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REVIEW

Plant Factories for the Production of Monoclonal Antibodies

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Abstract—Like animal cells, plant cells bear mechanisms for protein synthesis and posttranslational modification (glycosylation and phosphorylation) that allow them to be seriously considered as factories for therapeutic proteins, including antibodies, with the development of biotechnology. The plant platform for monoclonal antibody production is an attractive approach due to its flexibility, speed, scalability, low cost of production, and lack of contamination risk from animal-derived pathogens. Contemporary production approaches for therapeutic proteins rely on transgenic plants that are obtained via the stable transformation of plant cells as well as the transient (temporary) expression of foreign proteins. In this review, we discuss present-day approaches for monoclonal antibody production in plants (MAPP), features of carbohydrate composition, and methods for the humanization of the MAPP carbohydrate profile. MAPPs that have successfully passed preclinical studies and may be promising for use in clinical practice are presented here. Perspectives on using MAPPs are determined by analyzing their economic benefits and production rates, which are especially important in personalized cancer therapy as well as in cases of bioterrorism and pandemics.

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In recent years, immunotherapy has had great success in treating diseases that were previously considered incurable, owing to the development of efficient therapeutic monoclonal antibodies (TMA). More than 40 full-size TMAs are now widely used to treat various diseases [1]. Nonetheless, the production of TMAs in mammalian cell systems bears potential safety risks related to the end products, due to the contamination of TMAs with cell metabolism products, mammalian viruses, or nucleic acids with oncogenic activity. Plants are an ancient source of medicines for humans, and they have come under serious consideration as factories for producing therapeutic proteins, including antibodies, only after the development of biotechnologies [2, 3]. Plant cells possess similar mechanisms to those of animal cells for protein expression and posttranslational modification, including glyco-

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALG, asparagine-linked glycosylation; Asn297, asparagine residue at position 297 in IgG heavy chain; BeYDV, bean yellow dwarf virus; C_H, constant region of immunoglobulin heavy chain; C_L, constant region of immunoglobulin light chain; CMP-Neu5Ac, CMP-acetylneuraminic acid; CPMV, cowpea mosaic virus; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; EV, Ebola virus; Fab, fragment antigen binding; Fc, fragment crystallizable; Fuc, fucose; FUT11,12, α1,3-fucosyl transferase 11 and 12; FUT13, α1,4-fucosyl transferase 13; Fv, fragment variable; GalT, β1,4-galactosyltransferase; GALT1, β1,3-galactosyltransferase; Glc, glucose; GlcNAc, N-acetylglucosamine; GNT I, α1,3-mannosyl-glycoprotein 2β-N-acetylglucosamine transferase; GNT II, α1,6-mannosyl-glycoprotein 2β-N-acetylglucosamine transferase; GNT III, β1,4-*N*-acetylglucosamine transferase III; HIV, human immunodeficiency virus; Ig, immunoglobulin; IgG, class G immunoglobulin; JV, Junin virus; Man, mannose; MAPP, monoclonal antibodies produced in plants; MNS, mannosidase; Neu5Ac, N-acetylneuraminic acid; NHL, non-Hodgkin lymphoma; OST, oligosaccharyltransferase; PA, *Bacillus anthracis* protective antigen; P2G12, TMA 2G12 produced in tobacco plant; PVX, potato virus X; rituximab-P, plant-produced TMA rituximab; RSV, respiratory syncytial virus; scFv, single-chain variable fragment (fusion protein consisting of V_L and V_H connected with linker peptide); sIgA, secretory IgA; TMA, therapeutic monoclonal antibodies produced in animal cells; TMV, tobacco mosaic virus; trastuzumab-P, plant-produced TMA trastuzumab; V_H, variable region of Ig heavy chain; V_L, variable region of Ig light chain; WNV, West Nile virus; 2G12, TMA interacting with GP120 HIV.

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sylation. Therefore, they are suitable for producing animal and human proteins. Moreover, plant systems provide scalable antibody production, which is, importantly, safe, due to a lack of contamination with mammalian pathogens [4]. The first plant antibodies, which are occasionally called plantibodies [5], were obtained from transgenic *Nicotiana tabacum* plants that expressed IgG1 cDNA as isolated from a hybridoma [6]. Later, transgenic *N. tabacum* was used to produce hybrid secretory IgA and IgG-recognizing surface antigens, which were designed to treat dental caries in humans [7]. In addition to antibody fragments (ScFv and Fab), full-size MAPPs have also been produced using various technical solutions [8-10].

MAPP PRODUCTION SYSTEMS

Using various strategies, genes that encode animal proteins, including vaccines and antibodies, can be expressed in plant cells [11-13]. The first strategy is based on stable nuclear genomic integration, or nuclear transformation, by using one of the three following methods to introduce genetic material.

1) Indirect method with *Agrobacterium tumefaciens* based on gene transfer via Ti-plasmid [14].

2) Direct DNA delivery into the protoplast of the plant cell via osmotic or electric shock [15].

3) Direct DNA delivery into intact cells or tissues via high-velocity "bombardment" with metal microparticles (e.g., gold) covered with DNA [16].

Because antibodies are dimers composed of two monomers, each of which contains two different polypeptides (Fig. 1a), stably transformed plants can be obtained by two methods for introducing genes that encode Ig light and heavy chains [17]. The first approach is based on obtaining individual transgenic plants that are expressing light or heavy chain genes, which would then be crossed, and the offspring expressing both Ig chains are then selected. The second approach involves the subsequent or simultaneous introduction of genetic constructs encoding the two Ig chains. Notably, this was the approach that first allowed for the production of full-sized monoclonal antibodies [6], when cDNA molecules produced from murine hybridoma mRNA were used for the transformation of tobacco leaf segments and the subsequent regeneration of mature tobacco plants. IgG single γ- or κ-chain-expressing plants were hybridized, producing offspring that simultaneously synthesized both Ig chains, and the amount of functional antibodies reached up to 1.3% of the total leaf protein. The second approach, which employed the simultaneous introduction of genetic constructs encoding both Ig chains, was used for the production of MAK33 antibody in *Arabidopsis thaliana* [18]. The process of creating fertile transgenic plants is time consuming (~1 year), but it provides an advantage in that the offspring with the desired combination of immunoglobulin chains, e.g., IgA, can be obtained via cross-pollination [17, 19].

Seeds as well as leaves of transgenic plants are also attractive objects for the accumulation of antibodies, where they remain stable and functional for several years, even if maintained at room temperature [20]. Rice (*Oryza sativa*) that expresses the anti-HIV antibody P2G12 (the first letter stands for plant) in seed endosperm is an example of a successfully implemented transgenic approach [21]. In this study, zygotic embryos of rice were bombarded with gold particles covered with DNA encoding heavy and light chains from the 2G12 antibody under control of *glutelin-1* transcription promoter, with the subsequent selection of plants that were synthesizing full-sized P2G12 antibody.

Another strategy for antibody production is the transformation of the chloroplast genome and production of transplastomic plants, primarily microalgae [22, 23]. Owing to the safety of cultivation and the ability to control growth in closed photobioreactors, the single-cell eukaryotic algae *Chlamydomonas reinhardtii* is a good candidate. By using chloroplast transformation, stable microalgae lineages for antibody production can be rapidly obtained [24]. However, upon choosing this technology, the features of antibody posttranslational modifications that take place inside chloroplasts should be considered.

The third strategy that is now the most common one is based on the short-term (transient) expression of genes without inserting them into the host plant genome [9, 10]. Generally speaking, foreign DNA, e.g., from binary vectors (see below), enters plant cells, but only a small amount might be integrated into the host chromosomes. The remaining molecules, which are similar to episomal DNA, may stay transcriptionally active for several days. This short-term expression does not depend on chromosomal integration and position effects. For transient production of antibodies, the Australian tobacco plant *Nicotiana benthamiana* is used most frequently [25]. There are several different approaches to transient antibody expression, but the most efficient methods are based on agroinfiltration, in which a suspension of *A. tumefaciens* is placed on a leaf surface in the presence of a surfactant or introduced into the leaf intercellular space using a needleless syringe or vacuum infiltration. Agrobacterial transfer of genes from bacteria into plant cells, including those encoding the light and heavy chains of the antibody, is mediated by a special vector. This so-called binary vector functions in both *E. coli* and *A. tumefaciens*. For instance, a vector based on Bin 19 [26] consists of the following two parts: (i) a segment of agrobacterial T-DNA, multiple cloning sites, a marker gene for transformed plant cell selection, a reporter gene, and a foreign gene under the control of the transcriptional promoter for the expression of the antibody chain inside the

plant cell, and (ii) the remaining vector DNA to serve the replicative function of plasmids in two bacterial systems as well as selective marker genes for expression in bacteria.

Synthesis of antibody chains may be achieved in plant cells using non-amplifying vectors under the control of the transcription promoter, e.g., the 35S promoter from cauliflower mosaic virus (CaMV) [27] (Fig. 1b). However, vectors based on plant RNA viruses genomes, primarily from tobacco mosaic virus (TMV) and potato virus X (PVX), have become most common [9, 10] (Fig. 1c). Compared to stably transformed plants with target protein yields ranging from 1 to 40 μg/g leaf fresh weight, a transient expression system based on viral vectors reaches up to 300-500 μg/g leaf fresh weight [28].

To synthesize a multi-subunit protein, it is important to analyze the ratio at which its components are synthesized in plants. In the case of antibodies, an equimolar light-to-heavy chain ratio is required. If competition of templates for cell resources were avoided, then the synthesis ratio would primarily determine success in which both chains would be simultaneously synthesized in the same cell. While the synthesis of non-replicating templates is directed by the transcriptional promoter, e.g., the CaMV 35S promoter, competition between templates for ribosomes and translation factors may be neglected due to their excess in the cell. The yields of antibodies in plants that are agro-inoculated with vectors containing the CaMV 35S promoter depends on the stability of mRNA in the cell. The inhibition of silencing increases the yield of antibodies by many times. In particular, in the presence of a silencing inhibitor from P19 tomato bushy stunt virus, it is possible to obtain trastuzumab-P in an amount comparable to that of viral vectors [29, 30].

In contrast to non-replicating vectors, RNA virus genome-containing vectors exhibit the ability to compete with each other for "viral factories" formation sites, which eventually result in the displacement of one vector by another during replication and translation [28]. Therefore, while using vectors that contain a genome from only one virus, e.g., TMV, it is impossible to obtain an equimolar accumulation of Ig light and heavy chains in a plant cell. Thus, a pair of viruses exerting no competition in a natural setting would be preferred. As an example, TMV and PVX might be used together. The vectors based on such viruses do not compete with each other during the production of antibodies, but also reciprocally stimulate reproduction of each other [28].

By attempting to solve the issue with competition of viral vector, Hamorsky et al. [31] proposed a technique in which MAPP VRC01, intended for treatment and prevention of AIDS and binding to a CD4 site, was synthesized in leaf cells of *N. benthamiana* that were agro-inoculated with a single vector based on crucifer-infecting tobamovirus [32]. This vector encoded MAPP VRC01 as a single polyprotein precursor containing the fused sequences of Ig light and heavy chains as well as a recognition site for a kex2p-like protease [33] (Fig. 1d). During the maturation process, a single polyprotein was cleaved by the endogenous kex2p-like protease of *N. benthamiana* generating mature Ig light and heavy chains. It was demonstrated that this approach might give rise to accumulation of MAPP peaking as early as 5-7 days (150 mg/kg leaf fresh mass) after the leaves were inoculated with this vector.

Lomonossoff et al. [34] successfully combined two systems that were based on modified RNA-2 from cowpea mosaic virus (CPMV) for production of P2G12, which is another MAPP used to prevent AIDS, as follows: (i) non-amplifying vectors under the control of the CaMV 35S promoter and (ii) an amplifying system based on a deleted version of RNA-2 combined with a component based on CPMV RNA-1. This system yielded MAPP up to 100 mg/kg leaf fresh weight.

Vectors based on genomes of DNA-containing viruses can also be used for MAPP production. In particular, using BeYDV (bean yellow dwarf virus), Arntzen et al. [35, 36] created a vector controlling synthesis of both Ig chains against Ebola virus GP1 glycoprotein from a single DNA molecule (Fig. 1e).

Plant viruses, which are not pathogenic to humans, are also considered as nanoparticle carriers for TMAs that are routinely obtained in animal cells. Conjugate PVX–trastuzumab that was obtained using the chemical reagents 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/sulfo-N-hydroxysuccinimide markedly upregulated apoptosis in a HER2-positive cancer cell culture targeted by trastuzumab (SKOV-3 and SK-BR-3) compared to non-conjugated trastuzumab [37].

The stability of MAPP in plants, and its resistance to plant proteases, is an important factor determining yield and integrity of antibodies. Recently, a method for correcting the amino acid sequence in IgG was proposed to remove potential cleavage sites that are recognized by plant proteases [38]. In murine IgG that was produced in tobacco plants, cleavage sites were identified and were then removed using site-specific mutagenesis. This genetic engineering approach significantly decreased IgG fragmentation and increased its production yield in tobacco plants.

BRIEF ESSAY OF *N*- AND *O*-GLYCOSYLATION OF PROTEINS IN HIGHER PLANTS

As in all eukaryotes, glycosylation is one of the most important posttranslational modifications occurring in the secretory and membrane proteins in plants. To better understand the principles of MAPP humanization, it is necessary to briefly refer to protein *N*- and *O*-glycosylation occurring in higher plants. Plant glycoproteins belong to two main types: *N*-glycoproteins having carbohydrate moiety attached to asparagine (Asn), and *O*-gly-

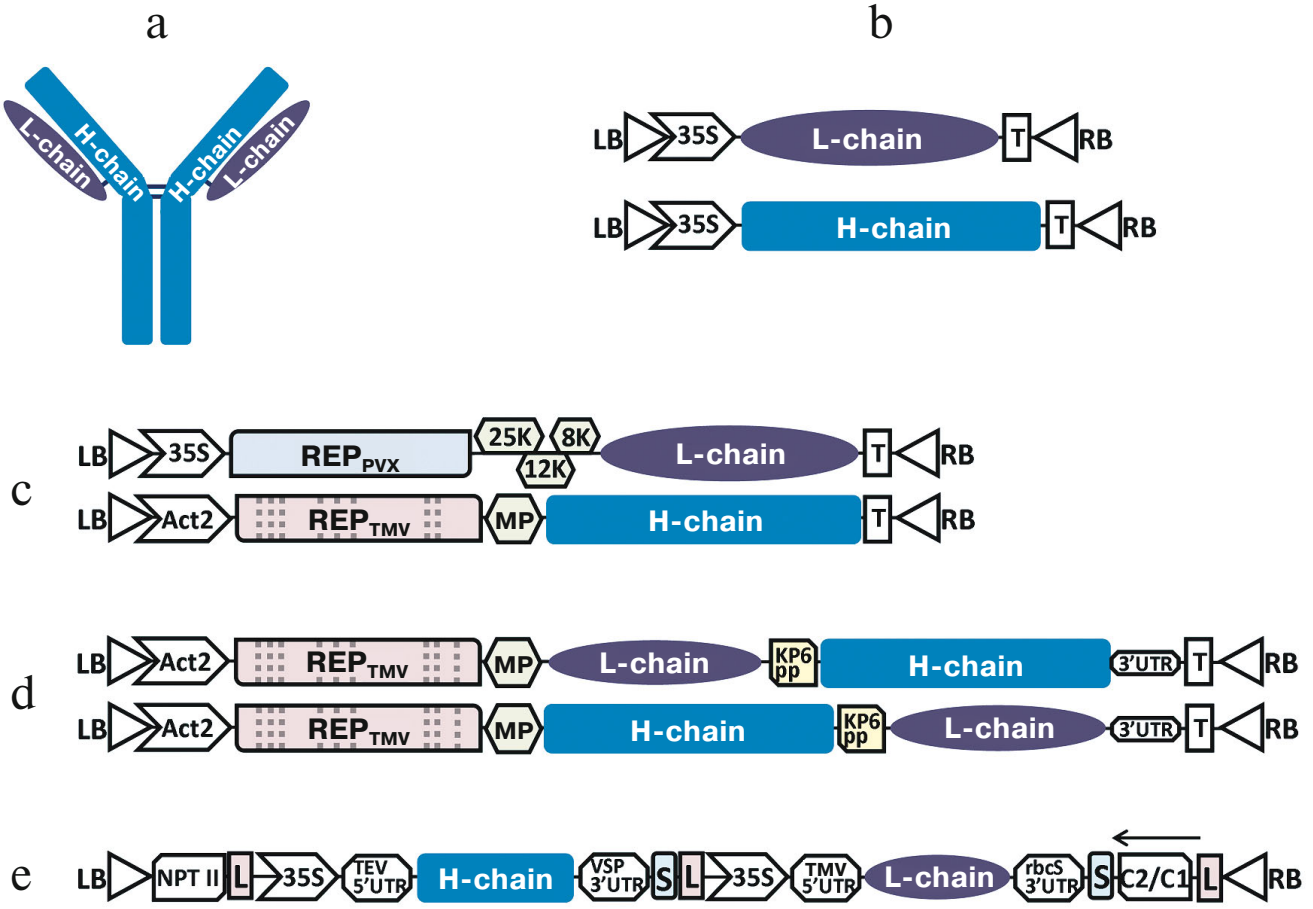

Fig. 1. Vector constructs used for IgG production in plants. a) A scheme depicting the IgG as two identical light chains (L-chains) and two identical heavy chains (H-chains). b) Vector constructs under control of the 35S promoter from cauliflower mosaic virus (CaMV) are schematically depicted. Heavy and light chain genes are located in two different vectors. c) Vector constructs based on tobacco mosaic virus (TMV) and potato virus X (PVX) genomes are schematically depicted. REP_{PVX}, PVX replicase gene; 25 K, 12 K, and 8 K genes of PVX triple gene block; Act2, *actin 2* gene promoter from *Arabidopsis thaliana*; REP_{TMV}, TMV replicase gene, dotted lines denote introns; and MP, TMV movement protein gene. d) Two vector constructs that control the synthesis of heavy and light chain genes from VRC01 MAPP are schematically depicted. KP6pp, a sequence encoding a recognition site for kex2p-like protease; and 3′-UTR, 3′-untranslated region. e) A vector construct based on DNA-containing bean yellow dwarf virus (BeYDV) genome is schematically depicted. NPT II, neomycin phosphotransferase II gene conferring resistance to kanamycin; L, long intergenic region (LIR) from the BeYDV genome; TEV 5′-UTR, 5′-untranslated region from the tobacco etch virus (TEV) genome; VSP 3′-UTR, 3′-untranslated region from soybean *vspB* gene; S, short intergenic region (SIR) from the BeYDV genome; TMV 5′-UTR, 5′-untranslated region of TMV; rbcS 3′-UTR, 3′-untranslated region from pea *rbcS* gene; C2/C1, sequences encoding replication initiation proteins Rep and RepA from the BeYDV genome; the arrow is the transcription direction from the L-promoter of genes *C1/C2*; T, transcription terminator; and RB and LB, right and left borders of Ti-DNA.

coproteins in which the oligosaccharide is bound to the oxygen atom in serine (Ser) or threonine (Thr). Ser and Thr residues may be modified by attaching various carbohydrate moieties, including fructose, glucose, mannose, and GlcNAc that occur inside the endoplasmic reticulum (ER) [39]; plant cell wall *O*-glycoproteins (arabinogalactan proteins) containing galactose, arabinose, fucose, rhamnose, and glucuronic acid [40] as well as *O*-glycosylation by GlcNAc moieties of cytosolic or nuclear proteins playing an important role in cell signaling have been relatively well described [41].

Structurally, *N*-glycans are divided into three categories: high-mannose, hybrid, and complex types. In contrast to mammals, plants produce less complex and varied oligosaccharides because they do not synthesize branched and sialylated *N*-glycans. High-mannose *N*-glycans in animals and plants contain five to ten mannose residues, whereas complex *N*-glycans in plants structurally differ from those of mammals (Fig. 2) [42-44]: (i) β1,2-xylose, that is never found in mammalian *N*-glycans, is linked to β-mannose from the plant glycan core; (ii) α1,3-fucose of proximal core GlcNAc is found instead of mammalian α1,6-fucose; (iii) β1,3-galactose and α1,4-fucose linked with terminal GlcNAc in plant *N*-glycans form a Lewis a (Le^a) oligosaccharide structure, whereas in mammals, β1,4-galactose is frequently combined with

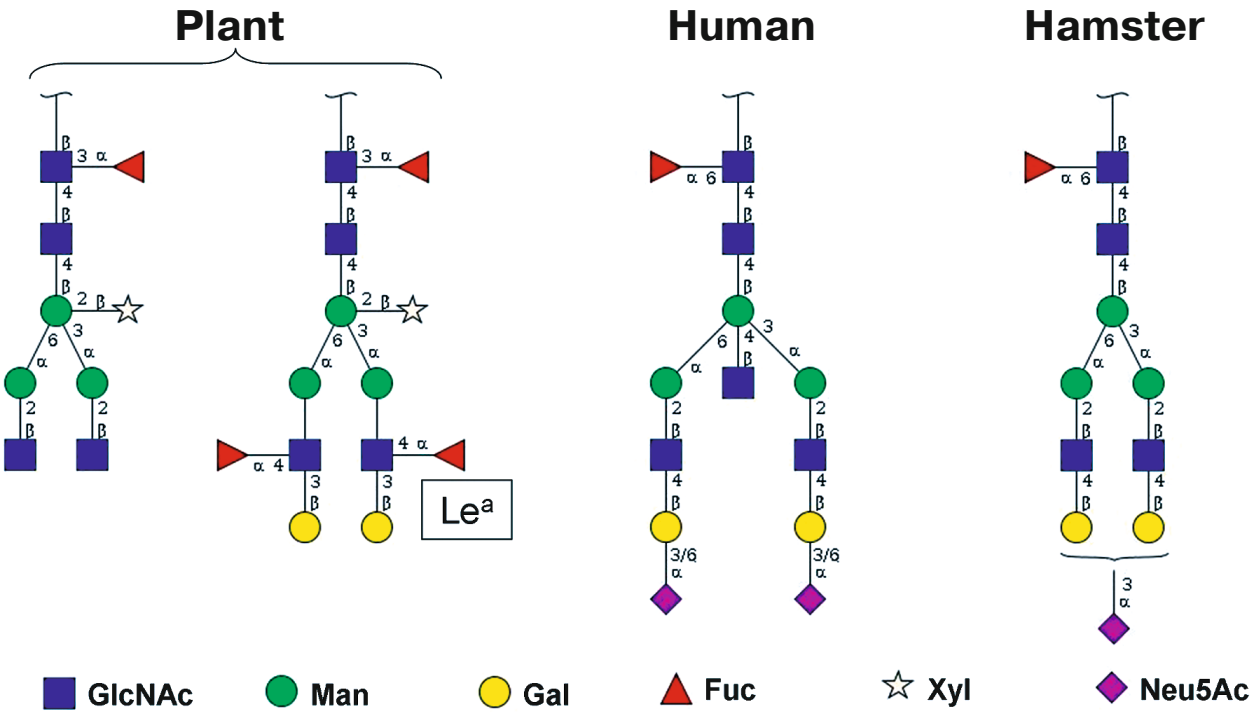

Fig. 2. Differences in complex *N*-glycans between plants and mammals. Typically, plants contain glycans with xylose attached to a mannose residue of the glycan core as well as α1,3-fucose on the core GlcNAc residue instead of α1,6-fucose, which is found in mammalian glycans. Moreover, a Lewis a (Le^a) structure may also be produced in plant cells. It contains β1,3-galactose and α1,4-fucose attached to the terminal GlcNAc residue. In mammals, this GlcNAc is often decorated with β1,4-galactose linked to the sialic acid.

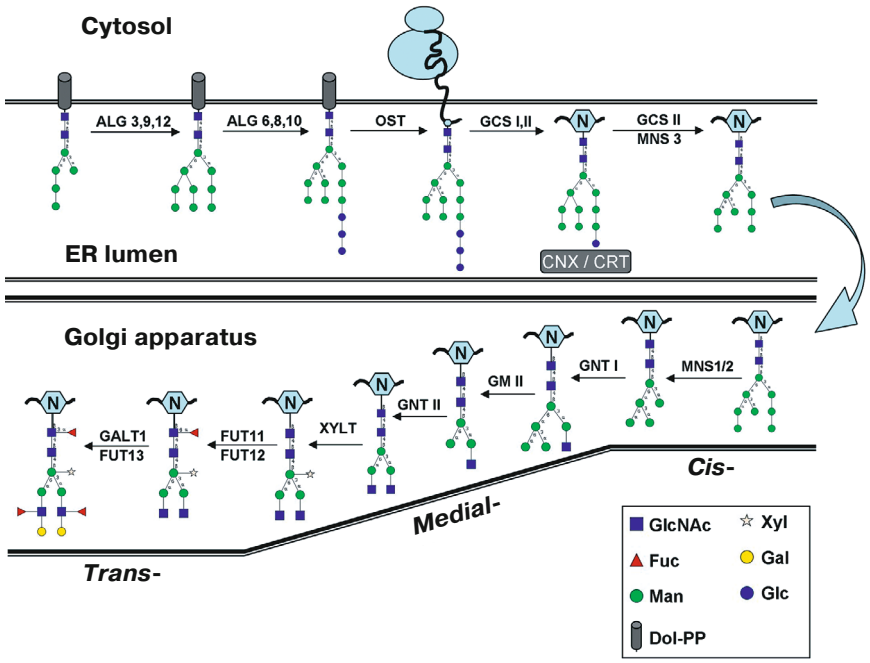

Fig. 3. Major steps in the formation of *N*-linked glycans in plants. Protein *N*-glycosylation begins inside the ER, where ALG-family glycosyltransferases are involved in the assembly of the "glycan-precursor" ($Glc_3Man_9GlcNAc_2$) on dolichol-phosphate. After that, oligosaccharide is transported from the Dol-PP by oligosaccharyltransferase (OST) onto an asparagine residue in the nascent target protein. Then, ER-glucosidases (GCS I, II) cleave the Glc residues, resulting in $Glc_1Man_9GlcNAc_2$. At this stage, proper protein folding is mediated by the chaperones calnexin and calreticulin (CNX/CRT) followed by the further modification of the attached glycan inside the ER: the cleavage of Man residues by mannosidases (MNS1/2), subsequent attaching of GlcNAc mediated by GlcNAc-transferases I and II (GNT I, II), Xyl by β1,2-xylosyltransferase (XYLT), and Fuc by α1,3-fucosyltransferases 11 and 12 (FUT11/12). An Le^a structure can be created in the *trans*-Golgi apparatus by β1,3-galactosyltransferase (GALT1) and α1,4-fucosyl transferase (FUT13).

sialic acid [45-47]. These features of plant glycans originate from their biogenesis.

Initial steps of *N*-glycosylation are highly conserved between plants, mammals, and yeasts. As in mammals, polysaccharide (glycan) biogenesis in plants occurs in the ER and Golgi apparatus, which includes the activity of numerous transmembrane enzymes such as glycosyltransferases and glycosidases [48]. The process proceeds with dolichol and synthesis of a dolichol-glycan precursor consisting of dolichol phosphate (Dol-P) bound to preassembled oligosaccharide containing 14 monosaccharides ($Glc_3Man_9GlcNAc_2$), and it is conserved in all eukaryotes [43]. *N*-glycosylation is initiated inside the ER with the transmembrane protein complex oligosaccharyltransferase (OST) and the transfer of preassembled glycan ($Glc_3Man_9GlcNAc_2$) onto an asparagine residue (located in an amino acid sequence containing an Asn-X-Ser/Thr motif, where X is any amino acid except proline) in the nascent polypeptide chain. This step is vital for plants. In particular, the absence of the catalytic subunit of OST results in death of *A. thaliana* plants [49]. Then, after the precursor is attached to the Asn residue in the ER lumen, a series of trimming reactions and structural arrangements that are common for all eukaryotic cells take place (Fig. 3). All genes homologous to yeast genes controlling *N*-glycosylation were found in the plant genome [50]. Numerous *A. thaliana* mutants have been created that are deficient in synthesis of glycosyltransferases involved in asparagine-linked glycosylation (ALG) (Fig. 3). Defects in genes encoding ALG3 [51], ALG12 [52], and ALG9 [53] have no significant impact on growth and development of plants. However, a mutation in ALG11 results in an inhibition of the synthesis of normal high-mannose glycans and an increased amount of complex glycans [54]. Consequently, it affects the development of plants, decreases their fertility, and impairs the synthesis of the cell wall. ALG10 deficiency lowers the efficacy of glycosylation and impairs the growth and development of plant leaves [55].

Initial steps of the maturation process consist of the sequential removal of three Glc residues by glucosidases I and II, resulting in formation of $Glc_2Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$, respectively. By recognizing proteins bearing $Glc_1Man_9GlcNAc_2$, chaperones calnexin and calreticulin assist in proper protein folding. In the next step, glucose and the terminal α1,2-mannose in one of the glycan branches (Fig. 3) are removed by α-glucosidase II and α-mannosidase 3 (MNS3), respectively, resulting in glycan $Man_8GlcNAc_2$, which is transported towards the Golgi apparatus to be modified by glycosyltransferases and glycoside hydrolases for further maturation of the *N*-glycans.

In plants, mannose trimming is mediated by α-mannosidases (MNS1-MNS3) that are located within the ER and *cis*-Golgi apparatus [56]. Recent data suggest that MNS3 and other α-mannosidases are required for trimming of *N*-glycans involved in biosynthesis of cellulose and the cell wall [43]. MNS3-deficient *A. thaliana* plants produce high amounts of immature *N*-glycans and very few complex mature *N*-glycans. The majority of these *N*-glycans contain terminal α1,2-mannose on one branch, thereby showing the participation of MNS3 in the trimming process [56].

Maturation and diversification of human $Man_5GlcNAc_2$ begin in the medial Golgi apparatus by adding β1,2-GlcNAc, which is mediated by GlcNAc-transferase I (GNT I). As in animals, this step is critical for further maturation of plant *N*-glycans. Mutant *A. thaliana cgl1* that is deficient in GNT I is unable to produce complex glycans, and, consequently, loses its ability for normal growth [55]. Similarly, rice plants (*O. sativa*) with mutant *gnt1* exhibit severe defects in organ development accompanied by impaired reproduction [57]. Plant GNT I uses the oligosaccharide $Man_5GlcNAc_2$ as a substrate acceptor to link one GlcNAc residue to the exposed α1,3-mannose residue (Fig. 3).

The next step in *N*-glycan maturation that occurs in the medial Golgi apparatus involves removal of α1,3- and α1,6-mannose moieties by Golgi-α-mannosidase II (GM II), which is required for formation of complex *N*-glycans. The final hybrid *N*-glycan $GlcNAc_1Man_3GlcNAc_2$ is a specific substrate for β1,2-*N*-acetylglucosaminyltransferase II (GNT II), which catalyzes transfer of another GlcNAc moiety onto α1,6-mannose, thereby highlighting the transition from hybrid to complex *N*-glycan [43, 58]. Then, in the medial Golgi apparatus, the glycan $GlcNAc_2Man_3GlcNAc_2$ may be transformed by β1,2-xylosyltransferase (XYLT) and the two core α1,3-fucosyltransferases 11 and 12 (FUT11 and FUT12) that fucosylate a core region of biantennary glycan. This glycan consists of the branched glycan heptasaccharide and two disaccharide moieties (antennae). The transferases XYLT and FUT11/FUT12 compete with each other for the same glycan substrates. The *FUT11* and *FUT12* genes found in *A. thaliana* seem to result from duplication, given that (i) they duplicate each other during fucosylation of the core region of glycans [59] and (ii) in monocots such as rice, a similar reaction is performed by the single enzyme α1,3-fucosyltransferase [60]. *Arabidopsis thaliana* plants with *xylt*, *fut11*, and *fut12* gene knockouts do not have an altered phenotype, which suggests that β1,2-Xyl and α1,3-Fuc moieties are not essential for plant development [43, 61].

The final steps of *N*-glycan modifications occur in the *trans*-Golgi apparatus and are mediated by β1,3-galactosyltransferase 1 (GALT1) and α1,4-fucosyltransferase 13 (FUT13), which thus create an oligosaccharide Lewis a structure [Fucα1-4(Galβ1-3)GlcNAc-R] bearing allergenic properties [43, 44]. In the first step, GALT1 transfers a Gal moiety via the β1,3-bond onto the terminal GlcNAc residue, which results in the synthesis of a Galβ1-3GlcNAc structure. In the second step, FUT13

transfers Fuc via the α1,4-bond onto GlcNAc, thereby completing the synthesis of the Le^a oligosaccharide. These complex *N*-glycans are absent in mammals, which may therefore cause adverse immune reactions upon their administration in humans [62]. Interestingly, mutant *A. thaliana* plants that are deficient in the enzymes GALT1 and FUT13 lack the Le^a oligosaccharide, which does not affect plant growth and development [63].

FEATURES OF MAPP CARBOHYDRATE PROFILE

Human blood serum polyclonal IgG mainly contains complex *N*-glycans. Fc- and Fab- fragments significantly differ from each other in terms of the amount of specific residues within *N*-glycans, although they have a common biantennary structure. In healthy people, no complex tri- and tetra-antennary multi-antigen glycans were found in IgG. An *N*-linked glycan in the C_H2 domain that was attached at Asn297 (according to the nomenclature based on enumeration of amino acids in IgG heavy γ-chain) influences antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC) [64], complement-dependent cytotoxicity [65], complement-dependent phagocytosis [66], and antiinflammatory activity [67]. As in human and animal cell cultures, posttranslational modifications may also be performed in plants, including (i) formation of disulfide bonds for antibody assembly and (ii) posttranslational *N*-glycosylation, which is essentially similar to the process occurring in animals [43]. The MAPP Asn297-linked glycan is mainly presented by a complex GnGnXF type glycan, which is found along with a small amount of oligomannose MMXF-type glycans [68] (according to the glycan nomenclature at http://www.proglycan.com/protein-glycosylation-analysis/nomenclature). The core region of GnGnXF-type and MMXF-type Asn297-linked glycan contains α1,3-fucose and β1,2-xylose, which, together with a lack of terminal galactose and sialic acid [68], distinguishes MAPP from human IgG (Fig. 2). By comparing MAPP with TMA, a carbohydrate profile with high homogeneity is observed in MAPP, which is required by regulatory organs. Indeed, if there are only two main types of MAPP (GnGnXF and MMXF), then the TMA preparation obtained in CHO cell culture may contain from five to seven structures of Asn297-linked glycan [69, 70]. Using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), the preparation of an anti-HIV 2G12 antibody obtained in CHO cell culture was found to contain a mixture of structures including glycans (i) bearing no galactose residues (GnGnD) and (ii) containing one (AGnF, GnAF) or (iii) two (AAP) terminal galactose residues as well as minor amounts of glycopeptides with terminal sialic acid (NAAF, NaNaF). Moreover, all the observed glycoforms contained a fucose moiety bound via α1,6-link to the GlcNAc that was adjacent to Asn297 [71].

MAPP Asn297-linked glycan is a specific plant glyco-epitope that is lacking in mammals, although antibodies against it may be found in the blood serum in approximately 50% of people [72]. An assessment of the immunogenicity of MAPP Asn297-linked glycan in animals yielded controversial results. The first approach involved the immunization of mice with IgG that was obtained in transgenic tobacco plants, which did not result in development of antibodies directed either against proteinaceous or glycan portions of the recombinant IgG [73]. Another study on rabbits that were immunized with MAPP preparation P2G12 demonstrated that they developed antibodies against xylose- and fucose-containing Asn297-linked glycan. Moreover, when using Western blots, IgE from patients suffering from allergies were able to react with P2G12 [74].

Recently, the biosafety of immunogenic MAPPs for humans was assessed by using a plant-produced idiotype vaccine conjugated with hemocyanin for the targeted treatment of follicular B-cell lymphoma [75]. A personalized vaccine was produced in leaves of *N. benthamiana* plants that were agroinjected with a construct encoding Fab regions of heavy and light chains isolated from the patient's tumor, fused with the constant domains of normal human IgG1. All recombinant anti-idiotype antigens used in this study were glycoproteins containing oligomannose MMXF type glycans and complex GnGnXF type glycans. This personalized idiotype vaccine induced production of idiotype antibodies, which suppressed tumor growth in 9 out of 11 patients. At the same time, no serious side effects related to vaccination, i.e., the administration of antibodies with MAPP-specific glycosylation, were documented [75]. Nonetheless, despite the safety of plant-produced antibodies demonstrated in these studies, there are still some concerns that certain plant glycans may be immunogenic for humans [76], which in turn necessitates MAPP "humanization".

HUMANIZATION OF THE MAPP CARBOHYDRATE PROFILE

By examining the role of carbohydrate moieties in functioning of human IgG and TMA obtained from animal cell cultures, the biological activity of antibodies was shown to depend on certain carbohydrate moieties contained in the *N*-glycan [1]. For instance, fucose attached via an α1,6-bond to core *N*-glycan lowers the binding efficacy of the Fc-fragment to the Fcγ receptor IIIA (FcγRIIIA) and, subsequently, ADCC activation. Defucosylation of TMA Asn297-linked glycan greatly increased the degree of its binding to FcγRIIIA, thereby enhancing ADCC effector function [77, 78].

In MAPPs, α1,3-fucose attached to the core of Asn297-linked glycan may also reduce the efficacy of interactions between the Fc-fragment and FcγRIIIA. By

removing the fucose residues from the constant region of antibodies, MAPPs are made more efficient at stimulating ADCC [3, 79].

It has long been recognized that methods and conditions for producing MAPPs with carbohydrate profile most closely resembling human IgG should be developed. Humanization of plant producers is aimed at: (i) eliminating MAPP immunogenicity, (ii) introducing human genes into plants as well as the ability to synthesize MAPPs with carbohydrate profile similar to that of human IgG, and (iii) inhibiting β1,2-xylosylation and α1,3-fucosylation of the core portion of the Asn297-attached glycan. Two methods for MAPP humanization have been proposed: (i) subcellular ER targeting using signal sequence (HDEL/KDEL), and (ii) glyco-engineering eliminating genes responsible for undesired glycosylation and/or inserting human genes providing synthesis of humanized *N*-glycan using gene engineering.

Historically, subcellular targeting was proposed earlier, suggesting that the signal sequence HDEL/KDEL allowed antibodies to stay inside the ER, thereby abandoning the attachment of β1,2-xylose and α1,3-fucose to the core region of the Asn297-linked glycan. This approach allowed for the synthesis of several KDEL-containing MAPPs in transgenic tobacco plants. Among them was cPIPP, a hybrid (mouse/human) IgG1 that binds to the β-subunit of human chorionic gonadotropin and contains a KDEL-sequence in the C-terminal portion in both antibody chains [80]. cPIPP was found to contain only *N*-glycans of a high-mannose type that is typical of glycoproteins that did not undergo maturation and trimming in the Golgi apparatus. On the contrary, cPIPP lacking a KDEL-sequence contained complex glycans consisting of core α1,3-fucose and β1,2-xylose. A similar study was also performed with KDEL-containing MAPP against the surface antigen of hepatitis B virus (HBsAg) [81]. Although the KDEL sequence was located in the C-terminal portion of both chains, nonetheless approximately 10-20% of the antibodies contained complex glycans. The discrepancy in the study results were explained by the conformational features of the Fc-fragment and the availability of the KDEL sequence [82].

Another approach, *N*-glyco-engineering of plants-producers, has been developed recently, and it has brought greater success in generation of glyco-modified MAPPs [76, 83]. This success was facilitated by the fact that plants are generally characterized by their tolerance to changes in the protein glycosylation profile. In particular, the elimination of complex *N*-glycans suppressing β1,2-xylosylation and α1,3-fucosylation in the core portion of Asn297-linked glycan usually does not result in significant effects on the growth and development of *A. thaliana* [43, 61] and *N. benthamiana* [69, 84] plants. This common tolerance to *N*-glyco-engineering is a requirement for the humanization of biosynthetic pathways for *N*-glycosylation in plants.

Elimination of plant glycans in MAPPs. This approach is aimed primarily at eliminating β1,2-xylosylation and α1,3-fucosylation from the core portion of MAPP Asn297-linked glycan, i.e., processes lacking in humans. The feasibility of this strategy was confirmed in *A. thaliana* plants, when the genes encoding XYLT and FUT were disrupted by inserting agrobacterial T-DNA [59]. The study first required the construction of a *xylt A. thaliana* plant through the knockout of the single gene *XYLT*. Next, *futa* and *futb* plants were sequentially generated, and then they were hybridized and selected for *fuct* offspring, with both genes being knocked out. In the final step, the plants were hybridized to obtain *xylt/fut* offspring with suppressed XYLT and FUT synthesis. This knockout plant was not affected in terms of viability, but it abandoned the synthesis of potentially immunogenic *N*-glycan. Instead, structures with the two terminal GlcNAc moieties ($GlcNAc_2Man_3GlcNAc_2$) were mainly synthesized.

Later, this glyco-modification technology was applied to other plant species including *N. benthamiana*, the main MAPP-producing plant. A gene knockout performed by RNA interference (RNAi) provided a double mutant *N. benthamiana ΔXTFT* [71]. By examining the glycoforms in P2G12 antibody, Asn297-linked glycan was demonstrated to lack xylose and α1,3-fucose. Plant-produced P2G12 antibodies do not differ from the 2G12 obtained from CHO cell culture in terms of antigen binding and HIV neutralization [71], but they display a superior ability to stimulate ADCC [85].

The *N. benthamiana ΔXTFT* plant turned out to be a good tool for humanization of MAPP cetuximab, which was originally produced in murine cell line Sp2/0 [86]. The problem is that cetuximab is a chimeric antibody (mouse/human) containing *N*-glycans in both Fc- and Fab-regions. When cetuximab was produced in wild type plants, it contained xylose and α1,3-fucose in the core portion of the *N*-glycans, but its synthesis in the *ΔXTFT* plant resulted in removal of these moieties. In addition, there is an alternative approach for removing β1,2-xylose and α1,3-fucose from *N*-glycan. A double mutant *N. benthamiana* ΔGMDΔXYLT plant with knocked out GTP-D-mannose 4,6-dehydratase and β1,2-XYLT genes [87] also produced MAPPs lacking plant-specific Asn297-linked glycans.

Introduction of mammalian genes into plant cells for the synthesis of humanized *N*-glycan containing a GlcNAc moiety at the mannose fork of the core in Asn297-linked glycan. Studies on TMA showed that the emergence of GlcNAc in the mannose fork of the core in Asn297-linked glycan inhibits the further attachment of the fucose residue, which subsequently results in the increased efficacy of antibodies to induce ADCC [77]. Thus, upregulated expression of β1,4-*N*-acetylglucosaminetransferase III (GNT III) may facilitate the augmented ability of antibodies to trigger ADCC. The problem is that plants

lack GNT III activity, and, therefore, the *N*-glycans in plants contain no GlcNAc at the mannose fork of the core in *N*-glycan (Fig. 3). By introducing mammalian GNT III into plant cells, it might be possible to overcome this "flaw". Indeed, after introducing rat [88] or human [89] GNT III gene into tobacco plant cells, a MAPP with the desired structure of the *N*-glycan was produced as well as some unusual glycans. For instance, this approach resulted in the emergence of terminal GlcNAc residues, which is atypical in wild type plants [89]. The use of chimeric mammalian GNT III containing sequences that targeted it towards the *trans*-Golgi apparatus resulted mainly in production of *N*-glycans containing GlcNAc at the mannose fork [76].

To synthesize complex tri- and tetra-antennary *N*-glycans, animal GNT IV and V should be introduced into plant cells. Use of enzymes with signal sequences providing correct location within the medial Golgi apparatus provided multi-antennary *N*-glycans [84].

MAPP β1,4-galactosylation. The terminal galactose in human IgG Asn297-linked glycan (i) is involved in the Fc-fragment binding to FcγRIIIA [90] and (ii) serves as an acceptor substrate for the formation of final complex Asn297-linked glycans in the *trans*-Golgi apparatus. Plant *N*-glycans do not contain terminal galactose because they lack the enzyme β1,4-galactosyltransferase (GalT). Inserting a sequence encoding human GalT into the genome of *N. benthamiana* plants provides transgenic plants with the ability to β1,4-galactosylate the terminal residues of *N*-glycans [70]. Moreover, it was shown that bi-galactosylated anti-HIV MAPP displays an increased ability to neutralize virus.

Terminal sialylation. The terminal sialylation of Asn297-linked glycan in human IgG is a final step in *N*-glycosylation. Nonetheless, the amount of blood serum-sialylated IgG1 in healthy humans ranges from 2 to 5%. Production of TMA in animal cell cultures such as the CHO cell line, murine myeloma J558L, and human HEK293 cell line increases the amount of sialylated forms up to 31, 10, and 33%, respectively [91]. Sialylation of TMAs leads to an antiinflammatory effect and increases their stability against proteolysis in the human bloodstream. In plants, no sialylated proteins are found [92] because plant cells lack the biosynthesis of CMP-Neu5Ac and the machinery for its delivery into the Golgi apparatus as well as sialyltransferase to transfer Neu5Ac onto terminal galactose in an Asn297- linked glycan [76]. Therefore, human genes that synthesize the missing enzymes should be introduced into plant cells for the sialylation of MAPP Asn297-linked glycan. The feasibility of this approach was confirmed in *N. benthamiana* plants that were transfected with the animal and human genes responsible for the entire sialylation process, including the activation, transport, and transfer of Neu5Ac onto terminal galactose [93]. Introduction of the murine gene encoding UDP-GlcNAc-2-epimerase/*N*-acetylmannosamine kinase, the transporter for CMP-Neu5Ac, human genes coding for phosphate-Neu5Ac synthase, CMP-Neu5Ac synthase, GalT, and rat genes encoding α2,6-sialyltransferase resulted in the sialylation of up to 80% of the *N*-glycans [94].

Lowering non-glycosylated forms of MAPP. It is known that the absence of Asn297-linked glycan decreases the ability of TMA to activate ADCC. For example, non-glycosylated cetuximab is unable to bind FcγRI and FcγRIIIA and, consequently, it cannot activate ADCC [95]. Rituximab and trastuzumab produced in CHO cells usually lack or contain less than 1% non-glycosylated antibodies. However, during production of MAPP trastuzumab-P and rituximab-P, the percentage of non-glycosylated antibodies increased to 20-30% [96]. A method lowering the amount of non-glycosylated MAPP to 5-7% by inserting an amino acid substitution adjacent to the Asn297 site has been developed [96].

MAPPs WITH PROMISE FOR USE IN CLINICAL PRACTICE

Several MAPPs at various developmental stages have been proposed [23], but they have not yet become available on the pharmaceutical market. MAPPs aimed at the treatment of viral and bacterial infections as well as cancer-related diseases hold promise for clinical use.

Antiviral and antibacterial MAPPs. *Human immunodeficiency virus (HIV).* Infection caused by lentiviruses HIV-1 and HIV-2 that destroy the human immune system results in increased susceptibility to a wide range of infections, cancer, and other diseases [97]. HIV remains one of the major medical problems worldwide. It is known that the use of preparations (ointments and creams) containing anti-HIV MAPPs as microbicidal agents for topical (vaginal and anal) application decreases sexual infection [98]. Demand for microbicidal agents increases every year, and it is currently estimated as five tons per 10 million people. HIV-neutralizing MAPPs have been created using (i) stably transfected maize [99], *A. thaliana* [100], tobacco [101], and (ii) transient expression in *N. benthamiana* [31, 102]. Local application of MAPP P2G12 promises to provide a relatively cheap and safe means to protect people. The safety of this approach has been confirmed in recent studies. P2G12 that was obtained from transgenic tobacco plants caused no side effects or adverse immunological effects after vaginal application as a microbicidal agent in 11 volunteers (aged 18-50).

The broad administration of P2G12 requires an expression system that provides inexpensive MAPP production in large amounts. Rice seeds containing P2G12 may serve as a source. A thorough examination of the properties of P2G12 antibodies that accumulated in rice seed endosperm [21] demonstrated that its heavy chain is

not glycosylated. Nonetheless, the heavy and light chains that assembled into functional P2G12 antibody provided stronger HIV-neutralizing activity than in P2G12 produced in other plant systems that were characterized by typical high-mannose and complex glycans.

Nicotiana benthamiana ΔXTFT transgenic plants [71] were also used to optimize and humanize the carbohydrate profile of P2G12. A MAPP with an almost homogenous carbohydrate composition of human-type *N*-glycans containing no xylose or α1,3-fucose in the core region of Asn297-linked glycan was obtained [85].

It is natural to consider the possibility of using secretory IgA (sIgA) because the prevention and the fight against AIDS with MAPPs are focused on topically applied drugs. sIgA is a complex hetero-deca-subunit protein complex containing dimeric IgA, including four light and four heavy chains as well as a joining (J) chain and a secretory component (Fig. 4a). Recently, a stably transformed tobacco plant and transient expression system were created for sIgA production. Using a set of binary pEAQ vectors [103], four genes encoding light and heavy chain as well as a J-chain and secretory component were introduced into the leaves of *N. benthamiana* (Fig. 4b). In both systems, sIgA P2G12 was produced [104]. The final hetero-deca-subunit protein complexes were not detected in the apoplast, but they were accumulated in the intracellular compartments, including vacuoles. The highest P2G12 sIgA yield was 15 μg/g (transgenic tobacco plant) and 25 μg/g (agroinjected *N. benthamiana*) of fresh green mass. Antibodies purified by affinity chromatography were able to specifically bind HIV GP140. Analysis of the carbohydrate composition mainly detected high mannose structures. Compared to P2G12 IgG, an important advantage of P2G12 sIgA is that it is more stable in secretions of the vaginal mucosa, which is promising for its future use.

Ebola virus (EV). Ebola hemorrhagic fever is a highly contagious disease that is common in western Africa and results in a mortality rate higher than 70%. People may catch EV after having contact with infected animal tissues or inhaling their fecal aerosol. Human-to-human transmission occurs via contact with infected blood or other biological fluids. EV infection results in damage to the vascular system with subsequent hemorrhages in the skin and internal organs. Ebola fever is caused by a small RNA-containing filovirus. Contemporary treatment methods consist in generation of antibodies against a transmembrane GP glycoprotein as the main EV virulence factor, which is involved in (i) EV entrance into host cells, (ii) development of cytopathic effects, and (iii) induction of protective antibodies in infected patients. A transient expression system in *N. benthamiana ΔXTFT* plants [71] turned out to be efficient in generation of MAPP MB-003 cocktail consisting of human and chimeric (human/mouse) monoclonal antibodies c13C6, h13F6, and c6D8 [105]. EV-infected rhesus monkeys

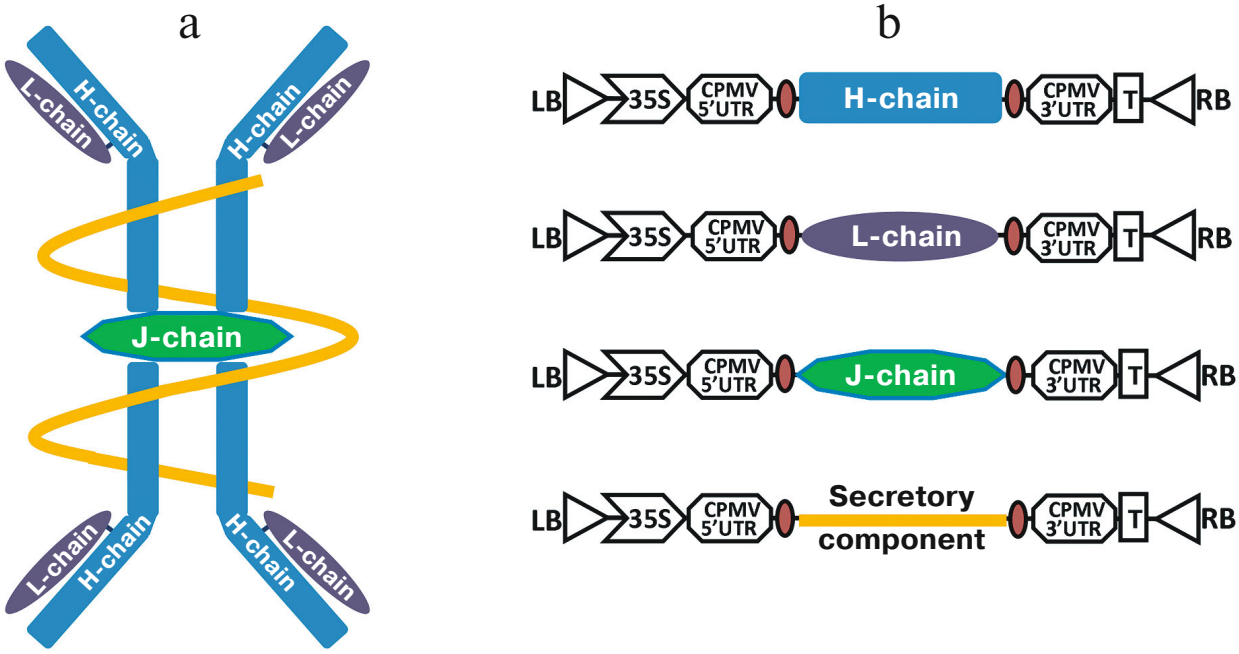

Fig. 4. Vector constructs used for sIgA production in plants are schematically depicted. a) The schematic representation of the sIgA: four identical light chains (L-chain) and four identical heavy chains (H-chain), a joining chain (J-chain), and a secretory component. b) pEAQ-family binary vectors are schematically depicted. A 35S, 35S promoter from cauliflower mosaic virus (CaMV); CPMV 5′-UTR, 5′-untranslated region from CPMV RNA-2; red ovals denote recombination sites; CPMV 3′-UTR, 3′-untranslated region from CPMV RNA-2; T, transcription terminator; and RB and LB, right and left border of Ti-DNA.

were found to be fully protected after they were given MB-003 antibodies via the intravenous route 1 h after infection with a lethal dose of EV, and the antibodies were repeatedly applied four and eight days later. The MB-003 antibody cocktail was superior to the antibodies produced in the CHO cell line because its components did not bear fucose moieties in the core portion of the Asn297-linked glycan, which stimulated ADCC [106]. Another approach based on production of ZMapp antibody cocktail (clone c13C6 from MB-003 together with humanized clones c2G4 and c4G7 from ZMAb) in *N. benthamiana* conferred protection to cynomolgus monkeys [107-109].

Regarding trials in humans, a ZMapp preparation was administered to seven patients during the 2014-2015 outbreak of Ebola hemorrhagic fever in western Africa, although its safety profile was not assessed in humans before administration. The general condition of patients was substantially improved in five patients, who then recovered, although treatment was started nine days after infection [110, 111]. Today, a systematic examination of the biosafety and therapeutic efficacy of ZMapp preparation has started after successful emergency aid to EV-infected patients (phase I/II).

Apart from that example, anti-EV MAPP can be used as a platform for the development of a vaccine immune complex [112]. Using a geminiviral replicon system, it became possible to create this complex in *N. benthamiana* plants. EV glycoprotein (GP1) was fused with the C-terminus of the heavy chain from humanized monoclonal antibody 6D8, which specifically binds to the epitope in GP1 protein. The simultaneous expression of genes encoding a fused protein "heavy chain–GP1" and light chain from 6D8 antibody resulted in formation of immune complexes that were able to stimulate the development of anti-EV antibodies in mice [112].

Junin virus (JV). Junin virus is an arenavirus that causes Argentine hemorrhagic fever. Although its spread is mainly restricted to Argentina, it has tended to extend its areal to other territories. Moreover, epidemiologists are concerned that JV might be used for bioterrorism because untreated JV-infection has a mortality rate reaching 20-30%. Treatment with immune serum for 8 days mitigates symptoms and decreases the mortality rate to 1% [113]. As early as 1989, monoclonal antibodies reacting with surface JV glycoproteins were prepared [114]. To create MAPP, genes encoding three clones of murine neutralizing monoclonal antibodies were expressed in *N. benthamiana ΔXTFT* plants [71] producing three clones of anti-JV MAPPs [115]. The *N*-glycosylation profile mainly indicated the presence of GnGn glycoforms. Administration of this MAPP to guinea pigs was used as a model to study Argentine hemorrhagic fever, and it gave rise to 100% protection after lethal infection with JV. Moreover, in terms of curative effects, anti-JV MAPP was superior to routine medicines (ribavirin, immune serum, etc.).

Human respiratory syncytial virus (RSV). RSV is a paramyxovirus that frequently causes severe lower respiratory tract infections in infants, young children, and elderly patients living in long-term care facilities. Passive immunization against RSV is efficient, which is evidenced by a widely used palivizumab that is produced in the NS0 murine cell line, a humanized TMA based on IgG1 against RSV F-protein. The high cost of this preparation motivates development of a plant production platform to decrease the cost of palivizumab by 20-fold [4]. Recently, a biosimilar palivizumab MAPP (palivizumab-P) was produced [4, 116] in *N. benthamiana ΔXTFT* plants [71]. By comparing the carbohydrate profile, it was found that palivizumab-P bears homogenous *N*-glycosylation with dominant GnGn-glycans and a small portion of high-mannose variants. By contrast, *N*-glycans in palivizumab are heterogeneous and mainly contain fucose [117]. By comparing palivizumab-P with commercially available palivizumab, it was shown that the two preparations had similar prophylactic and therapeutic efficacy in a cotton rat model [116].

West Nile virus (WNV). WNV is a flavivirus that is generally transmitted to humans through bites of infected *Culex* mosquitos (*Culex pipiens*); it causes fever, encephalitis, or meningitis. To date, no vaccine is yet available against WNV infection, but immunotherapy with antibodies is a promising approach. Glycoprotein GP-E from WNV contains epitopes in domain III that can interact with highly neutralizing antibodies. Monoclonal antibody E16 against GP-E has been isolated, and its humanized variant hE16 was prepared as well. Both antibodies are able to exert a curative effect after being administered to RSV-infected mice [118]. Using various vector systems, hE16 was synthesized in the leaves of *N. benthamiana* and lettuce, yielding 800 and 260 mg per kilogram of fresh biomass, respectively [119]. In addition, glyco-modified variants of hE16 from *N. benthamiana ΔXTFT* plants were obtained that exhibited high neutralizing activity [120, 121]. Overall, anti-WNV MAPP may become an efficient means for immunotherapy against WNV infection [23].

Anthrax. Anthrax is a dangerous infectious disease caused by the gram-positive anaerobic spore-forming bacterium *Bacillus anthracis*. An 83-kDa protective antigen (PA) is the main virulence factor, and it forms exotoxins that kill host cells. PA is the main target for making vaccines and TMA because it induces the development of specific neutralizing antibodies *in vivo*. MAPP against anthrax is primarily considered as a means for fighting against bioterrorism. MAPP against PA (MAPP-PA) was obtained in *N. benthamiana* and was shown to neutralize toxins both *in vitro* and *in vivo* at a level comparable to that of the original TMA [122]. A non-glycosylated form of MAPP-PA (NG-MAPP-PA) was found to be more efficient at protecting mice that were lethally infected with *B. anthracis* [123]. In primates, NG-MAPP-PA was

even more efficacious. It is believed that NG-MAPP-PA should be used to prevent the infection caused by the inhalation of *B. anthracis* spores.

Botulism. A neurotoxin produced by the anaerobic bacterium *Clostridium botulinum*, e.g., in canned food contaminated with its spores, causes lethal injury of the nervous system. Treatment of botulism requires that antitoxin be administered as soon as possible after establishing the diagnosis. A transgenic tobacco plant was created that produces toxin-neutralizing scFv antibodies [124], and, according to calculations, a plantation (1-2 ha) covered with these tobacco plants could yield up to 4 kg of antitoxin, which is sufficient for manufacturing one million therapeutic doses.

Anti-cancer MAPPs. *Rituximab*. As a sales champion among anti-cancer preparations, rituximab (MabThera®, Rituxan®) interacts with CD20, providing an efficient treatment for patients with lymphoma and leukemia. The original preparation is produced in the CHO cell line. Manufacture of rituximab in plants (rituximab-P) has been established by Caliber Biotherapeutics (USA) using a double vector (TMV and PVX) approach [28] that yields up to 650 mg of antibodies per kg of fresh leaf mass from *N. benthamiana* [3]. As a biosimilar drug, rituximab-P did not differ from the original rituximab in terms of triggering ADCC, whereas glyco-modified MAPP similar to glyco-modified TMA obinutuzumab (Gazyva®) [125] was 2-10-fold superior to the original product [3].

Trastuzumab. The original preparation of trastuzumab (Herceptin®) was aimed at treating HER2-positive breast cancer, and it is produced in the CHO cell line. A biosimilar plant product (trastuzumab-P) was obtained from both stably transformed tobacco plants [29] and a transient expression system in *N. benthamiana* [30, 126]. Trastuzumab-P is able to (i) bind the oncoprotein HER2/neu on the surface of cancer cells, (ii) inhibit cancer cell proliferation, and (iii) retard tumor growth in mice transplanted with SK-BR-3 human cancer cells [30, 127]. No data are available on the biological activity of glyco-modified trastuzumab-P.

Idiotype vaccine for treating non-Hodgkin's lymphoma (NHL). Rituximab is usually used to treat non-Hodgkin's lymphoma. However, 30-50% of patients are resistant to this therapy, which may be overcome by an anti-cancer vaccine based on personalized idiotype antibodies. Anti-idiotype antibodies are produced in the same person against hypervariable regions of their own antibodies. A unique tumor-specific surface antigen on malignant B cells that includes variable regions of IgG light and heavy chains serves as an idiotype during NHL. Development of personalized vaccine started with a tumor biopsy with which to perform nucleotide sequencing on the fragment encoding heavy and light chain-variable (Fv) regions of a tumor-specific antigen. Then, scFv-encoding cDNA was inserted into a TMV-based vector. Infecting tobacco plants with this vector construct allowed for the production of an idiotype vaccine against NHL containing a short polypeptide matching the Fv fragment [128]. Later, the feasibility of this technology was confirmed by examining 16 patients with NHL and creating 16 individualized vaccines with verified safety and immunogenicity [129]. Then, a technology of vaccine development for treating NHL was updated [130]. The nucleotide sequence of the Fv region from the heavy and light chains of the tumor-specific antigen was supplemented with human IgG1 constant regions, thereby resulting in a full-sized antibody synthesized in *N. benthamiana* using a double vector system [28]. This approach was applied to make an individualized vaccine for 11 patients with NHL. The development of each vaccine took less than 12 weeks (from biopsy to vaccine), with high expression levels in the plants (0.5-4.8 g/kg fresh leaf mass). In the next step, an idiotype vaccine was chemically conjugated with hemocyanin as a carrier protein, and together with granulocyte-macrophage colony stimulating factor, it was administered to patients over six sequential subcutaneous injections. It was demonstrated that in nine (82%) of the 11 patients who were injected with vaccine, an idiotype-specific cellular and humoral response were observed. None of the patients experienced serious side effects related to vaccination [75].

CONCLUSIONS

1. Plants are promising antibody factories because their cells have mechanisms for protein synthesis and posttranslational modification (glycosylation) similar to those in animal cells. Biotechnologists are attracted to the MAPP production strategy because of its flexibility, speed, scalability, and low manufacturing cost as well as the lack of animal pathogens contamination risk.

2. Like the majority of immunoglobulins in healthy people, MAPPs undergo glycosylation inside the ER and Golgi apparatus. The final steps of *N*-glycan modification involve β1,3-galactosyl transferase and α1,4-fucosyl transferase 13.

3. MAPP Asn297-attached glycan is mainly a complex type GnGnXF glycan along with a small amount of oligomannose type MMXF glycans. The core region of GnGnXF and MMXF type glycans contains one α1,3-fucose and one β1,2-xylose residue, which, along with a lack of terminal galactose and sialic acid, distinguish MAPP from human IgG.

4. MAPP Asn297-linked glycan is a specific plant-derived glyco-epitope that is lacking in mammals. MAPP biosafety assessments in patients suggest that it has no side effects, although it does not definitively alleviate concerns about the potential immunogenicity of some plant glycans in humans.

5. Methods for creating MAPPs that closely resemble human IgG in their carbohydrate profile have been

developed. Humanization of plants-producers is aimed at (i) elimination of MAPP immunogenicity, (ii) introduction of human genes into plants resulting in the acquisition of the ability to produce MAPP with the carbohydrate profile of human IgG, and (iii) inhibition of β1,2-xylosylation and α1,3-fucosylation in the core region of Asn297-linked glycan.

6. Several MAPPs at various developmental stages have been proposed but have not yet become available on the pharmaceutical market. Promise for their clinical use is held by (i) MAPPs aimed at treatment of viral (human immunodeficiency virus, Ebola virus, West Nile virus, Junin virus, and respiratory syncytial virus) and bacterial (anthrax and botulism) infections as well as (ii) anti-cancer MAPPs.

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