Glycosylation of plant-produced immunoglobulins

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Abstract

Many economically important protein-based therapeutics like monoclonal antibodies are glycosylated. Due to the recognized importance of this type of posttranslational modification, glycoengineering of expression systems to obtain highly active and homogenous therapeutics is an emerging field. Although most of the monoclonal antibodies on the market are still produced in mammalian expression platforms, plants are emerging as an alternative cost-effective and scalable production platform that allows precise engineering of glycosylation to produce targeted human glycoforms at large homogeneity. Apart from producing more effective antibodies, pure glycoforms are required in efforts to link biological functions to specific glycan structures. Much is already known about the role of IgG1 glycosylation and this antibody class is the dominant recombinant format that has been expressed in plants. By contrast, little attention has been paid to the glycoengineering of recombinant IgG subtypes and the other four classes of human immunoglobulins (IgA, IgD, IgE, IgM). Except for IgD, all these antibody classes have been expressed in plants and the glycosylation has been analyzed in a site-specific manner. Here, we summarize the current data on glycosylation of plant-produced monoclonal antibodies and discuss the findings in the light of known functions for these glycans.

Introduction to N-glycan processing in plants

N-glycosylation of secretory proteins is initiated by the en bloc transfer of a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) precursor in the lumen of the endoplasmic reticulum (ER). The assembly of the lipid-linked oligosaccharide precursor involves multiple Asn-linked glycosylation (ALG) enzymes that are all conserved in plants (Strasser 2016). The $Glc_3Man_9GlcNAc_2$ moiety is transferred by the oligosaccharyltransferase (OST) complex to asparagine residues in the sequence Asn-X-Ser/Thr (X can be any amino acid except proline) of newly synthesized polypeptides. In budding yeast, mammals and plants, OST is a multimeric membrane-bound protein complex consisting of one catalytically active subunit (STT3) and several different non-catalytic subunits that mediate interactions with the translocation channel and ribosome or might be required for glycosylation of specific sites (Shrimal and Gilmore 2019). Mammals harbor two different OST complexes. While the STT3A complex interacts with the translocon and mediates co-translational glycosylation, the STT3B complex catalyzes posttranslational glycosylation of proteins and glycosylates sites that have been skipped by the STT3A complex. Plants have also two catalytic subunits, termed STT3A and STT3B (Koiwa *et al.* 2003) that likely form two distinct heteromeric OST complexes (Niu *et al.* 2020). However, the function of individual OST subunits appears different in plants (Farid *et al.* 2011; Castilho *et al.* 2018) and our current understanding of the role of the STT3A and STT3B complexes in N-glycosylation of plant proteins is still limited (Jeong *et al.* 2018).

Once the oligosaccharide has been transferred by the OST complex, the N-glycan is subjected to stepwise processing. Removal of the terminal α1,2-linked glucose by α-glucosidase I and subsequent removal of the first α1,3-linked glucose by α-glucosidase II result in a glycan structure that can be recognized by the lectin chaperones calnexin (CNX) and calreticulin (CRT) that promote protein folding (Strasser 2018). In this ER-quality control process, misfolded glycoproteins are subjected to several rounds of interaction with CNX/CRT and monitoring of their folding status. Proteins that have acquired their native conformation are released from the CNX/CRT cycle and allowed to exit the ER to downstream compartments. Terminally misfolded proteins are recognized by a poorly understood process and directed towards ER-associated degradation (ERAD) to prevent the accumulation or secretion of potentially harmful proteins. The basic biological functions of the glycan-dependent ER-quality control process and clearance mechanism of aberrant glycoproteins are conserved in plants.

Correctly folded and assembled secretory glycoproteins leave the ER and transit through the Golgi apparatus where they encounter multiple glycosidases and glycosyltransferases that process oligomannosidic N-glycans to complex N-glycans (Strasser 2016). While in budding yeast and mammals, the first trimming reaction is catalyzed by an ER-resident α-mannosidase, MNS3 the corresponding plant enzyme is primarily located in the *cis*-Golgi (Schoberer *et al.* 2019). MNS3 catalyzes the trimming of a single α1,2-linked mannose from the middle branch of the $Man_9GlcNAc_2$ N-glycan to form $Man_8GlcNAc_2$. Subsequently, three additional mannose residues are cleaved off by Golgi α-mannosidase I. MNS1 and MNS2 are two functionally redundant Golgi α-mannosidases in *Arabidopsis thaliana* (Liebminger *et al.* 2009). The resulting $Man_5GlcNAc_2$ structure is used by β1,2-N-acetylglucosaminyltransferase I (GnTI) to initiate hybrid and complex N-glycan formation (von Schaewen *et al.* 1993; Strasser *et al.* 1999). The transfer of the GlcNAc residue to the α1,3-linked mannose by GnTI is required for further N-glycan modifications in the Golgi. In the next processing steps, Golgi α-mannosidase II (GMII) removes the α1,6- and α-1,3-linked mannose residues and β1,2-N-acetylglucosaminyltransferase II (GnTII) attaches a single GlcNAc to the α1,6-linked mannose to generate the complex N-glycan $GlcNAc_2Man_3GlcNAc_2$ (GnGn) (**Figure 1**). Until this step, the processing reactions are conserved between mammals and plants. Subsequently, a β1,2-linked xylose and a core α1,3-linked fucose are attached to GnGn to generate $GlcNAc_2XylFucMan_3GlcNAc_2$ (GnGnXF) the predominant complex N-glycan found on glycoproteins in plants (Wilson *et al.* 2001; Léonard *et al.* 2004; Strasser *et al.* 2004). Of note, the substrate specificities and the overlapping Golgi localization of the corresponding enzymes allow an alternative order of processing from $GlcNAcMan_5GlcNAc_2$ to GnGnXF with the β1,2-xylosyltransferase (XylT) activity preceding trimming by GMII (Strasser 2016). Further modifications of complex N-glycans are catalyzed by β1,3-galactosyltransferase 1 (GALT1) and α1,4-fucosyltransferase (FUT13). The two *trans*-Golgi resident enzymes generate the Lewis-a carbohydrate epitope [Fucα1,4(Galβ1,3)GlcNAc-R] that is ubiquitously found in plants, but occurs only on a very limited number of plant glycoproteins (Fitchette-Lainé *et al.* 1997; Wilson *et al.* 2001; Strasser *et al.* 2007). Truncated $Man_3XylFucGlcNAc_2$ (MMXF) N-glycans are generated from GnGnXF by removal of terminal GlcNAc residues. This reaction is catalyzed either by the vacuolar β-N-acetylhexosaminidase 1 (HEXO1) or by an apoplast located HEXO3 that is the major contributor to the formation of truncated N-glycans on secreted glycoproteins (Liebminger *et al.* 2011; Shin *et al.* 2017).

Apart from the formation of Lewis-a structures, no further complex N-glycan modifications have been described. Plants lack N-acetylglucosaminyltransferases for the formation of tri- or tetra-antennary N-glycans and the attachment of a bisecting GlcNAc. Common mammalian complex N-glycan modifications that are found on immunoglobulins such as core α1,6-fucose and terminal β1,4-galactose have not been described (Strasser *et al.* 2009). Moreover, plants lack the biosynthesis pathway for CMP-sialic acid, a Golgi

CMP-sialic acid transporter as well as α2,3- and α2,6-sialyltransferases (ST) that catalyze the transfer of CMP-Neu5Ac to complex N-glycans in the Golgi (Zeleny *et al.* 2006; Castilho *et al.* 2010). Due to the absence of these mammalian-type complex N-glycan modifications, the N-glycan heterogeneity on plant-produced glycoproteins is clearly reduced which is an enormous advantage for approaches aiming at the generation of defined homogenous N-glycans for different applications or glycan-structure function studies (Schoberer and Strasser 2018) (**Figure 1**).

Introduction to O-glycan biosynthesis in plants

In mammals, O-glycan biosynthesis occurs in a stepwise fashion involving the sequential transfer of single monosaccharide residues to secreted and membrane-bound proteins. During mucin-type O-glycan biosynthesis, which is the most common O-linked glycan in humans, polypeptide N-acetylglactosaminyltransferases catalyze the transfer of an N-acetylgalactosamine (GalNAc) residue from the nucleotide sugar UDP-GalNAc to hydroxyl side chains of Ser/Thr to generate GalNAcα1-O-Ser/Thr (Tn antigen) (Bennett *et al.* 2012). This initial step in mucin-type O-glycan biosynthesis can be carried out by one of 20 different polypeptide GalNAc-transferases in the Golgi apparatus of mammalian cells. After the initial glycosylation reaction, multiple monosaccharides are attached in the Golgi in a stepwise manner yielding in elongated and branched O-glycan core structures (Tran and Ten Hagen 2013). The most common extension is catalyzed by the core 1 β1,3-galactosyltransferase (T-synthase or C1GalT1), which adds galactose in a β1,3-linkage to generate the core 1 O-glycan structure Galβ1-3GalNAcα1-O-Ser/Thr (also known as T antigen). O-glycan structures can be further modified with the addition of sugars such as galactose, GlcNAc, fucose, and terminal sialic acid. These elongated and branched mucin-type O-glycans are typically found on mammalian glycoproteins.

A similar mucin-type O-glycan biosynthesis pathway does not exist in plants (Strasser 2012). However, specific proline residues are converted by prolyl-4-hydroxylases (P4Hs) to hydroxyproline (Hyp) followed by a glycosylation reaction. Two major types of O-glycans are attached to glycoproteins with Hyp residues. Unbranched chains composed of up to five arabinose (Ara) residues are added to clusters of Hyp residues in proteins such as extensins, whereas complex arabinogalactans (AGs) are attached to clustered non-contiguous Hyp residues on arabinogalactan proteins (AGPs) (Ellis *et al.* 2010). The O-glycosylation of AGPs is initiated by a set of Hyp-galactosyltransferases that add a single galactose to a Hyp residue in the Golgi of plants. The AGP glycan structures are not well characterized but include incorporation of multiple galactose residues and additional modifications with arabinose, xylose, fucose or glucuronic acid. On extensins, a single galactose can be attached to a Ser residue next to a Hyp repeat that is not further modified or elongated with other sugar residues.

Engineering of N-glycan processing pathways in plants

Initial attempts to engineer the N-glycan processing pathway in plants aimed to prevent the formation of complex or truncated N-glycans with β1,2-xylose and core α1,3-fucose. Both N-glycan modifications have been associated with an increased risk for immunogenicity and adverse allergic reactions in humans (Bardor *et al.* 2003; Jin *et al.* 2008; Paulus *et al.* 2011). The potential immunogenicity of the plant-specific N-glycans has been discussed extensively in the context of molecular farming, and there is still an ongoing debate whether β1,2-xylose and core α1,3-fucose modifications have an adverse effect when present on recombinant therapeutic proteins (Grabowski *et al.* 2014; Ward *et al.* 2014; Piron *et al.* 2015; Shaaltiel and Tekoah 2016; Rup *et al.* 2017). Furthermore, unwanted regulatory concerns make their elimination desirable.

Pioneering work in *A. thaliana* demonstrated that plants tolerate the complete removal of β1,2-xylose and core α1,3-fucose residues without any adverse effects on plant growth or development (Strasser *et al.* 2004). Based on this study, gene silencing approaches were successfully applied to almost completely remove these N-glycan modifications in the aquatic plant *Lemna minor* and in *Nicotiana benthamiana* (Cox *et al.* 2006; Strasser *et al.* 2008). *N. benthamiana* is currently used by academic groups and companies worldwide as a transient expression system for monoclonal antibodies, Fc-fusion proteins, virus-like particles and antigens used for therapy, prophylaxis and diagnostics (Stoger *et al.* 2014; Lomonossoff and D'Aoust 2016; Margolin *et al.* 2020; Sainsbury 2020). Multiplex CRISPR/Cas9 genome editing was recently used to generate *N. benthamiana* deficient in plant-specific core α1,3-fucosyltransferase and β1,2-xylosyltransferase activities (Jansing *et al.* 2019). Consistent with previous findings for *A. thaliana* (Strasser *et al.* 2004), no obvious phenotype was described for this multiple knockout line highlighting that *N. benthamiana* plants tolerate the removal of plant-specific complex N-glycans very well. Anti-HIV IgG antibodies produced in the ΔXT/FT knockdown line (Strasser *et al.* 2008) or in the recently described knockout line (Jansing *et al.* 2019) displayed primarily GnGn N-glycans (**Figure 1**). This human-type N-glycan structure is the preferred base for engineering of glycan extensions and introduction of mammalian-type complex N-glycan modifications (Montero-Morales and Steinkellner 2018). GnGn N-glycans can serves as acceptor substrates for the attachment of β1,4-linked galactose (Strasser *et al.* 2009), branching (Castilho *et al.* 2011b; Nagels *et al.* 2011), bisecting GlcNAc (Castilho *et al.* 2015), core α1,6-fucosylation (Castilho *et al.* 2011a) or the formation of immunomodulatory helminth N-glycans carrying Galβ1-4(Fucα1-3)GlcNAc (Lewis X) or GalNAcβ1-4GlcNAc (LDN) structures (Wilbers *et al.* 2017). The formation of complex N-glycans with β1,4-galactose in plants paves the way for subsequent sialylation which has been achieved by transient or stable expression of the mammalian sialylation machinery (Castilho *et al.* 2010; Kallolimath *et al.* 2016). Using these approaches, complex N-glycan branches were capped with single α2,3- or α2,6-linked sialic acid residues or further extended with α2,8-linked polysialic acid (Kallolimath *et al.* 2016).

Other glycoengineering approaches intended to eliminate interfering complex N-glycan modifications. The Lewis-a epitope formation interferes with β1,4-galactosylation and potentially presents an immunogenic epitope when highly abundant on recombinant therapeutic proteins. Lewis-a structures have, for example, been detected on recombinant human erythropoietin (EPO) produced in *N. benthamiana* and *Physcomitrella patens* (Weise *et al.* 2007; Castilho *et al.* 2011b). Knockout of the GALT1 orthologue in *P. patens* prevented the formation of the Lewis-a epitope on recombinant EPO (Parsons *et al.* 2012). Transient knockdown of HEXO3 in *N. benthamiana* enriched the amount of GnGn-containing N-glycans on recombinant glycoproteins (Shin *et al.* 2017) and depletion of a specific β-galactosidase from the apoplast of *N. benthamiana* prevented the removal of β1,4-galactose from recombinant glycoproteins (Kriechbaum *et al.* 2020). Complete knockout of the endogenous plant genes coding for these glycosyl hydrolases will further improve the *N. benthamiana* expression system resulting in the formation of recombinant glycoproteins with highly homogeneous glycans.

Besides differences in N-glycan processing, some recombinant proteins expressed in plants are underglycosylated (Van Droogenbroeck *et al.* 2007; Hamorsky *et al.* 2015; Castilho *et al.* 2018; Göritzer *et al.* 2019; Montero-Morales *et al.* 2019; Stelter *et al.* 2020). The reduced N-glycosylation efficiency is caused by yet unknown differences in the function of the plant OST complex. For some recombinant proteins including antibodies, the underglycosylation of N-glycosylation sites can be overcome by co-expression of a single subunit OST from *Leishmania major* (LmSTT3D) (Castilho *et al.* 2018; Montero-Morales *et al.* 2019; Göritzer *et al.* 2020).

Engineering of O-glycosylation processing pathways in plants

Despite the huge differences between mammalian and plant-type O-glycans, comparatively little attempts have been directed towards the production of human-type O-glycans in plants. The analysis of plant-produced recombinant proteins carrying mucin-type O-glycosylation sites revealed the presence of Hyp as well as several pentose residues corresponding to unbranched arabinose chains found on plant extensins (Karnoup *et al.* 2005; Pinkhasov *et al.* 2011; Castilho *et al.* 2012; Yang *et al.* 2012; Dicker *et al.* 2016; Göritzer *et al.* 2017). Hyp residues are not found on human proteins such as IgA1 or EPO. The presence of the arabinose chain may cause adverse effects and bears the risk of an unwanted immune response against plant-produced therapeutic proteins. Therefore, one aim of plant O-glycan engineering approaches is the elimination of specific P4H activities to prevent Hyp formation and subsequent plant-specific glycosylation. In *P. patens*, knockout of a single P4H completely abolished the production of Hyp on recombinant EPO (Parsons *et al.* 2013). Plants like *A. thaliana* or *N. benthamiana* have numerous potential P4H candidates that could be involved in the hydroxylation of proline on recombinant proteins (Velasquez *et al.* 2011). Consequently, the removal of the plant-specific modification will likely require the knockout of several P4H genes coding for enzymes with similar substrate specificities.

In addition to the removal of the unwanted native O-glycosylation repertoire of plants, other engineering strategies aimed to introduce mucin-type O-glycosylation. Human polypeptide GalNAc-transferase 2 has, for example, been transiently expressed in *N. benthamiana* to initiate O-GalNAc formation on different recombinant glycoproteins including peptides derived from human mucin 1 (MUC1), EPO-Fc or IgA1 (Pinkhasov *et al.* 2011; Castilho *et al.* 2012; Yang *et al.* 2012; Dicker *et al.* 2016). On the single O-glycosylation site of human EPO-Fc, the core 1 structure could be generated by expression of the *Drosophila melanogaster* core 1 β1,3-galactosyltransferase. Co-expression of the mammalian CMP-sialic acid biosynthesis pathway, the CMP-sialic acid transporter and the corresponding sialyltransferases led to production of IgA1 or EPO-Fc with sialylated mucin-type O-glycans (Castilho *et al.* 2012; Dicker *et al.* 2016). For the generation of defined O-glycan structures on recombinant proteins the absence of an endogenous mucin-type O-glycan biosynthesis pathway is of great advantage as it allows the stepwise modification of O-glycans with only the desired monosaccharides.

Glycosylation of plant-produced IgGs

In wild-type plant-produced IgGs, the Fc-resident GnGn N-glycan (**Figure 2**) is commonly modified with β1,2-xylose and core α1,3-fucose residues to produce GnGnXF which is not present in mammals. In glycoengineered *N. benthamiana,* IgG with humanized complex-type GnGn N-glycans as major glycoform were produced (Strasser *et al.* 2008; Jansing *et al.* 2019). Besides the conserved N-glycosylation sites on the Fc portion, additional carbohydrate chains can be linked to the hypervariable regions of IgG. For instance, up to 25% of IgG molecules isolated from the serum of healthy individuals as well as several therapeutic monoclonal antibodies like cetuximab have been reported to carry N-glycans on their variable domains which exhibit site-specific differences compared to the Fc-resident N-glycan on the same molecule. While the Fc N-glycan of such antibodies is less modified, the N-glycan in the variable region is more exposed and displays extensive processing (Teh *et al.* 2014). Similarly, such IgG1 antibodies produced in plants were shown to carry up to 30% α1,3-fucose residues in the Fab resident N-glycan revealing a leaky knockdown in the ΔXT/FT plants (Castilho *et al.* 2015).

On some plant-produced IgGs low amounts of truncated structures have been detected (Strasser *et al.* 2008; Stelter *et al.* 2020). Depending on the IgG idiotype expressed, also small amounts of not fully processed oligomannosidic structures can occur resulting from different secretion efficiency to the apoplast due to ER-retention of potentially incompletely folded IgGs (Westerhof *et al.* 2014). An issue that has only been

tackled recently are the differences in N-glycosylation occupancy of plant- and mammalian-produced glycoproteins. While the single N-glycosylation site present in the human IgG Fc region is almost 100% glycosylated when expressed in mammalian cells, 10-30% of plant-produced IgG is underglycosylated at this site due to yet unknown features of the plant OST complex (Castilho *et al.* 2018; Stelter *et al.* 2020). However, transient expression of the single-subunit OST from *L. major* successfully increases the N-glycan occupancy on the IgG Fc site. Using these different strategies, plants can produce IgGs with very little microheterogeneity carrying a homogenous glycosylation profile with mostly GnGn N-glycans.

Glycoengineering of IgG has focused mainly on the elimination of core-fucose from the N-glycan in the Fc region of the heavy chain as major contributions to antibody activities have been assigned to that N-glycan residue (Umaña *et al.* 1999; Shinkawa *et al.* 2003; Yamane-Ohnuki *et al.* 2004; Junttila *et al.* 2010). However, co-expression of the responsible mammalian core α1,6-fucosyltransferase in glycoengineered ΔXT/FT plants facilitated the generation of IgGs with and without fucose while retaining an otherwise identical N-glycosylation profile (Forthal *et al.* 2010; Castilho *et al.* 2011a). This led to a series of studies of plant-produced IgG showing that the absence of fucose increases the affinity for FcγRIII receptor binding and improved antibody-dependent cellular cytotoxicity (ADCC) on natural killer cells (Jez *et al.* 2012; Loos and Steinkellner 2012; Qiu *et al.* 2014; Marusic *et al.* 2018; Stelter *et al.* 2020). A similar glycosylation-dependent mechanism has an impact on antibody-dependent cellular phagocytosis (ADCP) by macrophages and influences the receptor-mediated effector function of virus-neutralizing antibodies (Forthal *et al.* 2010; Lai *et al.* 2014; Hayes *et al.* 2017). Furthermore, it has been suggested that α1,6-linked fucose could contribute to antibody dependent enhancement (ADE) of infection and therefore plant-produced IgGs with GnGn could be safer and more efficacious antibody-based therapeutics against dengue virus and other ADE-prone viral diseases (Dent *et al.* 2016; Hurtado *et al.* 2020). The success of afucosylated IgG antibodies produced in plants is highlighted with the case of ZMapp, an antibody cocktail for treatment of Ebola virus infections which was used during the Ebola outbreak in 2014/2015 (Qiu *et al.* 2014). Core fucose-free monoclonal antibody 13F6 which is one of the ZMAPP components displayed clearly enhanced potency against Ebola virus compared to 13F6 variants with core fucose (Castilho *et al.* 2011a).

Capping of both branches (>80%) of the IgG Fc glycan (Strasser *et al.* 2009; Stelter *et al.* 2020) with β1,4-galactosylated structures could be achieved by targeting of human β1,4-galactosyltransferase (B4GALT1) to a late Golgi compartment in *N. benthamiana* ΔXT/FT (Strasser *et al.* 2009; Castilho *et al.* 2011a; Jez *et al.* 2012). This is an improvement compared to CHO cell-produced IgG that frequently carry galactose residues only on one branch. Employing these glycoengineering approaches in plants it could be shown that β1,4-galactosylation can, although not improving interaction with Fcγ receptors, enhance neutralization activity of two anti-HIV antibodies (Strasser *et al.* 2009; Stelter *et al.* 2020).

The final and most complex step of human complex N-glycan processing is terminal sialylation. These negatively charged residues at the non-reducing end of N-glycans reduce protein turnover by preventing the exposure of galactose, GlcNAc or mannose to lectin receptors like the asialoglycoprotein receptor (Ashwell and Morell 1974). In addition, there is also long-standing evidence that IgG molecules can have an anti-inflammatory activity in autoimmune diseases and recent studies indicate that this activity is associated with the presence of sialic acid (Kaneko *et al.* 2006; Raju and Lang 2014; Wang and Ravetch 2019). The synthesis of sialylated N-glycans in plants involves the coordinated co-expression of several mammalian proteins acting in different subcellular compartments at different stages of the N-glycosylation pathway (Castilho *et al.* 2010). While the *in planta* sialylation works quite well for proteins like EPO, IgG sialylation is only possible in the presence of a core fucose residue (Castilho *et al.* 2015; Kallolimath *et al.* 2020).

The plant-based production of IgG1 to IgG4 subtypes has been reported recently and all of them display a quite similar N-glycan profile when expressed in glycoengineered *N. benthamiana* (Kallolimath *et al.* 2020). IgG3 has a second N-glycosylation site in the CH3 domain (**Figure 2**) and an extended hinge region that is very likely modified with Hyp and plant-specific O-glycans. When produced in *N. benthamiana*, IgG3 displayed degradation products of the heavy chain. Whether the cleavage takes place in the extended hinge region remains to be shown. Modification of the hinge region with human mucin-type O-glycans might be a valuable strategy to reduce the proteolytic vulnerability of the hinge region of recombinant IgG3 produced in plants. Altogether, glycoengineering in plant-based systems provide a reliable platform to generate human IgG antibodies with a controlled glycosylation pattern.

Glycosylation of plant-produced IgAs

IgAs are increasingly gaining attention as a possible biopharmaceuticals for treatment of infectious diseases and cancer especially in mucosal settings due to their unique structural and functional properties. The two IgA isotypes (IgA1 and IgA2) carry two to five N-glycosylation sites on the α-(heavy) chain. In addition, the IgA1 hinge region is elongated and modified with up to six O-linked glycans (Yoo and Morrison 2005). In serum, IgA occurs mostly as its monomeric structural unit, however, it can be further assembled into dimers through incorporation of the joining chain (JC), a small polypeptide with a single biantennary complex N-glycan, which along with the IgA tailpiece N-glycan contributes to correct dimer formation (Atkin *et al.* 1996; Yoo *et al.* 1999; Göritzer *et al.* 2020). Newly synthesized dimeric IgA can associate with the pIgR receptor that is expressed as integral membrane protein on the basolateral side of epithelial cells lining mucosal surfaces, after which it is transported across the epithelium and released into the lumen. At the luminal side, pIgR is cleaved and a part referred to as secretory component (SC) remains attached thereby forming SIgA (Johansen *et al.* 2001; Mostov *et al.* 1984). The secretory component is a hydrophilic and highly glycosylated polypeptide carrying seven N-glycosylation sites, which protect SIgA from degradation and can interact with various host cell receptors and pathogens (Brandtzaeg 2013).

The N- and O-glycans attached to IgA in circulation are very heterogenous and their function is often not well understood. Therefore, generating recombinant monomeric and multimeric IgA variants bearing well-defined glycans is challenging but desired to study their contribution to IgA function. Furthermore, aberrant glycosylation such as galactose-deficient IgA1 O-glycans that are involved in the pathogenesis of IgA nephropathy should be avoided in therapeutic settings to reduce the risk of adverse side effects like the formation of anti-glycan antibodies (Novak *et al.* 2011).

Successful functional expression of a fully assembled recombinant secretory IgA (CaroRX™) to prevent dental caries has first been shown in transgenic *N. tabacum* (Ma et al 1995). More recently, the production of IgA variants in different plant species displaying no apparent difference in assembly, integrity and functionality compared to mammalian-produced IgA has been reported (Karnoup *et al.* 2005; Paul *et al.* 2014; Göritzer *et al.* 2017; Dicker *et al.* 2016). The glycosylation efficiency is essentially the same in plant and mammalian expression systems with an almost complete occupancy of N-glycans on all sites of IgA except the one in the C-terminal tailpiece (Göritzer *et al.* 2017). The N-glycan diversity found on plant-produced recombinant IgAs is however, reduced compared to mammalian-derived IgA, with biantennary complex-type structures like $GlcNAc_1Man_3GlcNAc_2$ and GnGn as major glycoforms. In contrast, tri- and tetra-antennary structures, bisecting GlcNAc, and capping with sialic acid, which can be detected on mammalian-derived IgA (Rolye et al. 2003; Mattu et al. 1998), are missing in plants. Furthermore, it has been reported that IgA transiently produced in *N benthamiana* displays variable amounts of oligomannosidic structures indicating inefficient secretion (Paul *et al.* 2014; Westerhof *et al.* 2014; Göritzer *et al.* 2017). Site-

specific N-glycan analysis revealed major differences between the individual N-glycosylation sites of each IgA subtype. These distinct features are conserved among the different IgA subtypes and expression systems, although their glycosylation repertoire is very different. The most pronounced difference is the complete lack of α1,6- and α1,3-linked core-fucose in the CH2-resident N-glycosylation site in all IgA isotypes expressed in mammalian cells and wild-type plants, respectively (Göritzer *et al.* 2017; Dicker *et al.* 2016). N-glycans found on dimeric IgA produced in plants are similar but display a shift from paucimannosidic structures to more processed structures (GnGn) compared to their monomeric counterparts (Göritzer *et al.* 2020). This trimming likely occurs in a post-Golgi compartment by β-hexosaminidases and further differences between monomeric and dimeric IgAs can be explained by changes in the accessibility of the N-glycans due to dimer formation and incorporation of the JC. Like the JC of mammalian-derived IgA, the N-glycosylation site is fully occupied but displays high amounts of oligomannosidic N-glycans that are not commonly detected on the JC of dimeric IgA produced in mammalian cells (Paul *et al.* 2014; Göritzer *et al.* 2020). In humans, the SC of mucosal IgA is heavily glycosylated with branched complex N-glycans carrying high levels of sialic acid and the seven putative sites are occupied in varying degrees (Huang *et al.* 2015). A comprehensive site-specific and quantitative N-glycan analysis of the SC incorporated in plant-produced IgA is still lacking. Partial analysis revealed differing data on the N-glycan profile with either mostly complex-type structures or the presence of oligomannosidic structures indicating different subcellular trafficking routes of distinct IgA variants (Paul *et al.* 2014; Westerhof *et al.* 2014; Dicker *et al.* 2016).

The most significant difference between plant and mammalian expression hosts are the modification of the proline-rich hinge region. O-glycans found on IgA1 produced in mammalian cells are a combination of mucin-type core structures with a maximal occupation of six out of nine potential O-glycosylation sites (Göritzer *et al*. 2017; Royle *et al.* 2003). On the hinge region of plant-produced recombinant IgA1 the conversion of proline residues located next to O-glycosylation sites to Hyp and the presence of additional pentoses, presumably representing attached arabinose chains has been detected in different plant-based systems (Karnoup *et al.* 2005; Göritzer *et al.* 2017). These protein modifications increase the heterogeneity of plant-produced proteins, impede a detailed site-specific analysis of engineered O-glycan analysis, and may have adverse properties that affect the functionality or immunogenicity of therapeutic IgA.
One of the most important steps towards humanizing IgA1 antibodies produced in plants is the successful modification of the hinge-region with disialylated mucin-type core 1 O-glycans that largely resemble the human serum glycoform (Dicker *et al.* 2016). The recently completed sequencing of the glycoengineered *N. benthamiana* ΔXT/FT line (Schiavinato et al. 2019) allows now thorough mining of P4H candidates responsible for the conversion to Hyp to set up genome-editing approaches for their elimination. Other shortcomings of plant-produced IgA such as underglycosylation of the IgA tailpiece as well as the presence of paucimannosidic structures could be counteracted applying similar glycoengineering approaches as described for plant-produced IgG. It is possible to overcome the reduced glycosylation efficiency by co-expression of the single subunit OST from *L. major* (LmSTT3D) (Göritzer *et al.* 2020; Castilho *et al.*, 2018). A higher occupancy of the tailpiece N-glycan also increased the efficiency of dimeric IgA assembly in plants. Furthermore, through co-expression of human GnTII, the ratio of fully processed structures with two terminal GlcNAc residues (GnGn) can be substantially increased in *N. benthamiana* ΔXT/FT. In a recent study, the N-glycan core-structure of monomeric IgA produced in the *N. benthamiana* ΔXT/FT could be further extended carrying terminally galactosylated and sialylated N-glycans with high homogeneity at each N-glycosylation sites of all IgA isotypes through co-expression of the respective mammalian glycosylation enzymes (Göritzer *et al.* 2019). Additionally, the generation of monomeric IgA variants carrying mainly

truncated paucimannosidic glycans could be achieved through overexpression of two β-hexosaminidases targeted to the *trans* Golgi and apoplast. The produced glycovariants were then used to investigate the influence of distinct glycoforms on conformational and thermal stability as well as binding to FcαRI, the main IgA receptor. Consistent with data from human serum IgA, no effect on FcαRI binding was observed for the plant-produced IgA glycoforms (Mattu *et al.* 1998; Göritzer *et al.* 2019). On the other hand, a recent study reported that removal of terminal sialylation from serum IgA1 increases its pro-inflammatory capacities and distinct site-specific glycan modifications could play a role for effector functions (Steffen *et al.* 2020). Further studies with glycoengineered plant-produced monomeric and polymeric IgAs will contribute to shed light on the function of distinct IgA glycan modifications.

Glycosylation of plant-produced IgEs

IgE is the least abundant serum antibody and a central player in the allergic response. IgE antibodies directed toward allergens lead to symptoms of allergy through binding to the high-affinity IgE receptor FcεRI. The IgE structure differs from IgG in that IgEs contain four constant domains compared to three constant domains in IgG classes (Arnold *et al.* 2007) (**Figure 2**). IgE is the most heavily N-glycosylated antibody with seven N-glycosylation sites distributed across the constant domain of the human ε-(heavy) chain. Five sites are predominately occupied with complex N-glycans containing a core fucose and different levels of sialic acids (Arnold *et al.* 2004; Plomp *et al.* 2014; Shade *et al.* 2015; Montero-Morales *et al.* 2017). Asn383 is not glycosylated on recombinant, myeloma or serum IgE and Ans394 carries exclusively oligomannosidic N-Glycans. The N-glycan at this site corresponds to Asn297 from the IgG1 heavy chain and occupies the cavity between two Fc domains (Wurzburg *et al.* 2000). While removal of the N-glycan at Asn394 impairs effector functions (Shade *et al.* 2015; Jabs *et al.* 2018), specific modifications of the other N-glycans did not result in altered FcεRI binding on mast cells (Montero-Morales *et al.* 2019).

A recombinant human IgE antibody targeting HER2 has been transiently expressed in *N. benthamiana* and compared to the same antibody produced in HEK293 cells (Montero-Morales *et al.* 2017). Like the human cell-derived variant, plant-produced IgE carried complex N-glycans at the same N-glycosylation sites, Asn383 was not occupied and Asn394 was modified with oligomannosidic N-glycans. When produced in the glycoengineered ΔXT/FT line, the majority of the N-glycans on these sites correspond to GnGn. N-glycosylation sites Asn140, Asn168, Asn265 and Asn394 were essentially fully glycosylated. By contrast, Asn218 and Asn371 displayed underglycosylation with 18-48% occupancy compared to 75-90% in human cell-derived IgE (Castilho *et al.* 2018). Co-expression of the single subunit OST LmSTT3D increased the occupancy at both sites and resulted in more than 60% glycosylation of Asn383 with a complex-type N-glycan. Moreover, transient expression of recombinant IgE in *N. benthamiana* capable of protein sialylation resulted in N-glycans with terminal sialic acid ranging from 45-78% (Montero-Morales *et al.* 2019). The sialylation content of IgEs differ in individuals with specific allergies and allergic reactions may be attenuated by reduced levels of sialylated IgEs (Shade *et al.* 2020). Recombinant IgE variants with different amounts of sialic acid are therefore valuable for the characterization of distinct IgE functions.

Glycosylation of plant-produced IgMs

IgMs are the first antibodies produced during a humoral immune response and the third most abundant antibody subclass in humans. IgMs are heavily glycosylated oligomers containing five N-glycosylation sites on each IgM μ-(heavy) chain (Asn171, Asn332, Asn395, Asn402 and Asn563) (Arnold *et al.* 2007) (**Figure 2**). In human serum, IgMs circulate mainly as pentamers consisting of 10 μ-chains, 10 light chains and a single JC that are linked by disulfide bridges. Together with the single N-glycosylation site in the JC, a petameric IgM has 51 potential N-glycosylation sites. In addition, IgM can exist as a hexamer with in total

60 N-glycosylation sites. Like IgE, the μ-chain has four domains in the constant region. Asn171 is in the CH1 domain, Asn332 in the CH2 domain, Asn395 as well as Asn402 are located in the CH3 domain. Asn563 is located in the C-terminal tailpiece region which is required for JC incorporation and multimerization (Wiersma *et al.* 1998). On human serum IgM and recombinant pentameric IgM, Asn171, Asn332 and Asn395 carry predominately biantennary complex N-glycans with different degrees of sialylation (Loos *et al.* 2014; Pabst *et al.* 2015; Moh *et al.* 2016; Chandler *et al.* 2019; Hennicke *et al.* 2020). By contrast, Asn402 displays mainly $Man_5GlcNAc_2$ structures and Asn563 $Man_6GlcNAc_2$ to $Man_8GlcNAc_2$ oligomannosidic N-glycans. While sites Asn171, Asn332, Asn395 and Asn402 are typically fully occupied with N-glycans, there is some variation in the glycosylation efficiency of Asn563. On human serum-derived IgM or recombinantly produced IgM, full glycosylation (Loos *et al.* 2014; Pabst *et al.* 2015) as well as reduced N-glycosylation efficiency with only 17-60% occupancy were reported for Asn563 (Arnold *et al.* 2005; Moh *et al.* 2016; Chandler *et al.* 2019).

Previous studies have shown that IgM N-glycans are functionally important. Abolishing N-glycosylation impacts IgM secretion (Sitia *et al.* 1984) and immunomodulatory effects such as the internalization of IgM by T cells (Colucci *et al.* 2015) or complement activation (Wright *et al.* 1990; Gadjeva *et al.* 2008). On the other hand, distinct glycan modifications appear dispensable for the binding to the human Fcμ-receptor (Lloyd *et al.* 2017).

Transient co-expression of the μ, light and joining chains in *N. benthamiana* resulted in the expression of a functional IgM with a high proportion of hexamers (Loos *et al.* 2014). The type of N-glycans found on plant-produced IgM resembled that of recombinant IgM derived from human cells. Glycosylation sites Asn171, Asn332, Asn395 carried more than 50% of complex GnGn N-glycans when expressed in the glycoengineered ΔXT/FT line. On sites Asn402 and Asn563 96% of oligomannosidic N-glycans were detected. Upon co-expression of the pathway for *in planta* protein sialylation, complex N-glycans with mono- and disialylated structures were present on sites Asn171, Asn332 and Asn395 (Loos *et al.* 2014). Although the N-glycosylation efficiency at site Asn563 of plant-produced IgM was not reported, it is likely that the site in the tailpiece is incompletely glycosylated. As described for plant-produced dimeric IgA, the reduced N-glycan occupancy may affect the JC incorporation and leads to the higher proportion of hexameric IgM in plants (Loos *et al.* 2014).

Conclusion and outlook

In the last couple of years, a comprehensive glycosylation analysis of all antibody subclasses (except IgD) produced in plants has been performed. Overall, the analysis revealed that the type of N-glycans (complex vs. oligomannosidic) are conserved when expressed in plants. Differences are found due to the simplified N-glycan processing pathway, the sometimes-reduced N-glycosylation efficiency and the completely missing mucin-type O-glycosylation pathway. *N. benthamiana* plants are amenable to glycoengineering that resulted in the production of different recombinant antibodies with quite homogenous human-like glycans. These tailored structures are essential to investigate the biological function of distinct glycan modifications and make plants an attractive platform for the generation of recombinant antibodies with diverse activities and applications (Wang and Ravetch 2019).

Compliance with Ethical Standards:

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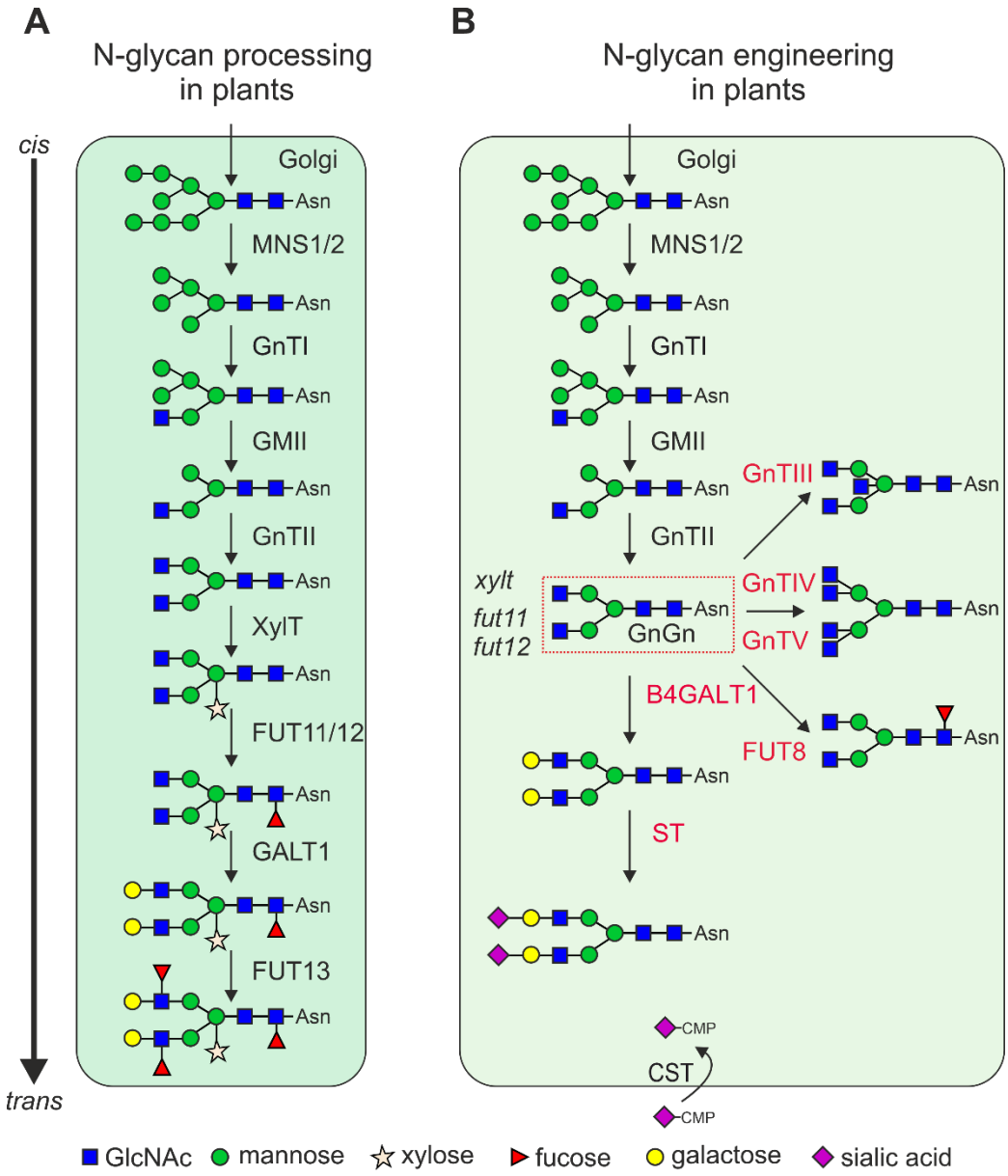

Figure 1. (A) Schematic representation of complex N-glycan processing in the plant Golgi apparatus. Golgi-α-mannosidase I (MNS1/2), N-acetylglucosaminyltransferase I (GnTI), Golgi-α-mannosidase II (GMII), N-acetylglucosaminyltransferase II (GnTII), β1,2-xylosyltransferase (XylT), core α1,3-fucosyltransferase (FUT11/12), β1,3-galactosyltransferase (GALT1) and α1,4-fucosyltransferase (FUT13) are indicated. (B) N-glycan engineering approaches to produce defined homogenous complex N-glycans on plant-produced recombinant antibodies: the generation of *xylt*, *fut11 fut12* knockouts results in the formation of the GnGn structure which can serve as acceptor substrate for N-acetylglucosaminyltransferase III (GnTIII), IV (GnTIV) and V (GnTV), core α1,6-fucosyltransferase (FUT8), β1,4-galactosyltransferase (B4GALT1) and α2,6-sialyltransferases (ST). Sialylation in plants requires the co-expression of the Golgi CMP-sialic acid transporter (CST) and proteins for CMP-sialic acid biosynthesis (not shown).

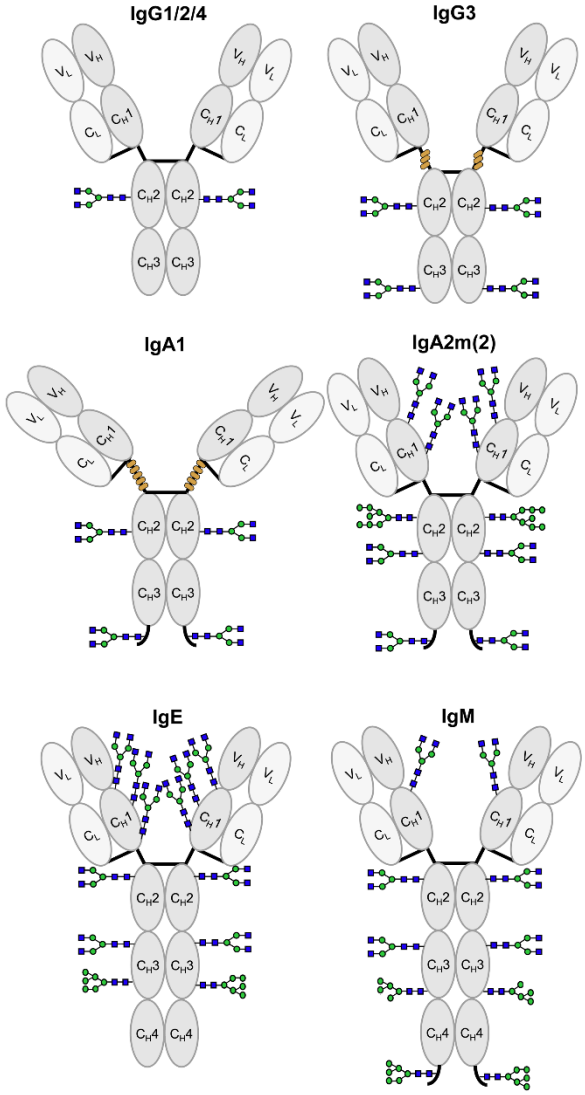

Figure 2. Schematic illustration of the structure and glycosylation sites of IgGs, IgAs, IgE and IgM. The light chain is colored in light gray and the heavy chain in dark gray. N-glycans found in the constant domains of the different antibody classes are indicated with symbols that are drawn according to the nomenclature from the Consortium for Functional Glycomics (http://www.functionalglycomics.org/). For each site, the predominant N-glycan structure (complex GnGn or oligomannosidic) found on ΔXT/FT *N. benthamiana* produced recombinant antibody is indicated. Potential O-glycosylation sites are marked in the hinge region of IgG3 and IgA1 (orange ellipse).