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N-Glycosylation in Plants: Scope and mechanism

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Abstract
(*N*)-linked protein glycosylation is a ubiquitous co- and post-translational modification which can alter the biological function of proteins and consequently affects the development, growth, and physiology of organisms. Despite an increasing knowledge of *N*-glycan biosynthesis and processing, very little is known about the biological function of individual *N*-glycan structures in plants. In particular, the *N*-glycan-processing steps mediated by Golgi-resident enzymes create a structurally diverse set of protein-linked carbohydrate structures. Some of these complex *N*-glycan modifications like the presence of β1, 2-xylose, core α1, 3-fucose or the Lewis a-epitope are characteristic for plants and are evolutionary highly conserved. In mammals, complex *N*-glycans are involved in different cellular processes including molecular recognition and signaling events. In contrast, the complex *N*-glycan function is still largely unknown in plants. Simple and complex carbohydrates (glycans) have long been known to play major metabolic, structural roles in biological systems. Targated microbial binding to host glycans has also been studied for decades. But such biological roles can only explain some of the remarkable complexity and organismal diversity of glycans in nature. It plays a critical role in host-pathogens interactions, antigenicity, and virulence determination and are therefore, considered as potential drug targets. The cell wall of Mycobacterium tuberculosis (Mtb) the causative agent of tuberculosis (TB), dominantly contains sugar and lipids. Despite the effort taken by the World Health Organization to reduce the incident rate, the prevalence of TB is increasing in certain regions.
This short focuses on important recent developments and discuss their implications for future research in plant glycobiology and plant biotechnology.

Keywords: Endoplasmic reticulum, Golgi apparatus, protein glycosylation, *N*-glycosylation, glycoprotein, *N*-acetylglucosaminyltransferase

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 (micro heterogeneity). Due to the large human glycome this micro heterogeneity may comprise several thousand glycoforms [2], the exact number remaining elusive. Reflecting this diversity manyfold functions have been attributed to the carbohydrate moiety of a protein, including folding, stability, conformation, solubility, quality control, halflife determination and oligomerization. Serum proteins are particularly well known for their high glycan-micro heterogeneity 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 1, 6-fucose, a residue normally present on human serum IgG [5]. Moreover, the presence/absence of sialic acid may reverse the function of IgG e.g. 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 Nglycosylation 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.

Another important issue is the presence of Gal1,3-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 1,3galactose) [9]. Indeed, this structure present on the therapeutic mAb cetuximab, induced a hypersensitive 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 tion of human-type glycosylation reactions and reduction of the micro heterogeneity to allow production of single glycoforms. However, the large glycome and the resulting high glycan-micro heterogeneity hamper the targeted manipulation of the Nglycosylation 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 plant.

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 B12 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 Nlinked glycans is initiated in the ER, where the oligosaccharide precursor Glc3Man9GlcNAc2 (Man9) is converted to Man8GlcNAc2 (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 GlcNAc2Man3GlcNAc2 (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 1, 2-xylose and core 1,3-fucose residues
(GnGnXF3, Fig. 1). Although core fucosylation is observed in mammals as well, the fucose residues are 1, 3-linked in plants as opposed to 1, 6-linkage in mammals. In some cases plant cells are able to further elongate the GnGnXF3 by attaching 1, 3-galatose and 1, 4-fucose residues to form Lewis-a epitopes (Lea) [19 20]. A plant peculiarity, common with insect cells, is the formation of paucimannosidic structures. This truncated oligosaccharide formation results from the removal of terminal GlcNAc residues from GnGnXF3 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 (GnGnXF3) or paucimannosidic oligosaccharides (MMXF3). 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/accumulation 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 (i.e. GnGnXF3) [23, 24], another secreted recombinant protein, follicle stimulating hormone, carries virtually exclusively paucimannosidic structures (MMXF3) [25], assumed typical for vacuolar proteins. The fact that this oligosaccharide formation carries core 1, 2-xylose and 1, 3-fucose residues indicates the proteins are processed through the Golgi, where the corresponding enzymes are active (XylT, 1,3-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 (GnGnXF3, MMXF3), different levels of oligo-mannosidic glycans and glycans carrying Lea 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 Lea motifs [27, 28]. This over-proportional synthesis of Lea epitopes is a surprise, they are absent on native hEPO and rhEPO derived from mammalian cells. Notably, all plant derived complex Nglycan formations carry 1, 2-xylose and core 1, 3-fucose residues, structures which are not present in mammals.

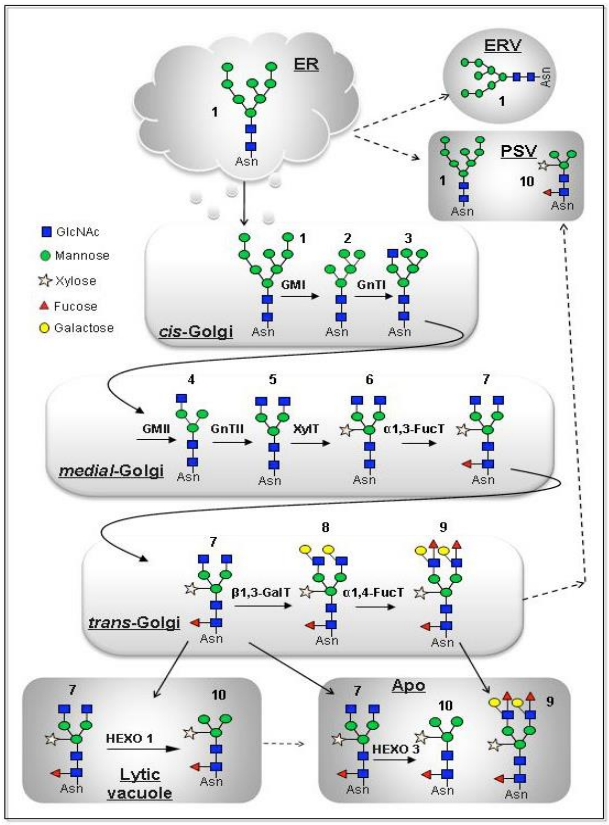

Fig 1

5. Immunological Relevance of Plant-Specific Carbohydrates

The possible adverse immune reactions of the "non-human" 1, 2-xylose and core 1, 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 Nglycans contribute to histamine release from basophil cells when incubated with sera of these patients. In contrast, skin prick testssuggest 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 TM, 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 1,3-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. 1, 2-xylosyltransferase and core 1, 3- fucosyltransferase (XylT, 1,3-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 1, 3-FucT. Those plants were viable without any obvious phenotype under standard growth conditions but produced proteins carrying complex N-glycan 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 1,3-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 (XTFT) [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 glycoengineered 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].

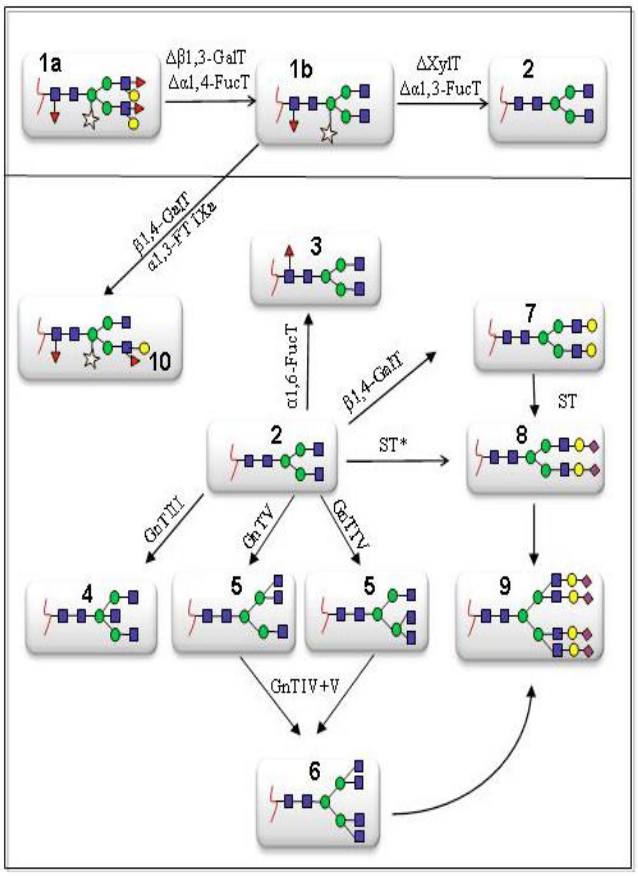

Fig 2

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 coexpressed 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 [114]. 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 glycovariants 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 success

fully 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 [115]. 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 glycoflexibility 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 [116]. 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 the APC 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 costimulatory 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, glycoengineering 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 (cisconfiguration), better immune responses may be evoked [117, 118]. Only now we are beginning to understand the factors that influence differential N-glycosylation and how N-glycans affect different protein functions. It has become apparent that a different application and a different mode of action may require a different Nglycosylation 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 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.

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