of the same protein in a homogeneous manner open new opportunities for research. In particular, transient expression systems can rapidly deliver purified, defined glycoproteins in sufficient amounts, certainly for research purposes. Thus, this technology has great potential to increase our understanding of the structure-function relationship of oligosaccharides in biological processes. This in turn, will undoubtedly have impact on the discovery and development of new therapies and drugs.

Perspectives for Application

For several reasons, plants have been considered as production platform for therapeutic proteins. A key success of their commercial application may very well be their superior glycosylation characteristics. The recently FDA approved glucocerebrosidase produced in carrot cells is an example. Targeting to the vacuole in these non-glycoengineered cells resulted in more than 90 % of its *N*-glycans being terminated with mannose residues [64]. Mannose ending *N*-glycans are necessary on glucocerebrosidase for uptake by macrophages, the cells that are deficient for this enzyme in Gaucher patients. The production of a glucocerebrosidase with a homogeneous *N*-glycosylation profile in plants is a clear advantage compared to commercial CHO produced glucocerebrosidase, which is galactosylated and sialylated and requires *in vitro* deglycosylation to expose terminal mannose residues.

The availability of glyco-engineered plant expression systems will further broaden the scope of different proteins that can successfully be produced by plants. An example of this, although not commercialised yet, is the production of highly homogeneously galactosylated antibodies by glyco-engineered *N. benthamiana* which outperformed the same antibody produced by CHO cell in a virus neutralization assay [102]. It has become apparent that different proteins may accumulate optimally in different plant species, depending on characteristics of the protein and the specifics of the plant platform. This, and Freedom To Operate issues, explain the many plant species currently under investigation as production platforms. Even mushrooms are evaluated for their capability to serve as a glycoprotein production platform [117]. However, not all relevant glyco-traits are already present in all of these plant species and in the near future humanisation of glycosylation will likely be established in the plant species of preference. On the other hand, consolidation to a limited number of commercially exploited plant expression systems can be expected as well.

Perhaps the most intriguing opportunity offered by the glyco-flexibility of plants, is to design *N*-glycans normally not found on the target proteins. By doing so, new *N*-glycans could provide features to the carrier glycoprotein such that it acquires improved therapeutic performance. This could be an important advantage for new subunit vaccines [118]. Subunit vaccines need a design such that they optimally interact with antigen presenting cells (APCs) to mount a protective immune response. Thereto, the vaccine should be taken up by an APC with the aid of so-called pattern recognition receptors (PRRs) such as toll like receptors and C-type lectin receptors (CLRs). As a consequence of receptor binding and subsequent vaccine uptake cytokines are secreted by the APC to stimulate or inhibit the differentiation or development of other immune cells. Parallel to cytokine secretion, fragments of the degraded vaccine are presented to T-cells on MHC molecules and co-stimulatory molecules such as CD80/86 are expressed on the surface of the APC to augment T-cell differentiation. Carbohydrate moieties, such as glycans on glycoproteins, play a pivotal role in antigen uptake by antigen presenting cells through CLRs. Since the specificity of CLRs for their carbohydrate ligands is known, glyco-engineering in plants holds the promise of producing subunit vaccines that are recognized by a specific CLR and another PRR. This dual recognition is important for an effective response. Interaction with a CLR often results in an adjuvant function. In many cases, adjuvants are added to vaccines as molecules to improve vaccine performance (in *trans*). By designing *N*-glycans that specifically target CLRs and which are covalently attached to the antigen (*cis*-configuration), better immune responses may be evoked [119, 120].

Only now we are beginning to understand the factors that influence differential *N*-glycosylation and how *N*-glycans affect protein functions. It has become apparent that a different application and a different mode of action may require a different N-glycosylation profile of a biopharmaceutical protein. Currently, mammalian cell lines are the system of choice for the production of many therapeutic glycoproteins. In the near future this leading position might be challenged by novel expression platforms with improved glycosylation machineries, including plants, bacteria, yeast and insect cells [29]. Plants have demonstrated a high degree of tolerance towards changes in the glycosylation pathway, allowing the modification of recombinant glycoproteins in a specific and controlled manner. This feature is currently unrivalled by alternative expression platforms. The outcomes have already contributed to and will continue to advance this field, ultimately underpinning the production of next generation biopharmaceuticals.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

ADCC = Antibody-dependent cell-mediated cytotoxicity

CDC = Complement-dependent cytotoxicity

GlcNAc = *N*-acetylglucosamine

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