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N-Glycosylation engineering of plants for the biosynthesis of glycoproteins with bisected and branched complex *N*-glycans

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Glycoengineering is increasingly being recognized as a powerful tool to generate recombinant glycoproteins with a customized N-glycosylation pattern. Here, we demonstrate the modulation of the plant glycosylation pathway toward the formation of human-type bisected and branched complex *N*-glycans. Glycoengineered *Nicotiana benthamiana* lacking plant-specific N-glycosylation (i.e. β1,2-xylose and core α1,3-fucose) was used to transiently express human erythropoietin (hEPO) and human transferrin (hTF) together with modified versions of human β1,4-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIII), α1,3-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIV) and α1,6-mannosyl-β1,6-*N*-acetylglucosaminyltransferase (GnTV). hEPO was expressed as a fusion to the IgG-Fc domain (EPO-Fc) and purified via protein A affinity chromatography. Recombinant hTF was isolated from the intracellular fluid of infiltrated plant leaves. Mass spectrometry-based *N*-glycan analysis of hEPO and hTF revealed the quantitative formation of bisected (GnGnbi) and tri- as well as tetraantennary complex *N*-glycans (Gn[GnGn], [GnGn]Gn and [GnGn][GnGn]). Co-expression of GnTIII together with GnTIV and GnTV resulted in the efficient generation of bisected tetraantennary complex *N*-glycans. Our results show the generation of recombinant proteins with human-type N-glycosylation at great uniformity. The strategy described here provides a robust and straightforward method for producing mammalian-type *N*-linked glycans of defined structures on recombinant glycoproteins, which can advance glycoprotein research and accelerate the development of protein-based therapeutics.

Keywords: erythropoietin / GnTIII / GnTIV / GnTV / *Nicotiana benthamiana* / N-glycosylation / transferrin

Introduction

The attachment of a bisecting GlcNAc residue and the formation of tri- and tetraantennary complex *N*-glycans by *N*-acetylglucosaminyltransferase III, IV and V are common extensions of oligosaccharides on mammalian glycoproteins. The branched structures are associated with various biological functions including cancer metastasis (reviewed by Zhao et al. 2008) and regulation of T-cell activation (Demetriou et al. 2001). In particular, branching increases the number of polylactosamine (Galβ1,4-GlcNAc-) structures on *N*-glycans, which are the ligands for galectins resulting in the formation of specific lattices with glycoproteins (Lau and Dennis 2008). In addition, galactosylated tri- and tetraantennary structures can be further elongated by terminal α2,6- or α2,3-linked sialic acid. The impact of these branched sialylated *N*-glycans on protein function has been impressively shown for one of the most prominent biopharmaceutical products, recombinant human erythropoietin (hEPO). hEPO is a glycoprotein hormone with three potential N-glycosylation sites. Structural analysis of recombinant hEPO produced in Chinese hamster ovary (CHO) cells exhibited a number of different sialylated structures; tetraantennary structures represent the major glycoforms (Hokke et al. 1995; Pabst et al. 2007). From a biological point of view, a high content of tetraantennary sialylated oligosaccharide chains is important since there is a positive correlation between the in vivo activity of recombinant EPO and the ratio of tetra- to diantennary oligosaccharides (Takeuchi et al. 1989; Yuen et al. 2003).

Glycoengineering of target proteins and host cells has proven to be a powerful tool for the generation of therapeutically relevant proteins with proper glycosylation (recently reviewed by Rich and Withers 2009). Some of these tailored glycoproteins, including hEPO, exhibit enhanced in vivo activities (Umaña et al. 1999; Egrie et al. 2003; Jeong et al. 2009). Engineering of the N-glycosylation pathway of various (putative) expression hosts led to remarkable success and resulted in the reconstruction of entire biosynthetic pathways (Aumiller et al. 2003; Hamilton et al. 2006; Castilho et al. 2010). However, comparatively little attempts have so far been made to increase the branching of complex *N*-glycans.

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Overexpression of the mammalian β1,4-*N*-acetylglucosaminyl transferase III (GnTIII) in different expression hosts (i.e. insect cells, CHO cells, tobacco plants) resulted in the generation of various glycoforms carrying a bisected GlcNAc accompanied by an overall heterogeneous N-glycosylation profile (Umaña et al. 1999; Rouwendal et al. 2007; Okada et al. 2010). The unwanted microheterogeneity of N-glycosylation is a serious limitation of these approaches and indicates the importance of precise subcellular targeting of recombinantly expressed glycosyltransferases for efficient *N*-glycan processing. For example, targeting of GnTIII to early instead of medial/late Golgi compartments resulted in an increase in incompletely processed bisected hybrid-type structures in CHO cells and in plants (Ferrara et al. 2006; Frey et al. 2009; Karg et al. 2010). Comparable studies that demonstrate the successful overexpression of *N*-acetylglucosaminyltransferase IV (GnTIV) or V (GnTV) in hosts suitable for the production of recombinant glycoproteins have not been described so far.

Plants are increasingly being recognized as an alternative expression platform for the production of complex therapeutically relevant proteins. Plants are able to carry out post-translational modifications like N-glycosylation, and the recent development of plant viral-based expression systems allows the efficient expression of recombinant proteins and a very rapid manufacturing process (Marrillonet et al. 2005; Sainsbury and Lomonossoff 2008; Bendandi et al. 2010). Glycoengineering of whole plants has led to the production of therapeutic glycoproteins with a rather uniform human-like N-glycosylation pattern (Schähs et al. 2007; Strasser et al. 2008, 2009; Castilho et al. 2010). Moreover monoclonal antibodies expressed in such plants with specifically altered N-glycosylation exhibited enhanced activities (Cox et al. 2006; Schuster et al. 2007; Strasser et al. 2009; Forthal et al. 2010). A crucial achievement in using plants as an expression platform was the generation of mutants that lack plant-specific N-glycosylation, i.e. β1,2-xylosylation and core α1,3-fucosylation. Such plants synthesize human-like *N*-glycans with two terminal β1,2-linked GlcNAc residues (GnGn structures: $GlcNAc_2Man_3GlcNAc_2$) at great uniformity (Koprivova et al. 2004; Strasser et al. 2004, 2008; Cox et al. 2006). In all higher eukaryotes, these oligosaccharides are the common core structures for further processing in the Golgi apparatus. Indeed, such GnGn *N*-glycans served as acceptor substrates for the generation of human-type structures, which are normally absent in plants, i.e. terminal β1,4-galactosylation and core α1,6-fucosylation (Strasser et al. 2009; Forthal et al. 2010). In mammals, GnGn is also the preferred acceptor substrate for the formation of branched or bisected *N*-glycans (Figure 1; Gleeson and Schachter 1983). These structures are not naturally present in plants due to the lack of the respective glycosyltransferases.

In this study, we aimed to modulate plant N-glycosylation toward the generation of bisected, tri- and tetraantennary complex *N*-glycans. To this end, we overexpressed human GnTIII, IV and V and modified versions thereof in the glycoengineered *Nicotiana benthamiana*, lacking plant-specific sugar residues (△XT/FT; Strasser et al. 2008). Co-expression of the three glycosyltransferases with two model glycoproteins, hEPO and hTF, resulted in the efficient attachment of bisecting GlcNAc residues and the formation of tri- and tetraantennary *N*-glycans.

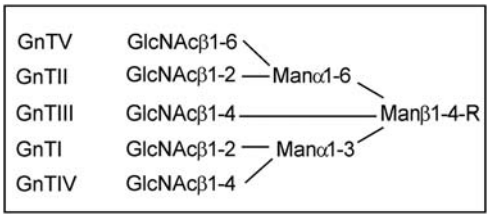

Fig. 1. Schematic presentation of the GlcNAc linkages catalyzed by α1,3-mannosyl-β1,2-*N*-acetylglucosaminyltransferase (GnTI), α1,6-mannosyl-β1,2-*N*-acetylglucosaminyltransferase (GnTII), GnTIII, GnTIV and GnTV.

Results

Generation of recombinant hEPO and human transferrin

In a recent study, we have demonstrated the efficient down-regulation of plant-specific glycosylation, i.e. β1,2-xylosylation and core α1,3-fucosylation, in *N. benthamiana*, a plant species widely used for recombinant protein expression. This was achieved by an RNAi approach, which suppresses the expression of the two respective glycosyltransferases β1,2-xylosyl- and core α1,3-fucosyltransferase (FT) (△XT/FT line; Strasser et al. 2008). In this study, these △XT/FT plants, which synthesize mainly human-type $GlcNAc_2Man_3GlcNAc_2$ (GnGn) structures, were used as expression host. For *N*-glycan modeling, two reporter glycoproteins were chosen, (i) hEPO, with three N-glycosylation sites that are decorated with substantial fractions of branched sialylated *N*-glycans and (ii) human transferrin (hTF), a serum protein with two *N*-glycans that are highly sialylated, however in its native form devoid of any branching (Yamashita et al. 1993; Pabst et al. 2007). The cDNAs encoding the reporter glycoproteins were transiently expressed in *N. benthamiana* using a potent viral-based expression system (magnICON, Marillonnet et al. 2005). hTF was expressed with a C-terminal strep-tag and hEPO was C-terminally fused to an IgG-Fc domain (EPO-Fc). Previous studies have demonstrated an enhanced stability of such EPO-Fc constructs (Bitonti et al. 2004). Due to a conserved N-glycosylation site within the Fc domain, this polypeptide can serve as an additional glyco-reporter. Both hTF and EPO-Fc were cloned into a tobacco mosaic virus (TMV)-based magnICON vector (Figure 2B), and △XT/FT leaves were infiltrated with appropriate agrobacterium strains. Leaves were harvested 4–5 days post infiltration, and recombinant protein expression was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. Cellular fractionation revealed hTF to be efficiently secreted to the intracellular fluid (IF). A clear band migrating at ~80 kDa, the expected size of glycosylated hTF, was present in the IF (Figure 3A). This band, which is absent in non-infiltrated leaves, reacted with an anti-strep antibody (data not shown).

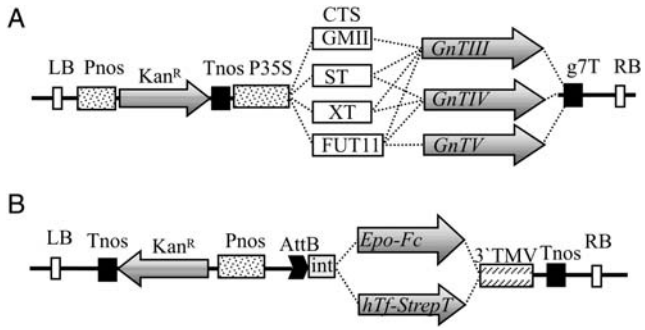

Fig. 2. Schematic presentation of the different plant expression cassettes used in this study. Binary vectors for the expression of mammalian glycosyltransferases (**A**). 3′-Modules of the TMV-based magICON vectors (pICH21595) for the expression of the EPO–Fc fusion protein and hTF (**B**). Pnos, nopaline synthase gene promoter; Tnos, nopaline synthase gene terminator; KanR, neomycin phosphotransferase II gene; P35S, Cauliflower Mosaic Virus promoter; g7T, agrobacterium gene 7 terminator; CTS, cytoplasmic tail-transmembrane-stem region; ST, rat α2,6-sialyltransferase; GMII, *A. thaliana* Golgi α-mannosidase II; XT, *A. thaliana* β1,2-xylosyltransferase; FUT11, *A. thaliana* α1,3-fucosyltransferase; GnTIII, human GnTIII; GnTIV, human GnTIV; GnTV, human GnTV; glycosylation enzymes lack their native cytoplasmic-transmembrane domain and parts of their stem region (see *Materials and methods*). 3′TMV, 3′-untranslated region; AttB, recombination site; int, intron; LB, left border; RB, right border.

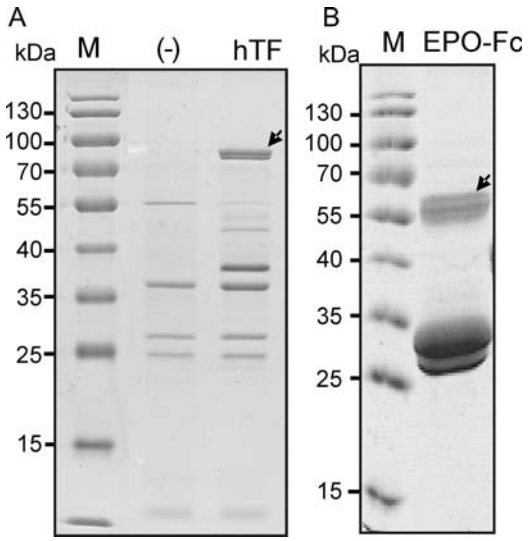

Fig. 3. Coomassie blue stained SDS–PAGE of plant-derived hTF present in the IF of ΔXT/FT leaves infiltrated with the hTF magnICON constructs, the position of hTF is marked by an arrow; (−) negative control: IF collected from leaves infiltrated with magnICON provectors without additional sequences; M, protein marker (**A**). Protein A purified EPO-Fc (indicated by an arrow); the bands at position 30 kDa represent free Fc; M, protein marker (**B**).

Protein A-purified EPO-Fc was monitored by SDS–PAGE and revealed a band corresponding to the expected size of ~55 kDa (Figure 3B). This band reacted with anti-EPO and anti-Fc antibodies (data not shown). An additional ~30 kDa band was also present on SDS–PAGE (Figure 3B), which reacted only with the anti-Fc antibody (data not shown). Tryptic digestion and subsequent mass spectrometry (MS) analysis revealed that the 30 kDa protein band refers to free Fc, an observation already made earlier upon expression of EPO-Fc in genetically modified chickens (Penno et al. 2010).

N-Glycosylation profile of EPO-Fc and hTF expressed in △XT/FT

N-Glycan composition of purified EPO-Fc was determined by liquid-chromatography-electrospray ionization-MS (LC-ESI-MS) analysis. MS spectra revealed that all three N-glycosylation sites of EPO are occupied by almost exclusively GnGn structures (Figure 4B and data not

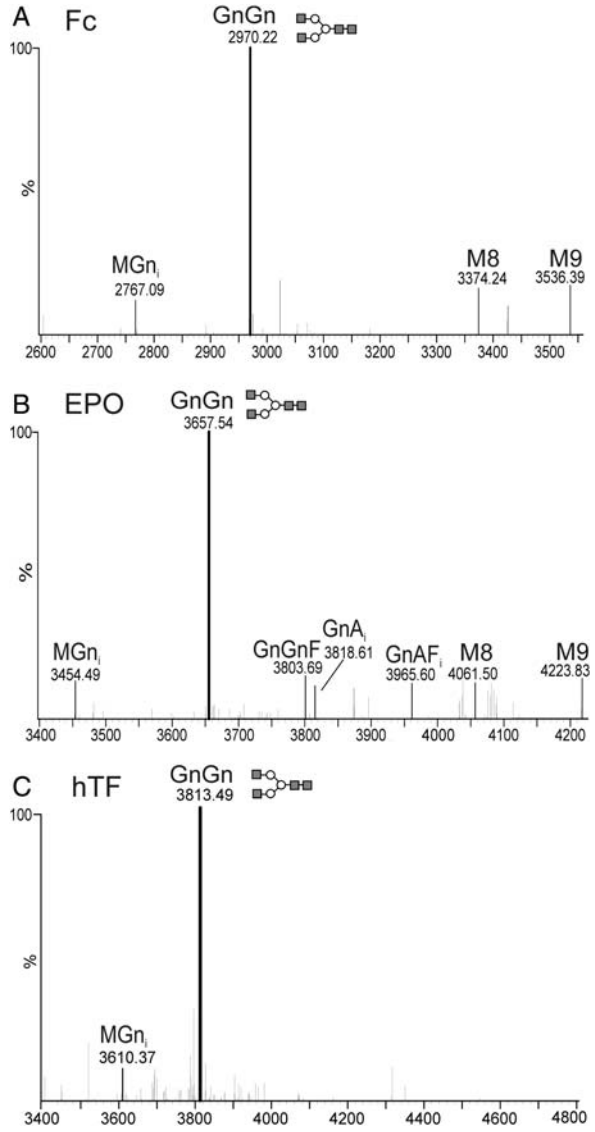

Fig. 4. Mass spectra of tryptic glycopeptides of EPO-Fc and hTF expressed in *N. benthamiana* △XT/FT line. N-Glycosylation profile of the Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**) and the EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) in the EPO–Fc fusion protein (**B**). N-Glycosylation profile of the hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS. Peak labels were made according to the ProGlycAn system (www.proglycan.com).

shown). This is also the predominant glycoform on the N-glycosylation site of the Fc fragment (Figure 4A). In addition to GnGn, the EPO glycopeptides displayed also minor peaks corresponding to oligomannosidic *N*-glycans (M8 and M9) and to fucose and galactose containing structures (GnGnF, GnAF, GnA). These peaks either arise from a slight leakiness of the silencing of FT (GnGnF) or are Lewis A epitope containing peaks, which have been previously described to be highly abundant on *Physcomitrella patents* produced hEPO (Weise et al. 2007). Indeed, purified EPO-Fc reacts on immunoblots with antibodies directed against core α1,3-fucose and Lewis A epitopes (data not shown). For *N*-glycan analysis of hTF, the band was extracted from the gel and tryptic-digested polypeptides were subjected to LC-ESI-MS. The MS spectra revealed a predominant peak assigned as GnGn structure for both *N*-glycan sites (Figure 4C and data not shown).

We also investigated whether plant-derived hEPO contains an *O*-linked oligosaccharide attached to Ser^{126}. MS analysis of peptides derived from the tryptic digestion of purified EPO-Fc did not show any evidence for the presence of *O*-linked glycan structures (data not shown). This result is consistent with recent studies showing that *N. benthamiana* lack the machinery for the formation of mucin-type O-glycosylation (Daskalova et al. 2010).

Generation of binary vectors for expression of glycosyltransferases

To obtain the attachment of bisecting GlcNAc residues and the formation of tri- and tetraantennary *N*-glycans on the recombinant glycoproteins, the corresponding human enzymes GnTIII, IV and V were transiently expressed using binary vectors. These vectors allow low-to-moderate protein expression, which is usually sufficient to achieve efficient modification of the N-glycosylation pattern (Strasser et al. 2009). As sub-Golgi targeting of recombinantly expressed glycosyltransferases has profound implications on the final glycosylation pattern of the glycoproteins, GnTIII, IV and V were fused to different Golgi targeting signals. The native cytoplasmic tail, transmembrane domain and stem (CTS) region, responsible for their sub-Golgi targeting in mammalian cells, was replaced by different CTS domains derived from plant *N*-glycan processing enzymes and the rat α2,6-sialyltransferase (ST), a well-known *trans*-Golgi targeting sequence in plants (Boevink et al. 1998; Wee et al. 1998; Schoberer et al. 2010; Figure 2A).

Generation of bisecting GlcNAc containing N*-glycans on hEPO and hTF*

In order to obtain the optimal amounts of bisected oligosaccharides and at the same time avoid interference of GnTIII activity with endogenous plant *N*-glycan processing enzymes as observed in previous studies, we evaluated different sub-Golgi targeting sequences. The catalytic domain of human GnTIII was fused to the CTS regions from early/medial (Golgi α-mannosidase II, GMII), medial (Arabidopsis alpha1,3-fucosyltransferase, FUT11; β1,2-xylosyltransferase, XT) and from one late acting enzyme (ST; Figure 2A). The resulting chimeric fusion proteins (^{GMII}GnTIII, ^{FUT11}GnTIII, ^{XT}GnTIII and STGnTIII) were expressed together with EPO-Fc. MS spectra from the recombinantly expressed glycoproteins displayed the presence of significant amounts of bisecting complex *N*-glycans (GnGnbi) on the EPO glycopeptides by using STGnTIII, ^{FUT11}GnTIII and ^{XT}GnTIII (Figure 5B and C and Supplementary data, Figure S1). GnGnbi was also the predominant peak when STGnTIII was co-expressed with hTF. Targeting of GnTIII to an early stage of the biosynthetic pathway (^{GMII}GnTIII) was less effective in the formation of GnGnbi structures on EPO-Fc. Note, in some cases, minor amounts of the bisected complex glycoforms present on the EPO glycopeptide, but not on hTF, were also fucosylated (GnGnbiF). In contrast, the GnGn structure of the Fc glycopeptide was only slightly processed toward GnGnbi structures (Figure 5A and Supplementary data, Figure S1).

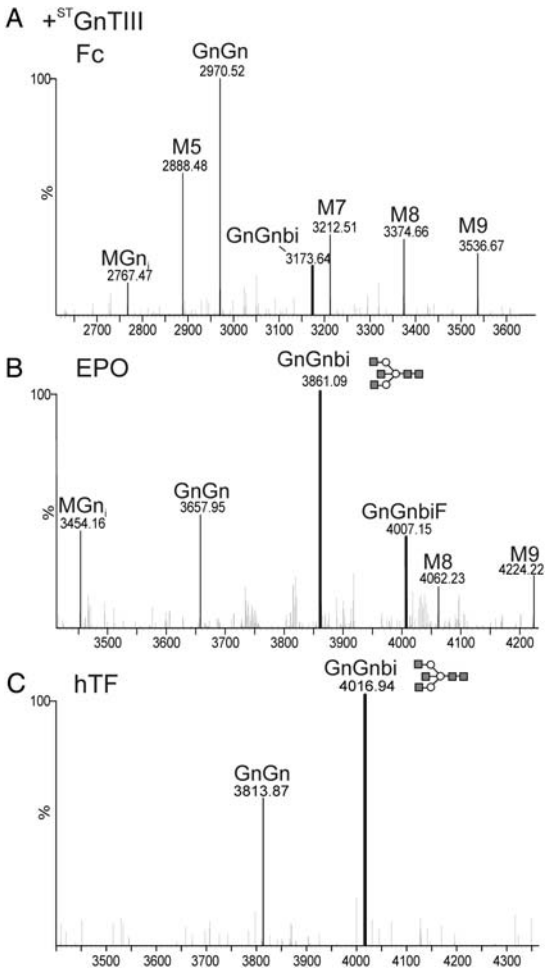

Fig. 5. N-Glycosylation profiles of EPO-Fc and hTF co-expressed with STGnTIII in △XT/FT mutants. Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**); EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) in the EPO–Fc fusion protein (**B**) and hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS. The peak assigned as GnGn could also contain a bisected structure lacking one of the two β1,2-linked GlcNAc residues (e.g. MGnbi).

Generation of triantennary N-*glycans on hEPO and hTF*

To produce triantennary complex *N*-glycans in plants human GnTIV (isozyme A), which adds a GlcNAc residue to the α1,3-mannose in β1,4-linkage (Figure 1), was co-expressed with the reporter proteins, and their *N*-glycans were analyzed by LC-ESI-MS. Apart from the native form, three different CTS–GnTIV fusions were generated potentially targeting the enzyme to different sub-Golgi compartments (Figure 2A). The native GnTIV (^{full}GnTIV) and the STGnTIV chimeric fusion proteins did not significantly change the Fc glycosylation profile. However, the constructs were able to modify to some extent the *N*-glycans from EPO resulting in a mixture of glycoforms that assign to di- and triantennary complex *N*-glycans (Supplementary data, Figure S2). On the other hand, co-expression of either ^{XT}GnTIV or ^{FUT11}GnTIV resulted in the formation of high amounts of structures that carry an additional GlcNAc residue on EPO and hTF glycopeptides (Figure 6 and Supplementary data, Figure S2). Even the Fc glycopeptide was found to carry significant amounts of triantennary complex *N*-glycans (Figure 6A). In addition, some of the minor peaks corresponding to fucosylated oligosaccharides displayed the incorporation of additional GlcNAc residues in both EPO and hTF.

To initiate branching at the α1,6-mannosyl-arm of GnGn human GnTV was transiently expressed together with EPO-Fc and hTF. The catalytic domain of GnTV was fused to the CTS region of FUT11 (^{FUT11}GnTV) since this targeting sequence resulted in highly efficient formation of triantennary complex *N*-glycans when fused to GnTIV (Figure 6). Upon ^{FUT11}GnTV expression, the formation of structures corresponding to [GnGn]Gn on EPO and hTF glycopeptides was detected (Figure 7B and C). A small amount of fucosylated [GnGn]Gn was also identified on EPO glycopeptides (Figure 7B). In contrast to that, the N-glycosylation site on

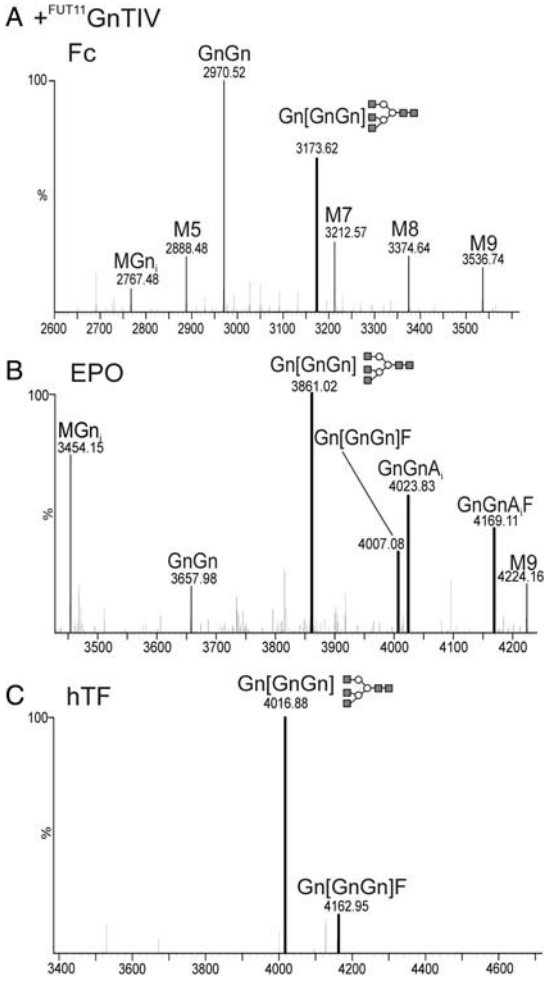

Fig. 6. N-Glycosylation profiles of EPO-Fc and hTF co-expressed with ^{FUT11}GnTIV in △XT/FT mutants. Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**); EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) in the EPO–Fc fusion protein (**B**) and hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS. The peak assigned as GnGn could also contain a GlcNAc-residue in β1,4-linkage.

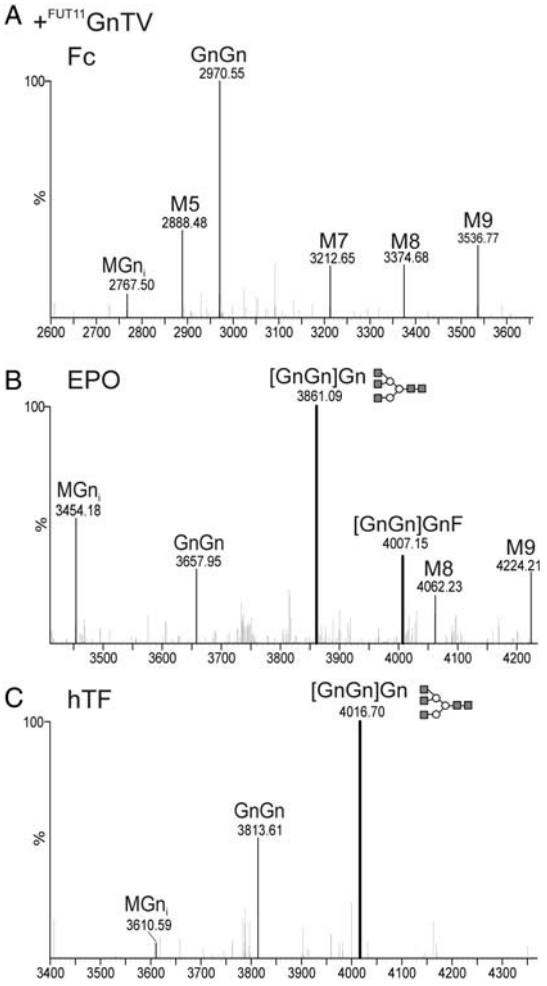

Fig. 7. N-Glycosylation profiles of EPO-Fc and hTF co-expressed with ^{FUT11}GnTV in △XT/FT mutants. Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**); EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) (**B**) and hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS. The peak assigned as GnGn could also contain a GlcNAc-residue in β1,6-linkage.

the Fc fragment did not show any peaks corresponding to putative branched complex *N*-glycans when co-expressed with ^{FUT11}GnTV (Figure 7A).

Generation of tetraantennary N-*glycans on hEPO and hTF*

The expression of GnTIV and GnTV has shown that plant complex *N*-glycans can be modified toward the formation of triantennary structures. To elongate both arms simultaneously, we co-expressed ^{FUT11}GnTIV and ^{FUT11}GnTV with EPO-Fc or hTF and performed LC-ESI-MS. The glycopeptides from EPO and hTF exhibited a predominant peak corresponding to a tetraantennary complex *N*-glycan structure ([GnGn][GnGn]) and minor amounts of triantennary structures ($GnGnGn_i$) as well as GnGn (Figure 8B and C). Consistent with the previous data for the expression of ^{FUT11}GnTV, the Fc glycopeptide carried mainly GnGn structures, accompanied with minor fractions of oligomannosidic structures. In order to evaluate whether the three mammalian glycosyltransferases can act in a synchronized mode, we co-expressed ^{FUT11}GnTIV and ^{FUT11}GnTV with different GnTIII constructs (^{GMII}GnTIII, ^{XT}GnTIII, ^{FUT11}GnTIII and STGnTIII). Co-expression of STGnTIII, which targets GnTIII to a late Golgi compartment, resulted in the formation of significant amounts of complex tetraantennary glycans also carrying a bisecting GlcNAc [GnGn][GnGn]bi on EPO and hTF (Figure 9). This oligosaccharide structure was also identified on the EPO glycopeptides when GnTIII was fused to a targeting signal for medial-Golgi location (^{XT}GnTIII and ^{FUT11}GnTIII) but was not detected when ^{GMII}GnTIII was co-expressed with ^{FUT11}GnTIV and ^{FUT11}GnTV (Supplementary data, Figure S3).

Structural identification of bisected and triantennary complex N-*glycans*

The transfer of a GlcNAc residue causes approximately a 203 Da mass shift of the respective peaks in MS spectra. We have used the hTF samples derived from co-expression with GnTIII, GnTIV and GnTV (Figures 5C, 6C and 7C) to identify the newly generated peaks by co-elution with known

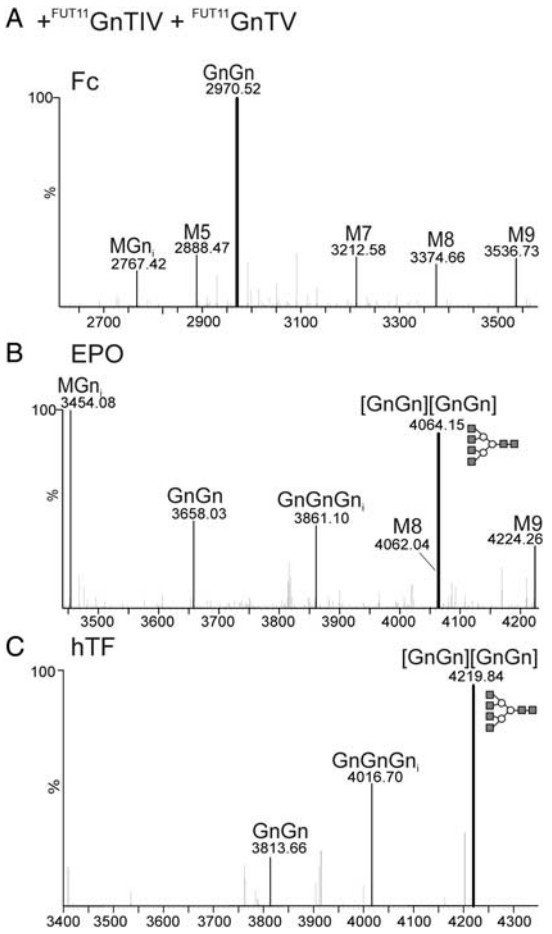

Fig. 8. N-Glycosylation profile of EPO-Fc and hTF co-expressed with ^{FUT11}GnTIV and ^{FUT11}GnTV in △XT/FT mutants. Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**); EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) in the EPO–Fc fusion molecule (**B**) and hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS.

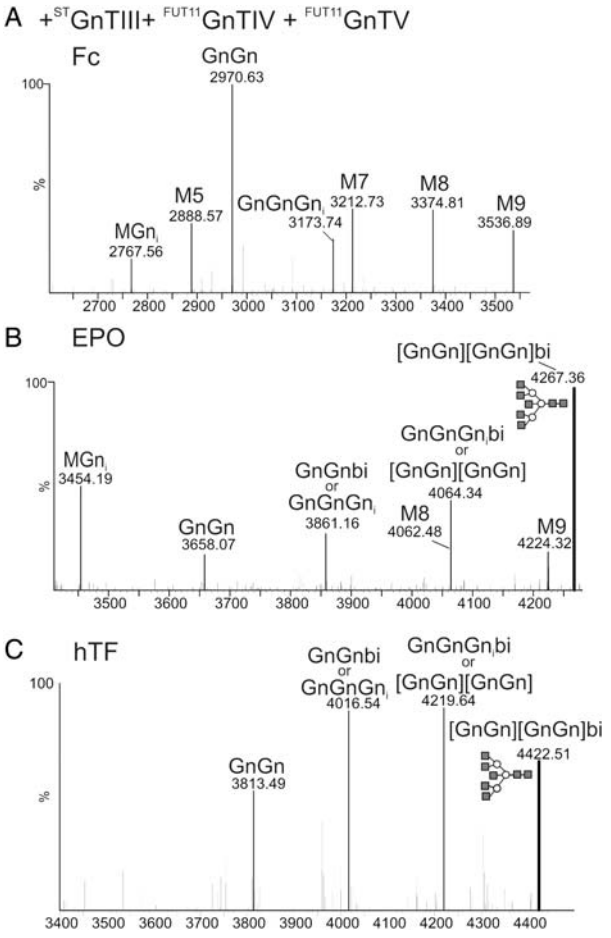

Fig. 9. N-Glycosylation profile of EPO-Fc and hTF co-expressed with STGnTIII, ^{FUT11}GnTIV and ^{FUT11}GnTV in △XT/FT mutants. Fc glycopeptide 2 (T^{289}KPREEQYNSTYR^{301}) (**A**); EPO glycopeptide 2 (G^{77}QALLVNSSQPWEPLQLHVDK^{97}) (**B**) and hTF glycopeptide 2 (Q^{603}QQHLFGSNVTDCSGNFCLFR^{623}) (**C**). "i" refers to the presence of putative isoforms of the same mass that cannot be distinguished by MS.

standards using chromatography on porous graphitic carbon with detection by ESI-MS (Pabst et al. 2007; Stadlmann et al. 2008). The peaks derived from the different GlcNAc modification reactions displayed co-elution with the respective standard peaks (Figure 10), confirming the successful generation of bisected and triantennary complex *N*-glycans in glycoengineered ΔXT/FT plants.

Discussion

In this study, we show the efficient formation of GnGn, tri- and tetraantennary *N*-glycans and/or the incorporation of a bisecting GlcNAc on two glycoproteins, hEPO and hTF. This was achieved by the overexpression of the respective mammalian enzymes in the expression host ΔXT/FT, a glycosylation mutant with a targeted down-regulation of XT and FT expression (Strasser et al. 2008). The expression of EPO-Fc and hTF in ΔXT/FT without additional mammalian glycosyltransferases resulted in the formation of virtually exclusively GnGn structures on all glycosylation sites particularly on hTF, which confirms the versatile utility of this N-glycosylation mutant as potential expression host for recombinant glycoproteins. The GnGn glycoform constitutes the pivotal intermediate for the formation of complex-type *N*-glycans in all higher eukaryotes. Upon the expression of mammalian β1,4-galactosyltransferase (GalT) and core α1,6-fucosyltransferasere in ΔXT/FT, we could show the efficient generation of

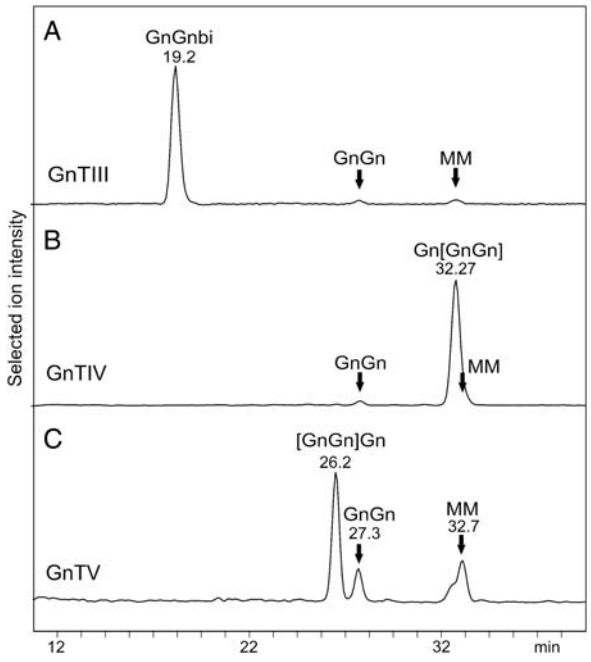

Fig. 10. Isomer assignment of bisected and triantennary complex *N*-glycans. *N*-Glycans of hTF generated upon co-expression of GnTIII (**A**), GnTIV (**B**) and GnTV (**C**), respectively, were enzymatically released, reduced and subjected to LC-ESI-MS with a carbon column. The elution position of different *N*-glycan standards is indicated by arrows. LC-MS data are shown as selected ion chromatograms for glycans with 0, 2 or 3 GlcNAc residues on the non-reducing side, for masses of 913.4, 1319.4 and the doubly charged ion at 761.8, respectively.

β1,4-galactosyled and core α1,6-fucosylated *N*-glycans on recombinantly expressed IgG (Strasser et al. 2008; Forthal et al. 2010). Here, we extended our efforts in humanizing the plant N-glycosylation pathway toward the generation of bisected and branched complex *N*-glycans, which are usually not synthesized in plants.

Previous studies have shown that the expression of GnTIII in wild-type plants resulted in the attachment of a bisecting GlcNAc (Rouwendal et al. 2007; Frey et al. 2009; Karg et al. 2010). However, as the native human GnTIII is very likely targeted to a medial-Golgi compartment overlapping mainly with FT, but also with *N*-acetylglucosaminyltransferase II (GnTII) and XT different incompletely processed glycoforms were generated (Rouwendal et al. 2007; Frey et al. 2009). This illustrates that the presence of a bisecting GlcNAc blocks further processing of *N*-glycans in plants just as in mammalian cells (Schachter 1986). Consistent with this finding, we found high levels of $GlcNAc_2Man_3GlcNAc_2$ peaks in addition to the presence of significant amounts of bisected complex *N*-glycans upon the expression of GnTIII targeted to an early sub-Golgi compartment (^{GMII}GnTIII). This result indicates that either GnTIII is not very active when fused to the GMII-CTS region or the bisecting GlcNAc is transferred to $GlcNAc_1Man_3GlcNAc_2$ which blocks further processing by GnTII.

The importance of proper sub-Golgi targeting of glycosyltransferases for appropriate *N*-glycan modification in plants has also been emphasized in other studies. Interference with the endogenous plant *N*-glycan processing pathway resulted in the generation of aberrant structures. For example, when native human GalT was expressed in plants, galactosylated and incompletely processed *N*-glycans were generated (Palacpac et al. 1999; Bakker et al. 2001) and a CTS–GalT fusion that directed the enzyme to an early stage of the pathway led to an increase in incompletely processed *N*-glycans (Bakker et al. 2006). However, targeting GalT to a late stage of the pathway using rat ST-CTS resulted in the generation of fully processed β1,4-galactosylated diantennary *N*-glycans (Strasser et al. 2009). Here, we demonstrate that GnTIV generates triantennary structures particularly efficiently upon targeting the enzyme to medial-Golgi compartments using FUT11- and XT-CTS sequences. Such oligosaccharides were synthesized at reduced levels when the full-length version of the human enzyme was used, indicating improper subcellular targeting of the native human GnTIV in ΔXT/FT plants.

Apart from the generation of tri- and tetraantennary complex *N*-glycans on EPO, we could also generate these structures on a glycoprotein (hTF), which normally does not contain branched oligosaccharides (Pabst et al. 2007), showing that different glycoproteins can be furnished with novel non-native *N*-glycan structures. However, the oligosaccharide of the Fc domain in the EPO–Fc fusion was not very efficiently branched and modified with a bisecting GlcNAc residue. In addition, we show the efficient generation of bisected tetraantennary oligosaccharides, which are not commonly found on mammalian glycoproteins. This is achieved by the sequential transfer of GlcNAc residues with GnTIII acting at the final stage in order to prevent blocking of GnTIV and V once the bisected GlcNAc is added. Our results may serve as an example that fine-tuning of the intracellular

targeting of glycosyltransferases facilitates the generation of naturally rare structures.

In this study, we describe a robust and straightforward method for producing glycoproteins with a tailor-made mammalian-type N-glycosylation pattern in plants at great homogeneity. Notably, due to the diverse endogenous N-glycosylation repertoire, such homogenous N-glycosylation pattern can hardly be achieved by any mammalian cell-based expression system. Glycoengineered plant-made glycoproteins allow the analysis of the impact of different glycoforms on glycoproteins more in detail and may advance the development of glycoprotein-based therapeutics. We have recently demonstrated the efficient in planta formation of human-type α2,6-sialylated *N*-glycans on recombinant proteins (Castilho et al. 2010). Together with the results described here, it appears possible to use glycoengineered plants in the near future for the generation of glycoproteins with branched and sialylated *N*-glycans, i.e. the structures needed for optimal efficacy of important therapeutic products such as EPO.

Material and methods

Binary vectors for expression of mammalian glycosyltransferases

GnTIII: For different targeting of the human β1,4-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIII), the catalytic domain and part of the putative stem region (comprising amino acids 35–531) were fused to the CTS region of different enzymes. For late Golgi targeting, the catalytic domain was fused to the CTS region of the rat ST. First, the catalytic domain was polymerase chain reaction (PCR) amplified from cDNA of HepG2 (human hepatocellular liver carcinoma) cells with the primer pair GnTIII F1/R1 (Supplementary data, Table S1). The PCR product was digested with *Xba*I/*Bam*HI and cloned into pPT2M binary vector (Strasser et al. 2005). Then, the α2,6-sialyltransferase CTS region (comprising amino acids 1–52) was PCR amplified from plasmid pGA482rST (Wee et al. 1998) with the primer pair ST 1F/R1. The PCR product was digested with *Xba*I/*Xho*I and cloned into the plasmid containing the GnTIII catalytic domain. The resulting plant expression vector was named STGnTIII (Figure 2). The other GnTIII expression vectors were generated as described in Supplementary data, Methods.

GnTIV: The plasmid containing the human α1,3-mannosyl-β1,4-*N*-acetylglucosaminyltransferase (GnTIV isoform A) cDNA was obtained from ImaGenes (clone: IRCMp 5012C0736D, http://www.imagenes-bio.de). For ^{FUT11}GnTIV, the CTS region was amplified from *A. thaliana* cDNA using the primer pair FUT11 F1/R1 (Supplementary data, Table SI). The PCR product was digested with *Xba*I/*Bam*HI and ligated into pPT2M to generate the vector pFUT11. The catalytic domain of GnTIV was amplified with the primer pair GnTIV F3/R1, digested with *Bgl*II/*Sal*I and ligated into *Bam*HI/*Sal*I digested pFUT11. The other GnTIV expression vectors were generated as described in Supplementary data, Methods.

GnTV: To amplify the fragment encoding a part of the stem region and the catalytic domain of human α1,6-mannosyl-β1,6-*N*-acetylglucosaminyltransferase (GnTV), total RNA was isolated from baculovirus-infected *Spodoptera frugiperda* Sf21 cells heterologously expressing a secreted human GnTV form (kindly provided by Lukas Mach) using the SV Total RNA Isolation System (Promega, Madison, WI). Reverse transcriptase–PCR was performed with the clone-specific primers pVTBacHis 1/2 and the cDNA was subcloned into pCR4 Blunt-TOPO vector (Invitrogen). To assemble the ^{FUT11}GnTV fusion construct, the pFUT11 plasmid was used as a template to amplify the FUT11-CTS region with the primers FUT11 F1/FUT11-GnTV R1. In parallel, the GnTV fragment (comprising amino acids 31–741) was amplified from the pCR4 Blunt-TOPO clone using the primers FUT11-GnTV F1/GnTV R2. The two overlapping amplification products were mixed together and used as template in a third PCR using the primers FUT11 F1/GnTV R2. The assembled product was digested with *Xba*I/*Xho*I and ligated into pPT2M. All binary vectors were transformed into *Agrobacterium tumefaciens* strain UIA 143.

MagnICON-based constructs for overexpression of glycoproteins

cDNA of the glycoproteins was cloned into the magnICON TMV-based module vector (TMV3': pICH21595, Bayer BioScience NV Research, Ghent, Belgium) containing two *Bsa*I sites designed for directional cloning of the target gene (Marillonnet et al. 2004, 2005; Giritch et al. 2006; Figure 2). The TMV5'α (pICH20999) module includes the signal peptide (SP) from the barley α-amylase sequence to target proteins to the secretory pathway. A binary vector (pICH14011) expressing the recombinase was used to allow in planta assembly of the two virus modules.

The EPO–Fc fusion was obtained by overlap extension PCR as follows: the cDNA encoding human IgG-Fc (amino acids 20–243) was amplified from clone pCEP4-Fc (f-star GmbH, Vienna, Austria) with primers Fc-EPO/Fc R1. EPO cDNA lacking the sequence that encodes the SP (amino acids 28–194) was amplified from the Ultimate ORF clone (IOH44362, Invitrogen, Carlsbad, CA) with primers EPO F5/EPO-Fc. Overlapping PCR products were used as a template for a third PCR to assemble the EPO–Fc fusion with primers EPO F5/Fc R1. The PCR product was digested with *Bsa*I and ligated into the TMV3' vector digested in the same way.

The cDNA-encoding hTF without the SP (amino acids 21–697) was amplified from clone IRATp970E0766D (ImaGenes, Berlin, Germany) with the primer pair hTF F5/R5 containing the sequence for the *Strep*-tag at the C-terminus and cloned into the *Bsa*I digested TMV3' vector. All viral-based vectors were transformed into the *A. tumefaciens* strain GV3101 pmp90.

Plant material and transient protein expression

Nicotiana benthamiana △XT/FT plants (Strasser et al. 2008) were grown in a growth chamber at 22°C with a16 h light/8 h dark photoperiod. Five-to-six-week-old plants were used for agroinfiltration experiments (Strasser et al. 2008; Castilho et al. 2010). To express the reporter proteins (EPO-Fc and hTF), the TMV3' vector containing the respective cDNA was co-infiltrated with the corresponding 5′ vector containing the SP and the binary vector containing the recombinase (Marillonnet et al. 2005). Binary vectors containing the

cDNA of the mammalian glycosyltransferases were co-infiltrated with the viral-based vectors (OD_{600} of ~0.15–0.2 for all agrobacteria).

EPO-Fc purification

Agroinfiltrated leaves (200–300 mg) were homogenized in liquid nitrogen and resuspended in 600 µL pre-cooled extraction buffer (100 mM Tris–HCl, pH 6.8, 40 mM ascorbic acid, 500 mM NaCl, 1 mM EDTA), incubated on ice for 10 min and subsequently cleared by centrifugation (9000 × g for 20 min at 4°C). The supernatant was incubated for 1.5 h at 4°C with 15–20 µL rProteinA Sepharose™ Fast Flow (GE Healthcare, Uppsala, Sweden) previously washed with 1× phosphate-buffered saline (PBS). After a brief spin down, the supernatant was discarded and the sepharose was washed three times with 1× PBS using Micro Bio-Spin chromatography columns (Bio-Rad, Hercules, CA). To extract the EPO–Fc fusion protein from the column, 20 µL of 2× Laemmli buffer (125 mM Tris–HCl, pH 6.8, 20% glycerin, 4% SDS; 10% mercaptoethanol, 0.1% bromphenol blue) was applied, incubated for 5 min at 95°C and centrifuged for 1 min (9000 × g). The samples were then directly used for SDS–PAGE.

Isolation of IF

Two to three infiltrated leaves were immersed in buffer solution (100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA) and subjected to vacuum (2×5 min). IF was collected by low-speed centrifugation (900 × *g* for 15 min). The IF was mixed with 2× Laemmli buffer and incubated for 5 min at 95°C prior to SDS–PAGE.

Analysis of glycopeptides

N-Glycan analysis of the reporter proteins was carried out by LC-ESI-MS of tryptic glycopeptides as described previously (Stadlmann et al. 2008; Strasser et al. 2008). Briefly, the SDS–PAGE bands corresponding to the EPO–Fc fusion protein (~55 kDa) and hTF (~80 kDa) were excised from the gel, S-alkylated, digested with trypsin and subsequently analyzed by LC-ESI-MS. During this procedure, four glycopeptides are generated for the EPO–Fc fusion protein: two glycopeptides for the Fc part are due to incomplete digestion (glycopeptide 1, E^{293}EQYNSTYR^{301}; glycopeptide 2, T^{289}KPREEQYNSTYR^{301}) and another two for EPO since two out of the three N-glycosylation sites (Asn^{24} and Asn^{38}) are found on the same glycopeptide (glycopeptide 1, E^{21}AENITTGCAEHCSLNENITVPDTK^{45}; glycopeptide 2, G^{77}QALLVNSSQPWEPLQLHVDK^{97}). For hTF, the two glycosylation sites at Asn^{432} and Asn^{630} are discriminated by two glycopeptides (glycopeptide 1, C^{421}GLVPVLAENY NKSDNCEDTPEAGYFAVAVVKK4^{53}; glycopeptide 2, Q^{603} QQHLFGSNVTDCSGNFCLFR^{623}).

Structural identification of N*-glycans*

The identification of the GlcNAc linkage to β1,4-, α1,3- and α1,6-mannosyl residues as found on bisected and triantennary *N*-glycans was carried out by chromatography on porous graphitic carbon with detection by ESI-MS (Pabst et al. 2007). The elution order of free, reduced *N*-glycans was compared with that of specific standards prepared using glycosidase digests of asialo-EPO, agalacto-EPO and human IgG *N*-glycans (Pabst et al. 2007; Stadlmann et al. 2008).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

CHO, Chinese hamster ovary; CTS, cytoplasmic tail, transmembrane domain and stem; ESI, electrospray ionization; FUT11, core α1,3-fucosyltransferase; GMII, Golgi α-mannosidase II; GnTII, *N*-acetylglucosaminyltransferase II; GnTIII, β1,4-mannosyl-β1,4-*N*-acetylglucosaminyltransferase; GnTIV, α1,3-mannosyl-β1,4-*N*-acetylglucosaminyltransferase; GnTV, α1,6-mannosyl-β1,6-*N*-acetylglucosaminyltransferase; hEPO, human erythropoietin; hTF, human transferrin; IF, intracellular fluid; LC, liquid chromatography; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SP, signal peptide; ST, α2,6-sialyltransferase; TMV, tobacco mosaic virus; XT, β1,2-xylosyltransferase.

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