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CHAPTER 2
Sialoglycans and genetically engineered plants

1 Introduction

N-glycosylation proteins enable proper protein folding and provides stability to the protein, efficient protein targeting for activity of lysosomal enzymes, protein–protein or protein–carbohydrate interaction, effector functions and biological activity of proteins, and control of protein half-life (Fig. 1).

Plants are being engineered to develop large quantities of biopharmaceutical proteins (Table 1) in a cost-effective manner and several technical, veterinary, and pharmaceutical proteins made in plants have been successfully made available commercially [2]. Since plant N-glycosylation pathway differs at some stages with the human N-glycosylation pathway, it is important to manipulate the plant N-glycosylation pathway to render them more appropriate expression systems for human N-glycosylated proteins of desired properties.

Solanaceous species under genus Nicotiana, such as Nicotiana tabacum (tobacco) and Nicotiana benthamiana (N. benthamiana, Fig. 2) have been extensively exploited in molecular farming due to the following advantages: (i) ease of cultivation, (ii) high biomass, (iii) genetic tools for trait manipulation, (iv) application of new plant breeding techniques (CRISPR/Cas9); and (v) nonfood status of the plant minimizing chances of contamination, (vi) possibility of natural insertion in the RNA-dependent RNA polymerase 1 gene [1, 3], which leads to a reduced level of gene silencing [1, 4].

Although the recombinant proteins of human origin are generated in plants with proper folding and it is possible to assemble complex proteins within the plant machinery, conventional expression systems for the production of recombinant biopharmaceutical proteins suffers from the limitation of proper synthesis of glycan structure in glycoconjugated molecules leading to the production of aberrant mixture of glycoforms that bear no resemblance to human glycans or are important from the point of view of therapy.
Sialic acids and sialoglycoconjugates in the biology of life, health and disease

Asn

MnTs

Mnsl

Och1

GnTI

GnTII

ST

GaIT

HEXO

Common precursor: Man8

Yeast-type high-mannosidic N-glycans

Insect-type pauci-mannosidic N-glycans

Plant-type complex N-glycans

Mammalian-type complex N-glycans

Fig. 1 N-glycosylation pathway represented from common precursor in plant human, yeast, and insect cells. The common endoplasmic reticulum (ER)-resident oligosaccharide precursor Man8 acts as initiating point for further modifications in the Golgi apparatus. Och1: a1,6-mannosyltransferase; MnTs: mannosyltransferases; Mns: mannosidase; GnT: N-acetylglucosaminyltransferase; GaIT: a1,4-galactosyltransferase; ST: a2,6-sialyltransferase; HEXO: hexosaminidase (N-acetylglucosaminidase); XT: β 1,2-xyllosyltransferase; and FT: core fucosyltransferase. Fucose can be transferred in a1,3-linkage (typical of plant) and a1,6-linkage (typical of mammal). (Reproduced with permission from Loos A, Steinkellner H IgG-Fc glycoengineering in non-mammalian expression hosts, Arch Biochem Biophys 2012;526:167–173.)
| Product                                           | Host                        | Application            | Clinical trial | Status                                      | Sponsor                                         |
|--------------------------------------------------|-----------------------------|------------------------|----------------|---------------------------------------------|------------------------------------------------|
| Taliglucerase alfa; Recombinant glucocerebrosidase (prGCD) | Carrot cell culture         | Gaucher disease        | NCT00376168    | Phase 3 completed (2012); FDA approved (2012) | Protalix, Karmiel, Israel                      |
| ZMapp                                            | Tobacco                     | Ebola Virus            | NCT02363322    | Phase 1 and 2 (2015)                        | National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA |
| PRX-102                                          | Tobacco cell culture        | Fabry Disease          | NCT01769001    | Phase 1 and 2 (2014)                        | Protalix, Karmiel, Israel                      |
| VaccinePh25 VLP                                   | Tobacco                     | Malaria                | NCT02013687    | Phase 1 (2015)                              | Center for Molecular Biotechnology, Plymouth, MI, USA |
| Vaccine                                          | Tobacco                     | Anthrax                | NCT02239172    | Phase 1 (2014)                              | Center for Molecular Biotechnology, Plymouth, MI, USA |
| Recombinant protective antigen                    | Tobacco                     | H5N1 Vaccine           | NCT01250795    | Phase 1 (2011)                              | Center for Molecular Biotechnology, Plymouth, MI, USA |
| H5-VLP + GLA-AF Vaccine                           | Tobacco                     | Influenza A Subtype H5N1 Infection | NCT01657929   | Phase 1 Completed (2014)                    | Infectious Disease Research Institute, Seattle, WA, USA |
| P2G12 Antibody                                   | Tobacco                     | HIV                    | NCT01403792    | Phase 1 Completed (2011)                    | University of Surrey, Guildford, UK           |

Reproduced with permission from open access article under the Creative Commons Attribution License (CC BY 4.0): Yao J, Weng Y, Dickey A, Wang KY. Plants as factories for human pharmaceuticals: applications and challenges. Int J Mol Sci 2015;16(12):28549–65.
Thus the synthesis of glycans resembling human glycan structure and its huge complexity remain a major challenge in glycoengineering of plant cells [6]. This is all more challenging due to the complexity and diversity of the glycans in different therapeutic recombinant proteins.

Targeted manipulation of the plant N-glycosylation pathway has enabled the production of human-like oligosaccharides and enabled the generation of functional and effective biopharmaceuticals. In the recent years plant revealing a simple N-glycosylation pathway but lacking the O-glycosylation pathway have been reported to be better and potential glycan expression systems over the conventional ones. Plant systems are being used as effective expression systems of complex sialoglycans and N-glycans and different strategies are being used for the expression of complex therapeutic sialylated glycoforms in plant systems.
2 N-glycosylation in plants

Recently different posttranslational modifications (PTMs) have been reported for peptide maturation and activation, including proteolytic processing, tyrosine sulfation, proline hydroxylation, and hydroxyproline glycosylation [7] in plants. While glycan epitopes of human complex N-glycans are often targets of lectins important for cell-cell communication, the role of plant N-glycans finds importance in protein folding, and other biological functions including salt stress responses, cellulose biosynthesis, microtubule association, and biogenesis of several receptor-like kinases [8].

N-glycosylation is a major post-translational modification PTM in eukaryotes and is important in maintaining cell viability, where the attached core N-glycans enables proper protein folding of secreted glycoproteins and membrane proteins in the endoplasmic reticulum (ER).

Although the studies of N-glycosylation in human is extensive, the knowledge in plants is restricted due to limited N-linked glycan and mutant phenotypes, limited methods to modify and target N-glycans at specific sites, and limited understanding of protein dynamics within secretory system. Glycoproteins move from the ER to the Golgi apparatus, where the N-glycan moieties undergo further maturation or may exit the ER via an alternative route to vacuoles retaining high-mannose N-glycan structures bypassing the modifications in the Golgi.

In eukaryotes, N-glycans processing is initiated in the ER where the precursor Glc3Man9GlcNAc2 (Man9) is converted to Man8GlcNAc2 (Man8) and processing of Man8 in Golgi leads to the formation of complex N-glycans (Fig. 1). N-glycan processing is identical in plants and mammals till the formation of vital intermediate GlcNAc2Man3GlcNAc2 (GnGn). In mammals, GnGn oligosaccharides enables diversification of N-glycosylation but in plants, the GnGn structures are arranged with 1,2-xylose and core 1,3-fucose residues (GnGnXF3). Although in mammals core fucosylation occurs in 1,6-linkage, the fucose residues in plants (Fig. 3) are in 1,3-linkage. Plant cells extend the GnGnXF3 by attaching 1,3-galactose and 1,4-fucose to form Lewis-a epitopes (Lea). Plants reveal formation of paucimannosidic structures due to the removal of terminal GlcNAc residues from GnGnXF3 by endogenous hexosaminidases similar to insects. As compared to human complex N-glycans, N-glycans of the plant systems lack sialic acid but contain core α1,3-fucose (Fuc) and β1,2-xylose (Xyl) modifications, and may contain terminal Lewis-a epitopes (β1,3-galactose (Gal) and α1,4-Fuc.
Fig. 3 (A) Schematic overview of complex N-glycan processing in plants. Golgi-\(\alpha\)-mannosidase I (MNS1/2), N-acetylglucosaminyltransferase I (GnT1), Golgi-\(\alpha\)mannosidase II (GMII), N-acetylglucosaminyltransferase II (GnTII), \(\beta\)1,2-xylosyltransferase (XylT), core \(\alpha\)1,3-fucosyltransferase (FUT11/12), \(\beta\)1,3-galactosyltransferase (GALT1), and \(\alpha\)1,4-fucosyltransferase (FUT13). (B) Representative view of N-glycan processing in mammalian cells. Golgi \(\alpha\)-mannosidase I (GMI), core \(\alpha\)1,6-fucosyltransferase (FUT8), N-acetylglucosaminyltransferase IV (GnTIV) and V (GnTV), \(\beta\)1,4-galactosyltransferase (B4GalT1), and \(\alpha\)2,6-sialyltransferases (ST). (C) Optimized N-glycan engineering approach: generation of xylt, fut11, fut12, and galt1 knockouts results in the formation of GnGn structure which serves as acceptor for GnTIV, GnTV, B4GalT1, and ST resulting in fully processed complex N-glycans. Sialylation in plants requires the co-expression of the Golgi CMP-sialic acid transporter (CST) and proteins for CMP-sialic acid biosynthesis. (Reproduced with permission from open access article under a Creative Commons Licence: Schoberer J Strasser R, Plant glyco-biotechnology. Semin Cell Dev Biol. 2018 80:133-141.)
linked to terminal N-acetylglucosamine, GlcNac (Fig. 3). Human complex N-glycans are often sialylated containing different epitopes, including Lewis x, N-acetyllactosamine (LacNAc), and N,N′-di-N-acetyllactosediamine (LacDiNAc).

Although the plants and mammals reveal differences in the N-glycan structures they share high degree of homology in the secretory pathway.

3 Sialylation and recombinant proteins produced in plants

Glycoengineering aims at the production of recombinant glycoproteins with a defined glycosylation profile, in order to study the impact of glycosylation and for the production of therapeutic agents. The plant expression systems are being designed to generate therapeutically important glycoproteins. Plant systems find importance as they are biologically safe, cost effective, and convenient. However, as plant N-glycosylation pathway differs in many aspects as compared to human N-glycosylation, modification of N-glycosylation pathway in plants is needed to avoid immunological challenges and get humanized authentic N-glycosylated molecules.

Plants reveal highly conserved secretory pathway with folding, assembly, and posttranslational modifications of proteins similar to the mammals. Animal sialyltransferases (STs) consist of four conserved motifs, namely large (L), small (S), very small (VS), and motif III. Although sialic acid has not been detected in plants, three orthologues containing sequences similar to the ST motifs have been identified in Arabidopsis thaliana L. The At3g48820 gene with gene id 824,043 codes for a Golgi resident protein but lacks the ability to transfer sialic acid to asialofetuin or Galβ1,3GalNAc and Galβ1,4GlcNAc oligosaccharide acceptors [10]. Strategies to produce humanized therapeutic glycoproteins in plants involves (i) retaining of the recombinant glycoproteins in ER, where N-glycans undergo modification, (ii) inhibiting the plant endogenous Golgi glycosyltransferase, and (iii) adding new glycosyltransferase from mammals.

Different approaches have been used to modify the N-glycosylation pathway in different plant species, using T-DNA insertion mutants [11], RNA interference (RNAi) [12–14], chemical mutagenesis [15], and targeted nuclease [16–18] approaches. N. benthamiana finds importance in molecular farming as the transient expression of proteins is fast and yields antibodies [19] by different transient expression systems, including the MagnICON system [20], the pEAQ vector [21], and the pTRA vector [22]. Zinc finger nucleases (ZFNs) [23] transcription activator-like effector nucleases
TALENs [24] have enabled easy knockout of multiple genes. In *N. benthamiana*, the two *XylT* genes and two of the five *FucT* genes were knocked out with TALENs to completely eliminate the $\beta$-1,2-xylosyltransferase activity and reduce core $\alpha$-1,3-fucosyltransferase activity by 60%. CRISPR/Cas9 system has been used to knockout two $\beta$-1,2-xylosyltransferase and four $\alpha$-1,3-fucosyltransferase genes in *N. benthamiana* [25].

Sia and polysialic acid (polySia) play a vital role in biological functions and therapeutic use. Expression system in plants has been designed with multigene vectors enabling the controlled in vivo synthesis of sialylated structures in the human sialylation pathway (Fig. 4) that sialylate glycoproteins in $\alpha$2,6- or $\alpha$2,3-linkage and transient coexpression of human $\alpha$2,8-polysialyltransferases lead to the production of active and functional polySia structures [26].

![Fig. 4 Strategy to engineer human sialylation pathway in plants using the endogenously present metabolite UDP-GlcNAc. Enzymes involved are: UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine-kinase (GNE), N-acetylneuraminic acid phosphate-synthase (NANS), CMP-sialic acid (Neu5Ac) synthetase (CMAS), CMP-Neu5Ac transporter (CST), $\beta$1,4-galactosyltransferase (GT), and $\alpha$2,6-sialyltransferase (ST). In planta protein sialylation was achieved by the coordinated expression and correct subcellular deposition of genes/proteins for (i) biosynthesis (GNE, NANS), (ii) activation (CMAS), (iii) transport (CST), and (iv) transfer of Neu5Ac to terminal galactose (ST). (Reproduced with permission from open access article under a Creative Commons License: Loos A, Steinkellner H IgG-Fc glycoengineering in non-mammalian expression hosts, Arch Biochem Biophys 2012;526:167–173.)](image-url)
4 Fc glycoengineering in plants

The conserved secretory pathway between plants and mammals enable the production of IgGs and IgAs efficiently. En block transfer of the Glc3Man9GlcNAc2 precursor onto the growing protein and subsequent trimming in ER and cis/medial–Golgi compartments reveal similarities between mammals and plants up to the synthesis of GnGn structures (Fig. 1). After this, in mammals, GnGn structures undergo intensive elongation/modification processes unlike in plants, which add xylose in β1,2-position to the innermost mannose residue and fucose in α1,3-position to the innermost GlcNAc residue of the GnGn core oligosaccharide (Fig. 1) which are absent in mammalian cells. Monoclonal antibodies (mAbs) in plants exhibit a N–glycosylation profile with a single dominant oligosaccharide structure, GnGnXF3.

The humanization of the plant pathways were thoroughly investigated by Palacpac et al. [27] and Bakker et al. [28]. They overexpressed the human β1,4-glactosyltransferase (GalT) in tobacco plants to elongate the plant–typical GnGnXF3 by β1,4-galactose leading to the formation of galactosylated structures and drastically reduced the degree of xylosylation and fucosylation. Nut production of mAbs (mAbs) suffered from challenges of formation of unexpected glycoforms and incompletely processed and hybrid structures [29, 30].

Mutants lacking plant-specific β1,2-xylose and core α1,3-fucose achieved by the elimination of endogenous enzymes, β1,2-xylosyltransferase (XT) and core α1,3-fucosyltransferase (FT3, Fig. 2) by knockdown and knockout approaches for the respective genes, and generated mutant plant lines of A. thaliana, Lemma minor, N. benthamian, moss Physcomitrella patens, DXT/FT plants (N. benthamiana glycosylation mutants lack plant-specific core β1,2-xylose and α1,3-fucose residues) were generated and found importance in the production of different mAbs and therapeutics. A schematic diagram of Fc glycoengineering is represented in Fig. 5.

Fc–N–glycosylation profiles of these mAbs achieved by the elimination of β1,2-xylose and core α1,3-fucose leading to the synthesis of human-type structures containing dominant GnGn with no detectable β1,2-xylose or α1,3-fucose residues revealed unaltered antigen binding and complement–dependent cytotoxicity CDC activity and enhanced antibody–dependent cell-mediated cytotoxicity ADCC, effector functions of antibody. This also enabled the generation of increased galactosylation, sialylation, branching, bisecting GlcNAc, or fucosylation.
GalT when targeted to a late Golgi compartment significantly improved β1,4-galactosylation in DXT/FT, transgenic plants. mAbs produced in such glycoengineered plants exhibited a single dominant Fc-N-glycan, digalactosylated AA structures which is predominant in serum IgG and mAbs as against HIV produced in these glycoengineered plants which exhibited improved anti-viral activity. GlcNAc bound in b1,4-position to the innermost mannose residue called bisecting GlcNAc is reported to enhance ADCC activity of mAb CAMPATH-1H, glycoengineered Rituxan and Herceptin with increased bisecting structures due to decreased 1,6-fucosylation caused by the blocking of the fucosyltransferase.

Contrasting reports exist that in CHO cells the overexpression of N-acetylglucosaminyltransferase III (GnTIII) done with the hypothesis to increase bisecting GlcNAc is reported to enhance ADCC activity of mAb CAMPATH-1H, glycoengineered Rituxan and Herceptin with increased bisecting structures due to decreased 1,6-fucosylation caused by the blocking of the fucosyltransferase.

In the DXT/FT mutant lacking plant-specific core modifications, less of bisecting glycoforms were synthesized as compared to Wild type plants. Glycomodified DXT/FT plants produced mammalian-type core α1,6-fucosylation by overexpressing core α1,6-fucosyltransferase, generating mAbs with and without fucose with identical N-glycosylation.

Plant based antibody 2G12 batches exhibited glycosylation profiles containing a predominant N-glycan structure, and GnGnXF3, GnGnF6, GnGn, and digalactosylated AA structures with binding similar to FccRI,

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**Fig. 5** Fc-glycoengineering in plants. Overview of mAb glycoforms generated in glycoengineered *N. benthamiana*. IgG N-glycans generated (1) in Wild type plants: GnGnXF3 [14]; (2) in DXT/FT3 plants: GnGn [14]; (3) in DXT/FT + FT6: GnGnF6 [31]; (4) in DXTFT + GalT: AA [32, 33]; (5) in DXT/FT along with six mammalian genes of the mammalian sialic acid pathway: NaNa [29]; and (6) in Wild type + GnTIII: GnGnXF3bi [33]. FT6: α1,6-fucosyltransferase, GalT: β1,4-galactosyltransferase, ST: α2,6-sialyltransferase, and GnTIII: N-acetylglucosaminyltransferase III (1). (Figure and legend reproduced with permission from open access article under a Creative Commons License: Loos A, Steinkellner H. IgG-fc glycoengineering in non-mammalian expression hosts. Arch Biochem Biophys 2012;526:167–73.)
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FccRIIa, and FccRIIb. 2G12 glycoforms lacking core fucose mediated antiviral activity against various lentiviruses including HIV-1.

The most complex step of human N-glycosylation is terminal sialylation and difficult to accomplish in plants as they lack the enzyme cascades. But in planta the sialylation of mAbs has recently become possible [12, 34] by the introduction of enzymes of the mammalian pathway into plants, allowing the biosynthesis of sialic acid its activation, its transport into the Golgi, and finally its transfer onto terminal galactose and mAbs coexpressed with engineered human sialylation pathway carried up to 80% sialylated structures [30]. Six mammalian enzymes were overexpressed in plants [34].

5 Applications

Advancement has been made in the design and development of plant expression systems for the generation of recombinant N-glycans glycoproteins by glycoengineering. N-glycosylation affects many properties of recombinant glycoproteins produced in planta including efficient plant-made antibodies for passive immunization but with shorter half-life in the blood due to a higher clearance rate [35–38]. The removal of the core fucose residue from mammalian α-1,6-fucose or the plant α-1,3-fucose from the N-glycan of an antibody has been reported to increase the antibody-dependent cellular cytotoxicity (ADCC) [1, 36, 37] thus proving as an effective biopharmaceutical. The Food and Drug Administration (FDA) has approved first plant-made pharmaceutical protein for human parenteral administration including taliglucerase alfa [38], also named as Elelyso, produced by Protalix Biotherapeutics for the application as a replacement therapy for Gaucher disease, which is advantageous due to the structure of the exposed terminal mannose residues on α-1,3-fucose- and β-1,2-xylose-containing N-glycan structures generated in plant cell vacuoles [1] that are required for the efficient uptake of the enzyme into macrophages. N. benthamiana has been extensively researched for the production of mucin-type O-glycans [39] and N-glycans [40] of recombinant proteins.

As plant cells lack β1,4-galactosylated and sialylated glycan, which have important biological functions in animal cells [1, 26], transgenic human β1,4-galactosyltransferase producing tobacco BY2 suspension-cultured cells were developed [1, 27]. Two genes encoding human CMP-N-acetylneuraminic acid synthetase and CMP-sialic acid transporter expressed in tobacco suspension-cultured cell to enable sialic acid biosynthesis in plants can act as bioreactor for mammalian glycoprotein production.
Human butyrylcholinesterase (BChE) is a tetrameric human serum sialylated protein that finds therapeutic importance as a candidate bioscavenger of organophosphorus nerve agents. *N. benthamiana* has been engineered for the expression of sialylated protein by transient co-expression of BChE cDNA by vectors [41] leading to the generation of rBChE expressing mono- and di-sialylated N-glycans in the intracellular fluid with similarity to the human protein orthologue. β1,4-N-acetylglucosaminyl-transferase IV overexpression in the recombinant engineered plant enabled the generation of branched N-glycans, with tri-sialylated structures with better and effective novel therapeutic role [42].

Glycoprotein hormone erythropoietin (EPO) finds importance in the maintenance of hematopoiesis and providing tissue protection and recombinant human EPO (rhuEPO) find application in the treatment of anemia. However, rhyEPO at higher doses can cause harmful increase in the RBC masses and reveals limited role in tissue protection. Asialoerythropoietin (asialo-rhuEPO), which is a desialylated form of rhuEPO, has been reported to lack hematopoietic activity, but retain cytoprotective activity. But chemically enzymatic desialylation of rhuEPO suffers from not being cost effective. Although plants are known to synthesize complex N-glycans, they lack enzymes to transfer sialic acid and β1,4-galactose to N-glycan chains, therefore serve as a potential expression for generation of asialoerythropoietin.

Asialo-rhuEPO is being designed to be produced in plants by introducing human β1,4-galactosyltransferase as the penultimate β1,4-linked galactose residues regulating its in vivo biological activity. Co-expression of human β1,4-galactosyltransferase and EPO genes in tobacco plants has been reported to accumulate asialo-rhuEPO confirmed by its specificity to Erythrina cristagalli lectin column, revealing expression of N-glycan structures with terminal β1,4-galactose residues and a functional co-expressed GalT. Asialo-rhuEPO has been reported to interact with the EPO receptor (EPOR) with similar affinity as rhuEPO with desired biological function [43].

N-glycans with terminal Neu5Ac residues are important for the biological activities and half-lives of recombinant therapeutic glycoproteins in humans but the fact that plants express negligible amounts of free or protein-bound Neu5Ac presents a major disadvantage for their application as biopharmaceutical expression system. Thus to synthesize Neu5Ac-containing N-glycans, plants need to synthesize Neu5Ac and its nucleotide-activated derivative, cytidine monophospho-N-acetylneuraminic acid. Transgenic *A. thaliana* plants expressing three key enzymes of the mammalian Neu5Ac biosynthesis pathway, UDP-N-acetylgalacosamine
2-epimerase/N-acetylmannosamine kinase, N-acetylneuraminic acid phospho-
te synthase, and CMP-N-acetylneuraminic acid synthetase, has been
designed and developed and their simultaneous expression has led to the
generation of significant Neu5Ac amounts in planta, which could be fur-
ther converted to cytidine monophospho-N-acetylneuraminic acid by the
coeexpression of CMP-N-acetylneuraminic acid synthetase leading to the
production of Neu5Ac-containing glycoproteins in plants [44].

Neu5Ac could be synthesized in the plant cytosol by the expression
of microbial Neu5Ac-synthesizing enzymes including Neu5Ac lyase from
Escherichia coli and Neu5Ac synthase (neuB2) from Campylobacter jejuni
in two model plants including Bright Yellow 2 (BY2) tobacco cells and
Medicago sativa [45].

Human CMP-N-acetylneuraminic acid (NeuAc) synthase (HCSS)
and α2,6-sialyltransferase (HST) enable sialylation of N-linked glycans in
mammalian cells. HCSS synthesizes CMP-NeuAc, which HST uses as a
donor substrate to transfer NeuAc to the terminal position of N-linked
glycans. HCSS and HST genes could be inserted and expressed by the
suspension-cultured tobacco BY2 cells to enable sialylation pathway in
plants, producing mammalian-type sialoglycoproteins with terminal NeuAc
residues in plants [46].

Two engineered constructs containing either the native signal peptide
from human lactoferrin or the signal peptide from sweet potato sporamin
fused to human lactoferrin has been reported to produce N-terminal se-
quences of rhLf purified from tobacco identical to Lf from human milk for
both constructs [47].

The natural insertion of N. benthamiana into the RNA-dependent RNA
copolymerase 1 gene [1, 49] enables rapid production of high-value hormones,
enzymes, and antibodies, and is successful in the production of ZMapp which
is a cocktail of neutralizing mAb c13C6 and two chimeric antibodies c2G4
and c4G7, which were applied during the 2014–15 Ebola outbreak [48], and
for the efficient production of vaccines against seasonal flu [49].

The intravenous immunoglobulin therapeutic application of ZMapp in-
volves direct reaction to the virus and bind as lock and key leading to its
deactivation and provides simulated immune response against Ebola
dand proteins Ebolavirus. Genes of the Ebola antibodies needed for the drug are
inserted into Agrobacterium, then tobacco plants are injected or infused
with the engineered viral vector-encoding Ebola antibodies, and plants pro-
duce the antibodies which are later isolated to form the drug known as
ZMapp (Figs. 6 and 7, Table 2) [48].
Fig. 6 Production of ZMapp through tobacco plant. (Reproduced with permission from Zahara K, Bibi Y, Ajmal M, Sadaf HM, Bibi F, Sardar N, Riaz I, Laraib S. J Coast Life Med 2017;5:206–11.)

Fig. 7 (A) Ebola and (B) Ebola and interaction with ZMapp. (A) Reproduced with permission Ebola picture Source ViralZone, SIB Swiss Institute of Bioinformatics.)
Table 2  Plant produced human pharmaceuticals and industrial production

| Company                                                        | Host                      | Lead product                                         | Expression technology                        | Advantage                | Website references |
|---------------------------------------------------------------|---------------------------|------------------------------------------------------|----------------------------------------------|--------------------------|--------------------|
| Mapp Biopharmaceutical/LeafBiol, USA                          | Tobacco leaves            | ZMapp for Ebola crisis                              | MagnICON Transient expression               | Speed                    | [52]               |
| Protalix, Carmiel, Israel                                     | Carrot or tobacco cell culture | ELELYSO (taliglucerase alfa) Enzyme replacement Vaccine for non-Hodgkin's Lymphoma | ProCellEx Stable Expression                 | Quality                  | [53]               |
| Icon Genetics, München, Germany                               | Nicotiana benthamiana leaves | Vaccine for non-Hodgkin’s Lymphoma                  | MagnICON Transient expression               | Speed and Personalization | [54]               |
| Ventria Bioscience, Junction City, KS, USA                    | Rice seeds                | VEN150 for HIV-associated chronic inflammation      | Express Tec Stable Expression               | Scale Cost               | [55]               |
| Greenovation Biotech GmbH, Heilbronn, Germany                 | Moss                      | Moss-GAA for Pompe Disease, Moss-GBA for Gaucher’s Disease, Moss-AGAL for Fabry Disease | Moss Physcomitrella patens-based Broytechnolgy | Speed Scale and Customized | [56]               |
| Kentucky BioProcessing, Owensboro, KY, USA                    | Nicotiana benthamiana leaves | Contract service                                     | Geneware Transient expression               | Speed                    | [57]               |
| PhycoBiologics Inc. Bloomington, IN, USA                      | Algae                     | Vaccines Growth Factor and enzymes                   | Microalgae expression                       | Speed Scale              | [58]               |
| Medicago, Québec, QC, Canada                                  | Nicotiana benthamiana Alfalfa | Vaccine for influenza, Pandemic market, Rabies and Rotavirus | Proficia Transient Expression; Stable Expression | Speed                    | [59]               |

Continued
| Company                      | Host                     | Lead product                                      | Expression technology                          | Advantage          | Website references |
|------------------------------|--------------------------|---------------------------------------------------|-----------------------------------------------|--------------------|--------------------|
| Synthon, Nijmegen, The      | Duckweed LeafyBiomass    | Antibody for non-Hodgkin’s Lymphoma               | LEX system Stable expression                  | Speed Quality      | [60]               |
| Netherlands                  | Tobacco leaves           | HIV Antibody                                      | Stable Nuclear Expression                     | Scale Cost         |                    |
| Fraunhofer IME, Aachen,     | Nicotiana benthamiana    | Influenza vaccine                                 | Transient expression                          | Speed              | [61]               |
| Germany                      | leaves                   |                                                   |                                               |                    |                    |
| Fraunhofer CMB/iBio,        | Rice seed                | Serum albumin                                     | Stable Expression                              | Quality Scale      | [62]               |
| Newark, DE, USA              | Tobacco leaves           | CaroRx for dental caries; PBI-220 antibody for    | Stable Expression                              |                    |                    |
| Healthgen, Wuhan, Hubei,    |                          | anthrax; DPP4-Fc for MERS coronavirus infection  |                                               |                    |                    |
| China                        |                          |                                                   |                                               |                    |                    |
| PlanetBiotechnology,        |                          |                                                   |                                               |                    |                    |
| Hayward, CA, USA             |                          |                                                   |                                               |                    |                    |

Reproduced with permission from open access article under Creative Commons Attribution License (CC BY 4.0): Yao J, Weng Y, Dickey A, Wang KY. Plants as Factories for Human Pharmaceuticals: Applications and Challenges. Int J Mol Sci. 2015;16(12):28549–65.
6 Mucin type O-glycans and plant expression

As plants lack endogenous glycosyltransferase that lead to Ser/Thr glycosylation as in mammals, plants find importance for the engineering of O-glycosylation as there are no endogenous glycosyltransferases that can act upon engineered enzymes for the synthesis of O-glycans. Transient expression of mucin-type O-GalNAc and core 1 O-linked glycan structures on recombinant human erythropoietin fused to an IgG heavy chain fragment (EPO-Fc) have been reported to be synthesized in N. benthamiana plants. Sialylated core structure constructs encoding human polypeptide: N-acetylgalactosaminyltransferase, Drosophila melanogaster core 1 β1,3-galactosyltransferase, human α2,3-sialyltransferase, and Mus musculus α2,6-sialyltransferase have been reported for their transient co-expression in N. benthamiana together with EPO-Fc leading to the synthesis of mono- and disialylated O-linked glycans and biantennary structures with terminal sialic acid residues [65].

Engineering of O-linked glycans is not much developed in plant systems as the O-glycosylation pathways in plants are different from that in human. In mammals O-glycans on secretory proteins are formed by the attachment of N-acetylgalactosamine (GalNAc) to serine or threonine residues (mucin-type O-glycosylation) which are further modified by the addition of different monosaccharides such as galactose, GlcNAc, sialic acid, forming mucin-type core O-glycan structures that is important in different biological processes [66] (Fig. 8).

In plants, unlike mammals, proline residues are converted to hydroxyproline (Hyp) by prolyl-4-hydroxylases (P4H) that are linked with arabinose residues. Knockout of P4H genes could eliminate O-glycosylation in P. patens, thereby helping in the modification of recombinantly expressed EPO [1, 6, 67]. Overexpression of human polypeptide GalNAc-transferase 2 (GalNAcT2) in Arabidopsis, tobacco BY2 cells, and N. benthamiana [68–70], initiating O-GalNAc formation on different recombinant glycoproteins (including EPO and IgA1 antibodies) [71], has been reported. This GalNAc residue acts as a substrate for subsequent elongation with β1,3-galactose by overexpressing β1,3-galactosyltransferase (C1GalT1) and expression of C1GalT1 and genes for the human sialylation pathway enabled the synthesis of sialylated O-glycans [6, 72].

7 Introducing helminth glycosylation into plants

Parasitic helminths secrete immunomodulatory with certain N-glycan epitopes including Lewis X and LDN-F glycan motives that find importance in treatment of allergies and autoimmune diseases. Overexpression of glycosyltransferases including FucTs, GaITs, and GalNAcTs in N. benthamiana
enabled the reconstruction of Lewis X and LDN-F motives [73] that find importance in the development of anti-helminthic vaccines.

8 Detection

Detection of low-level monosaccharides in the glycoprotein hydrolyzate are accomplished by derivatization prior to high-performance liquid chromatography (HPLC)-fluorescence and liquid chromatography (LC)-sonic spray ionization (SSI)-mass spectrometry (MS) analyses. LC-SSI-MS has been employed to identify the compositional monosaccharides including glucosamine, glucose, mannose, arabinose, xylose, and sialic acid found in the transgenic corn [74].

Fig. 8 (A) Schematic representation of plant-type O-glycosylation. Proline residues adjacent to O-glycosylation sites are converted to hydroxyproline (Hyp) by prolyl-4-hydroxylases (P4Hs). Hyp residues are further elongated (e.g., by arabinosyltransferases—AraTs). (B) Mucin-type O-glycan biosynthesis pathway in mammals. Polypeptide GalNAc-transferases (GalNAc-Ts), β1,3-galactosyltransferases 1 (C1GalT1), Cosmc (chaperone), sialyltransferases (ST6GalNAcIII/IV, ST3GalI). (C) Mucin-type O-glycan-engineering in plants. Strategies involve the knockout of P4Hs to prevent Hyp formation and expression of mammalian GalNAc-Ts, Drosophila melanogasterc1galt1, and STs. (Reproduced with permission from Schoberer J Strasser R, Plant glyco-biotechnology. Semin Cell Dev Biol. 2018 80:133-141.)
9 Discussion

In plant biomanufacturing of human proteins of importance, glycosylation, is one of the most addressed PTMs, as it affects protein homogeneity and functionality. Different engineering expression systems have been designed to control glycosylation and generate engineered N- and O-linked glycans with targeted sugar profiles and their various applications in the generation of human therapeutics [1–78].

Despite advances in the study of N-glycosylation pathways in plants, the study is far from complete and not completely known as compared to the human N-glycosylation pathway. The N-glycosylation pathway is not completely known for model plant organism A. thaliana and other different plant species. Although intra-Golgi glycosyltransferases are reported in A. thaliana, their functions remain unknown [79] but is assumed to play a vital role in synthesis of O-glycosylated proteins like arabinogalactan proteins. Studies from A. thaliana and rice have indicated that N-glycans enable growth under stress. However, complete genome sequencing of different plants will enable better understanding of the N-glycan pathway in plants and their efficient modifications and research in this exciting field of biology with human applications in the generation of therapeutics compatible to the human body is increasing across the globe.

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