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(54) Title: NICOTIANA BENTHAMIANA PLANTS DEFICIENT IN FUCOSYLTRANSFERASE ACTIVITY

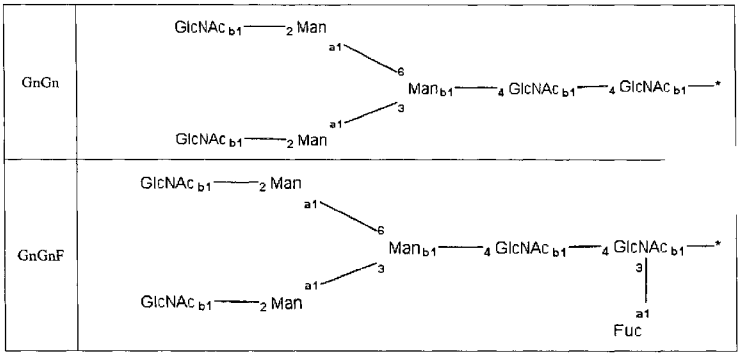


Fig. 10

(57) Abstract: The invention provides methods for reducing the levels of alfa (1,3)-fucosylated N-glycans on glycoproteins produced in plants or plant cells. In addition, the invention provides alfa(1,3)-fucosyltransferase genes from Nicotiana benthamiana, and mutant N. benthamiana plants in which the levels of alfa(1,3)-fucosylated N-glycans are reduced.

Nicotiana benthamiana plants deficient in fucosyltransferase activity

Field of the invention

The current invention relates to the field of molecular farming, i.e. the use of plants and plant cells as bioreactors to produce peptides and proteins, including biopharmaceuticals, particularly polypeptides and proteins with pharmaceutical interest such as therapeutic proteins, which have an altered N-glycosylation pattern resulting in a lower level of immunogenic protein-bound N-glycans, particularly a lower level of beta(1,2)-xylose residues and core alfa(1,3)-fucose residues on the protein-bound N-glycans, than counterpart unmodified plants. The invention relates to plants of the genus *Nicotiana* which are deficient in alfa(1,3)-fucosyltransferase and beta(1,2)-xylosyltransferase activity, which plants may be applied as host plants or host cells to produce heterologous glycoproteins.

Background

Glycosylation is the covalent linkage of an oligosaccharide chain to a protein resulting in a glycoprotein. In many glycoproteins, the oligosaccharide chain is attached to the amide nitrogen of an asparagine (Asn) residue and leads to N-glycosylation. Glycosylation represents the most widespread post-translational modification found in natural and biopharmaceutical proteins. It is estimated that more than half of the human proteins are glycosylated and their function frequently depends on particular glycoforms (glycans), which can affect their plasma half life, tissue targeting or even their biological activity. Similarly, more than one-third of approved biopharmaceuticals are glycoproteins and both their function and efficiency are affected by the presence and composition of their N-glycans.

Leafy crops, such as the tobacco plant *Nicotiana benthamiana*, are an attractive system for the production of therapeutic proteins, as plants are generally considered to have several advantages, including the lack of animal pathogens such as prions and viruses, low cost and the large-scale production of safe and biologically active valuable recombinant proteins, the case of scale-up, efficient harvesting and storage possibilities. However, N-linked glycans from plants differ from those of mammalian cells. In plants, beta(1,2)-xylose and alfa(1,3)-fucose residues have been shown to be linked to the core Man3GlucNAc2-Asn of glycans, whereas they are not detected on mammalian glycans, where sialic acid residues and terminal beta(1,4)-galactosyl structures occur instead. The

unique N-glycans added by plants could impact both immunogenicity and functional activity of the protein and, consequently, may represent a limitation for plants to be used as a protein production platform. Indeed, the immunogenicity of beta(1,2)-xylose residues and alfa(1,3)-fucose in mammals has been described (Bardor et al., 2003, Glycobiology 13: 427).

The enzyme that catalyses the transfer of xylose from UDP-xylose to the core β-linked mannose of protein-bound N-glycans is beta(1,2)-xylosyltransferase ("XylT", EC 2.4.2.38). The beta-1,2-xylosyltransferase is an enzyme unique to plants and some non-vertebrate animal species and does not occur in human beings or in other vertebrates. WO2007107296 describes the identification and cloning of beta-1,2-xylosyltransferases from the genus *Nicotiana* such as *Nicotiana benthamiana*.

The enzyme that catalyses the transfer of fucose from GDP-fucose to the core β-linked N-acetyl glucosamine (GlcNAc) of protein-bound N-glycans is alfa(1,3)-fucosyltransferase ("FucT", EC 2.4.1.214). WO2009056155 describes an alfa(1,3)-fucosyltransferase cDNA sequence from *Nicotiana benthamiana*.

Various strategies have been applied to avoid alfa(1,3)-fucosyl and beta(1,2)-xylosyl structures on glycoproteins produced by plants. WO2008141806 describes knock-outs in two alfa(1,3)-fucosyltransferase genes and in one beta(1,2)-xylosyltransferase gene in *Arabidopsis thaliana*. WO2009056155 describes an RNA interference strategy for the generation of *Nicotiana benthamiana* plants which are deficient in the formation of beta-1,2-xylosyl structures as well as devoid of alfa-1,3-fucosyl structures on heterologous glycoproteins. Yin et al. (2011, Protein Cell 2:41) report downregulation of the expression of the endogenous xylosyltranferase and fucosyltransferase in *Nicotiana tabacum* using RNA interference (RNAi) strategy. They found that xylosylated and core fucosylated N-glycans were significantly, but not completely, reduced in the glycoengineered lines. WO2010145846 describes knock-outs of the two beta(1,2)-xylosyltransferase genes in *Nicotiana benthamiana*. The homozygous combination of the four beta(1,2)-xylosyltransferase null alleles proved to be sufficient for the elimination of the complete beta-1,2-xylosyltransferase activity in *Nicotiana benthamiana*.

Knock-out alleles of the alfa(1,3)-fucosyltransferase genes of *Nicotiana benthamiana* have not been described thus far.

The current invention provides methods and means to reduce the levels of core alfa(1,3)-fucose residues on N-glycans on glycoproteins in *Nicotiana benthamiana*, as will become apparent from the following description, examples, drawings and claims provided herein.

Summary of the invention

In a first embodiment, the invention provides a method to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues in *Nicotiana benthamiana*, said method comprising the steps of providing a plant or plant cell comprising at least three knock-out alfa(1,3)-fucosyltransferase genes, and cultivating said cell and isolating glycoproteins from said cell. In another embodiment, said method further comprises a reduction of the level of beta(1,2)-xylosyltransferase activity. In yet another embodiment, said reduction of the level of beta(1,2)-xylosyltransferase activity is the result of a knock-out mutation in endogenous beta(1,2)-fucosyltransferase genes.

In another embodiment of the invention, a method is provided to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues in *Nicotiana benthamiana*, said method comprising the steps of providing a plant or plant cell comprising at least five knock-out alfa(1,3)-fucosyltransferase genes, and cultivating said cell and isolating glycoproteins from said cell. In a further embodiment, said knock-out alfa(1,3)-fucosyltransferase genes occur in a homozygous state in the genome.

In yet another embodiment, the methods according to the invention are further characterized in that the expression of at least five endogenous alfa(1,3)-fucosyltransferase encoding genes is reduced through transcriptional or post-transcriptional silencing. In a further embodiment, the plant or plant cell according to the invention further comprises at least one chimeric gene comprising the following operably linked DNA fragments: a plant-expressible promoter, a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene, and a DNA region comprising a transcription termination and polyadenylation signal functional in plants. In yet a further embodiment, said DNA region comprises the sequence of SEQ ID No. 19.

In yet another embodiment of the method of the invention, said glycoprotein is a heterologous protein. In yet a further embodiment, said heterologous glycoprotein is expressed from a chimeric gene comprising the following operably linked nucleic acid molecules: a plant-expressible promoter, a DNA region encoding said heterologous

glycoprotein, and a DNA region involved in transcription termination and polyadenylation. In yet another embodiment, the method according to the invention further comprises the step of purification of said heterologous glycoprotein.

In another embodiment of the invention, a glycoprotein is provided which is obtained by the methods according to the invention. In yet another embodiment of the invention, a glycoprotein with reduced levels of core alfa(1,3)-fucose residues is provided which is obtained by the methods according to the invention. In yet a further embodiment, a glycoprotein with reduced levels of core alfa(1,3)-fucose and beta(1,2)-xylose residues is provided which is obtained by the methods according to the invention.

Another embodiment of the invention provides a *Nicotiana benthamiana* plant, or a cell, part, seed or progeny thereof, comprising at least three knock-out alfa(1,3)-fucosyltransferase genes. Yet another embodiment of the invention provides a *Nicotiana benthamiana* plant, or a cell, part, seed or progeny thereof, comprising at least five knock-out alfa(1,3)-fucosyltransferase genes. In yet a further embodiment, said plant or plant cell is homozygous for the knock-out alfa(1,3)-fucosyltransferase genes. In another embodiment, said plant or plant cell further comprises at least one knock-out beta(1,2)-xylosyltransferase gene, wherein said knock-out beta(1,2)-xylosyltransferase gene comprises a mutated DNA region consisting of one or more inserted, deleted or substituted nucleotides compared to a corresponding wild-type DNA region in the beta(1,2)-xylosyltransferase gene and wherein said knock-out beta(1,2)-xylosyltransferase gene does not encode a functional beta(1,2)-xylosyltransferase protein.
In yet another embodiment, the said plant or plant cell further comprises at least one chimeric gene comprising the following operably linked DNA fragments: a plant-expressible promoter; a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene; and a DNA region comprising a transcription termination and polyadenylation signal functional in plants. In a further embodiment, said DNA region comprises the sequence of SEQ ID No. 19.
In a further embodiment, said plant or plant cell further comprises a glycoprotein foreign to said plant or plant cell. In yet another embodiment, said glycoprotein is expressed from a chimeric gene comprising the following operably linked nucleic acid molecules: a plant-expressible promoter, a DNA region encoding said heterologous glycoprotein, and a DNA region involved in transcription termination and polyadenylation.

In another embodiment of the invention, knock-out alleles of alfa(1,3)-fucosyltransferase genes are provided.

Yet another embodiment provides the use of the methods according to the invention to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues. A further embodiment provides the use of the methods according to the invention to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues and with a reduced level of beta(1,2)-xylose residues.

Brief description of the Figures

Figure 1: Results from Southern blot hybridization of *N. benthamiana* genomic DNA hybridized with a cDNA probe of FucTA from *N. benthamiana*. lane 1 = lambda marker, lanes 2-7: *N. benthamiana* genomic DNA digested with EcoRV (lane 2), HindIII (lane 3), EcoRI (lane 4), NsiI (lane 5), AseI (lane 6), PstI (lane 7); lane 8 = *Nicotiana tabacum* cv. SR1 digested with EcoRV and HindIII.

Figure 2: Example of a Southern blot comparing hybridization patterns of BAC clones (lanes 1-15) with the hybridization pattern of *N. benthamiana* genomic DNA (c).

Figure 3: Determining optimum EMS dose for production of M2 seeds in *N. benthamiana*. Seeds were treated with different concentrations of EMS. A: Germination rate 6 days (black bars) and 12 days (white bars) after sowing. B: Seed survival. C: plant fertility.

Figure 4: Crossing scheme used to obtain homozygous seven-fold knock out plants. x14: mutant allele XYL001 (XylTg14-1 as described in WO2010145846), x19: XYL002 (XylTg19-1 as described in WO2010145846), a: FucT004, b: FucT006, c: FucT007, d: FucT009, e: FucT003. The "x14/x14 x19/x19" refers to the double knock XylT mutant previously described in WO2010145846.

Figure 5: Setting up and testing the complementation assay for functionality of *N. benthamiana* FucT genes and mutant genes. WT: *A. thaliana* wildtype; 3KO: *A. thaliana* triple mutant (T-DNA-insertion knock-out mutant for XylT and FucTA and FucTB); At3KO + NbFucTA: triple mutant transformed with T-DNA carrying *N. benthamiana* FucTA cDNA; At3KO + mut FucTA: triple mutant transformed with T-DNA carrying *N. benthamiana*

FucTA cDNA carrying a point mutation creating a stop codon in exon 1 at position 217 of SEQ ID No. 1.

Figure 6: Comparison of fucosylation levels of protein samples from *N. benthamiana* plants in which different FucT genes have been knocked out. Western blot analysis of leaf protein samples from plants in which different FucT genes have been knocked out. Probed with anti-α1,3 fucose antibody (1/500 dilution); 3 min. exposure for chemoluminescence. WT: Wild Type plant; M: Protein Marker. Knocked-out versions of the gene are indicated in the table as lower case; wild type version as upper case.

Figure 7: Comparison of relative glycan levels on leaf proteins from *N. benthamiana* plants carrying null mutations for four or five FucT genes. Total protein was isolated from leaves of plants in which different FucT genes were mutated. Glycans were isolated and analyzed by MALDI-TOF. Relative levels are expressed as percentage of the total peak area as determined from the MALDI-TOF spectra. White bars: wild-type; Black bars: 4KO: FucTA (FucT004), -B (FucT006), -C (FucT007), and –D (FucT009) knocked out (average of three lines); Gray bars: 5KO: all FucT genes knocked out (FucT004, -006, -007, -009, and -003) (average of three lines).

Figure 8: Comparison of relative glycan levels on leaf proteins from *N. benthamiana* plants in which all XylT and/or FucT genes have been knocked out (FucT004, -006, -007, -009, and -003, and XylTg14-1 and XylTg19-1 as described in WO2010145846). Total protein was isolated from leaves of plants in which all XylT and/or FucT genes were mutated. Glycans were isolated and analyzed by MALDI-TOF. Relative levels are expressed as percentage of the total peak area as determined from the MALDI-TOF spectra. White bars: wild-type. Dark gray bars: 5KO: all FucT genes knocked out (average of three lines); Black bars: 7KO: all FucT and XylT genes knocked out (average of three lines); Light gray bars: RNAi: plants expressing XylT and FucT RNAi genes (Strasser et al. 2008, Plant Biotech J 6:392).

Figure 9: LC-MS analysis of glycans on an IgG1 expressed in a full knock-out N. benthamiana plant using magnICON®.
In the full knock-out *N. benthamiana* plant, all XylT and/or FucT genes have been knocked out (FucT004, -006, -007, -009, and -003, and XylTg14-1 and XylTg19-1 as described in WO2010145846). IgG1 was expressed in these full knock-out plants using magnICON®. IgG1 was isolated from leaf extract nine days after infiltration using protein G. The heavy

chain of the purified antibody was isolated by cutting the corresponding band from a reducing SDS-PAGE. The heavy chain protein in this band was used for glycan analysis by LC-MS as described by Kolarich et al. (2006) Proteomics 6:3369.

The upper panel shows a wider mass spectrum to illustrate the presence of non-glycosylated peptides. Peptide 1 (EEQYNSTY) and peptide 2 (TKPREEQYNSTYR) are two variants from the same trypsin digestion. They differ in length caused by steric hindrance of the trypsin by the presence of N-glycans. As a result, all peptide-glycans produce two peaks in this LC-MS spectrum; those for glycopeptide 2 in the lower panel are indicated with an arrow.

Figure 10: Structure of N-glycans (See also http://www.proglycan.com for a current nomenclature of N-glycans). * indicates the bond between the indicated sugar chain and an asparagine of the peptidic part of the resulting glycoprotein.

Figure 11: Comparison of fucosylation levels of protein samples from *N. benthamiana* plants in which 6 or 7 genes have been knocked out. Plants containing the FucT RNAi gene are compared with plants which do not contain this gene. Western blot analysis of leaf protein samples. Probed with anti- α1,3 fucose antibody (1/500 dilution); 1 hour exposure for chemoluminescence. WT: Wild Type plant; M: Protein Marker. Knocked-out versions of the gene are indicated in the table as lower case; wild type version as upper case.

Figure 12: Quantitative overview of fucosylated respectively xylosylated N-glycans present on the endogenous proteins of WT, 4-, 5-, 7-fold KO, RNAi and 7KO/ FucT RNAi plants. Total protein was isolated from leaves of plants and glycans were isolated and analyzed by MALDI-TOF. Glycan levels are expressed as the sum of all different fucosylated respectively xylosylated N-glycan peaks as determined from the MALDI-TOF spectra. WT: wild-type (average of two lines). RNAi: plants expressing XylT and FucT RNAi genes (Strasser et al. 2008, Plant Biotech J 6:392) (average of two lines). 4KO: all FucT genes except FucTE knocked out (average of six lines). 5KO: all FucT genes knocked out (average of three lines). HOM7KO: all FucT and XylT genes knocked out (average of three lines). HET7KO + RNAi: XylT and FucTA genes knocked out and other FucT genes are heterozygously knocked out combined with the FucT RNAi gene (average of four lines). HOM7KO + FucT RNAi: plants homozygous for all seven knock-out genes and containing the FucT RNAi gene (average of four lines).

Detailed description of different embodiments of the invention

The current invention is based on the identification of five genes encoding alfa(1,3)-fucosyltransferase in *Nicotiana benthamiana*, and that knocking-out more of these genes progressively reduces the levels of core alfa(1,3)-fucose residues on proteins produced in said plant.

In a first embodiment, the invention provides a method to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues in *Nicotiana benthamiana*, said method comprising the steps of providing a plant or plant cell comprising at least three knock-out alfa(1,3)-fucosyltransferase genes, and cultivating said cell and isolating glycoproteins from said cell.

"Reduced levels of core alfa(1,3)-fucose residues" or "a reduced level of core alfa(1,3)-fucose residues" as used herein is meant to be a reduction of levels of core alfa(1,3)-fucose residues with respect to levels as obtained in control plants. The "control plant" is generally a selected target plant which may be any plant, and may advantageously be selected among tobacco and related species like *Nicotiana*, including *N. benthamiana*, *N. tabacum*, and *S. tuberosum*, or other plants such as *M. sativa*. Generally, in the control plant the alfa(1,3)-fucosyltransferase gene is unmodified and it has wild-type levels of alfa(1,3)-fucosyltransferase activity.
"Wild type levels of alfa(1,3)-fucosyltransferase activity" (also written "wildtype" or "wild-type"), as used herein, refers to the typical level of alfa(1,3)-fucosyltransferase activity in a plant as it most commonly occurs in nature. Said control plant has thus not been provided either with a silencing nucleic acid molecule targeted to the endogenous alfa(1,3)-fucosyltransferase encoding gene or with an allele of an alfa(1,3)-fucosyltransferase gene associated with a low level of α-1,3-fucosyltransferase activity, such as a knock-out allele.

Said reduced levels of core alfa(1,3)-fucose residues can consist of a reduction of at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 97%, or at least 99%. The amount of alfa(1,3)-fucosylated glycan structures associated with a produced glycoprotein can be determined according to the methods described in this invention.

"Core alfa(1,3)-fucose residues", also "alfa(1,3)-fucose residues", or "alpha(1,3)-fucose residues" or "α(1,3)-fucose residues" as used herein refers to a fucose that is alpha 1,3-linked to the core region of N-glycans.

"Alfa(1,3)-fucosyltransferase" or "alpha(1,3)-fucosyltransferase", or α(1,3)-fucosyltransferase", or "FucT" is an enzyme that catalyses the transfer of fucose from GDP-fucose to the core β-linked N-acetyl glucosamine (GlcNAc) of protein-bound N-glycans (EC 2.4.1.214).

Genes encoding alfa(1,3) fucosyltransferase (FucT) in plants include the following database entries identifying experimentally demonstrated and putative FucT cDNA and gene sequences, parts thereof or homologous sequences: NM 112815 (*Arabidopsis thaliana*), NM103858 (*Arabidopsis thaliana*), AJ 618932 (*Physcomitrella patens*) At1g49710(*Arabidopsis thaliana*), At3g19280 (*Arabidopsis thaliana*). DQ789145 (*Lemna minor*), AY557602 (*Medicago truncatula*) Y18529 (*Vigna radiata*) AP004457 (*Oryza sativa*), AJ891040 encoding protein CAI70373 (*Populus alba* x *Populus tremula*) AY082445 encoding protein AAL99371 (*Medicago sativa*) AJ582182 encoding protein CAE46649 (*Triticum aestivum*) AJ582181 encoding protein CAE46648 (*Hordeum vulgare*), and EF562630.1 (*Nicotiana benthamiana*) (all sequences herein incorporated by reference).

A "Knock-out alfa(1,3)-fucosyltransferase gene" or "knock-out alfa(1,3)-fucosyltransferase allele" or "knock-out allele of the alfa(1,3)-fucosyltransferase gene" or "knock-out FucT gene" or "knock-out FucT allele" as used herein refers to a gene or an allele of said gene which does not complement the *Arabidopsis thaliana* triple knock-out as described by Kang et al. (2008, Proc Natl Acad Sci USA 105: 5933), using the methods as described in this invention. Said "knock-out alfa(1,3)-fucosyltransferase gene" is a wild-type alfa(1,3)-fucosyltransferase gene or allele, which comprises one or more mutations in its nucleic acid sequence. Said knock-out gene can, for example, be a gene that is not transcribed into a functional mRNA, or a gene of which the encoded RNA is not spliced correctly, or a gene not encoding a functional protein. Knock-out genes may thus comprise, for example, genes with mutations in promoter regions, with mutations in splice-sites, or with mutations coding sequences resulting in amino acid substitutions or resulting in premature translation termination.

A mutation can be a deletion, an insertion or a substitution of one or more nucleotides. Mutations can be either "natural mutations" which are mutations found in nature (e.g.

produced spontaneously without human application of mutagens) or "induced mutations", which are induced by human intervention, e.g. by mutagenesis and are called non-natural mutant null alleles.

"Mutagenesis", as used herein, refers to the process in which plant cells (e.g., a plurality of *Nicotiana benthamiana* seeds or other parts, such as pollen, etc.) are subjected to a technique which induces mutations in the DNA of the cells, such as contact with a mutagenic agent, such as a chemical substance (such as ethylmethylsulfonate (EMS), ethylnitrosourea (ENU), etc.) or ionizing radiation (neutrons (such as in fast neutron mutagenesis, etc.), alpha rays, gamma rays (such as that supplied by a Cobalt 60 source), X-rays, UV-radiation, etc.), or a combination of two or more of these. Thus, the desired mutagenesis of one or more alfa(1,3)-fucosyltransferase genes may be accomplished by use of chemical means such as by contact of one or more plant tissues with ethylmethylsulfonate (EMS), ethylnitrosourea, etc., by the use of physical means such as x-ray, etc, or by gamma radiation, such as that supplied by a Cobalt 60 source. While mutations created by irradiation are often large deletions or other gross lesions such as translocations or complex rearrangements, mutations created by chemical mutagens are often more discrete lesions such as point mutations. For example, EMS alkylates guanine bases, which results in base mispairing: an alkylated guanine will pair with a thymine base, resulting primarily in G/C to A/T transitions. Following mutagenesis, *Nicotiana benthamiana* plants are regenerated from the treated cells using known techniques. For instance, the resulting *Nicotiana benthamiana* seeds may be planted in accordance with conventional growing procedures and following self-pollination seed is formed on the plants. Additional seed that is formed as a result of such self-pollination in the present or a subsequent generation may be harvested and screened for the presence of mutant alfa(1,3)-fucosyltransferase genes. Several techniques are known to screen for specific mutant genes, e.g., Deleteagene™ (Delete-a-gene; Li *et al.*, 2001, Plant J 27: 235-242) uses polymerase chain reaction (PCR) assays to screen for deletion mutants generated by fast neutron mutagenesis, TILLING (targeted induced local lesions in genomes; McCallum *et al.*, 2000, *Nat Biotechnol* 18:455-457) identifies EMS-induced point mutations, direct sequencing, etc.

Mutant alfa(1,3)-fucosyltransferase genes may be generated (for example induced by mutagenesis) and/or identified using a range of methods, which are conventional in the art, for example using PCR based methods to amplify part or all of the alfa(1,3)-fucosyltransferase genomic or cDNA and direct sequencing.

Following mutagenesis, plants are grown from the treated seeds, or regenerated from the treated cells using known techniques. For instance, mutagenized seeds may be planted in accordance with conventional growing procedures and following self-pollination seed is formed on the plants. Additional seed which is formed as a result of such self-pollination in the present or a subsequent generation may be harvested and screened for the presence of mutant alfa(1,3)-fucosyltransferase genes, using techniques which are conventional in the art, for example polymerase chain reaction (PCR) based techniques (amplification of the alfa(1,3)-fucosyltransferase genes) or hybridization based techniques, e.g. Southern blot analysis, BAC library screening, and the like, and/or direct sequencing of alfa(1,3)-fucosyltransferase genes. To screen for the presence of point mutations (so called Single Nucleotide Polymorphisms or SNPs) in mutant alfa(1,3)-fucosyltransferase genes, SNP detection methods conventional in the art can be used, for example oligo-ligation-based techniques, single base extension-based techniques, techniques based on differences in restriction sites, such as TILLING, or direct sequencing and comparing the sequences to wild-type sequeces using, for example, NovoSNP (Weckx et al, 2005, Genome Res 15: 436).

As described above, mutagenization (spontaneous as well as induced) of a specific wild-type alfa(1,3)-fucosyltransferase gene results in the presence of one or more deleted, inserted, or substituted nucleotides (hereinafter called "mutation region") in the resulting mutant alfa(1,3)-fucosyltransferase gene. The mutant alfa(1,3)-fucosyltransferase gene can thus be characterized by the location and the configuration of the one or more deleted, inserted, or substituted nucleotides in the wild type alfa(1,3)-fucosyltransferase gene.

Once a specific mutant alfa(1,3)-fucosyltransferase gene has been sequenced, primers and probes can be developed which specifically recognize the mutant alfa(1,3)-fucosyltransferase gene in biological samples (such as samples of plants, plant material or products comprising plant material).

As used herein, the term "allele(s)" means any of one or more alternative forms of a gene at a particular locus. In a diploid (or amphidiploid) cell of an organism, alleles of a given gene are located at a specific location or locus (loci plural) on a chromosome. One allele is present on each chromosome of the pair of homologous chromosomes.

In another embodiment, a method is provided to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues and reduced levels of beta(1,2)-xylose residues in *Nicotiana benthamiana*, said method comprising the steps of: providing a plant cell comprising at least three knock-out alpha(1,3)-fucosyltransferase genes; and having a reduced level of beta(1,2)-xylosyltransferase activity; and cultivating said cell and isolating glycoproteins from said cell.

"Reduced levels of beta(1,2)-xylose residues" as used herein is meant to be a reduction of levels of core beta(1,2)-xylose residues with respect to levels as obtained in control plants. The "control" plant is generally a selected target plant which may be any plant and may advantageously be selected among tobacco and related species like *Nicotiana*, including *N. benthamiana*, *N. tabacum*, and *S. tuberosum*, or other plants such as *M. sativa*. Generally, in the control plant the beta(1,2)-xylosyltransferase gene is unmodified and it has wild-type levels of beta(1,2)-xylosyltransferase activity. "Wild type levels of beta(1,2)-xylosyltransferase activity" (also written "wildtype" or "wild-type"), as used herein, refers to the typical level of beta(1,2)-xylosyltransferase activity in a plant as it most commonly occurs in nature. Said control plant has thus not been provided either with a silencing nucleic acid molecule targeted to the endogenous beta(1,2)-xylosyltransferase encoding gene or with an allele of an beta(1,2)-xylosyltransferase gene associated with a low level of beta(1,2)-xylosyltransferase activity, such as a knock-out allele.

Said reduced levels of beta(1,2)-xylosyltransferase residues can consist of a reduction of at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 97%, or at least 99%. The amount of beta(1,2)-xylosylated glycan structures associated with a produced glycoprotein can be determined according to the methods described in this invention.

"Reduced levels of core alfa(1,3)-fucose residues and reduced levels of beta(1,2)-xylose residues" can consist of a reduction of the levels of glycans comprising alfa(1,3)-fucose residues, beta(1,2)-xylose residues, or alfa(1,3)-fucose and beta(1,2)-xylose residues. Said reduction can consist of a reduction of at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 97%, or at least 99%. The amount of alfa(1,3)-fucosylated and beta(1,2)-xylosylated glycan structures associated with a produced glycoprotein can be determined according to the methods described in this invention.

The level of beta(1,2)-xylosyltransferase activity can be reduced by reducing the expression of endogenous beta(1,2)-xylosyltransferase encoding genes.

By "reducing the expression" of a stated integer it is meant that transcription and/or translation and/or post-translational modification of the integer is inhibited or prevented or knocked-down or knocked-out or interrupted such that the specified integer has a reduced biological effect on a cell, tissue, organ or organism in which it would otherwise be expressed.

Those skilled in the art will be aware of whether expression is inhibited, interrupted or reduced, without undue experimentation. For example, the level of expression of a particular gene may be determined by polymerase chain reaction (PCR) following reverse transcription of an mRNA template molecule. Alternatively, the expression level of a genetic sequence may be determined by northern hybridisation analysis or dot-blot hybridisation analysis or in situ hybridisation analysis or similar technique, wherein mRNA is transferred to a membrane support and hybridised to a "probe"molecule which comprises a nucleotide sequence complementary to the nucleotide sequence of the mRNA transcript encoded by the gene-of-interest, labeled with a suitable reporter molecule such as a radioactively-labelled dNTP (eg [alpha-32P] dCTP or [alpha-35S] dCTP) or biotinylated dNTP, amongst others. Expression of the gene-of-interest may then be determined by detecting the appearance of a signal produced by the reporter molecule bound to the hybridised probe molecule.

Alternatively, the rate of transcription of a particular gene may be determined by nuclear run-on and/or nuclear run-off experiments, wherein nuclei are isolated from a particular cell or tissue and the rate of incorporation of rNTPs into specific mRNA molecules is determined. Alternatively, the expression of the gene-of-interest may be determined by RNase protection assay, wherein a labelled RNA probe or "riboprobe" which is complementary to the nucleotide sequence of mRNA encoded by said gene- of-interest is annealed to said mRNA for a time and under conditions sufficient for a double-stranded mRNA molecule to form, after which time the sample is subjected to digestion by RNase to remove single-stranded RNA molecules and in particular, to remove excess unhybridised riboprobe. Such approaches are described in detail by Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: a laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1989. 1659 p. ISBN 0-87969-309-6.

Those skilled in the art will also be aware of various immunological and enzymatic methods for detecting the level of expression of a particular gene at the protein level, for

example using rocket immunoelectrophoresis, ELISA, radioimmunoassay and western blot immunoelectrophoresis techniques, amongst others.

The level of beta(1,2)-xylosyltransferase activity can conveniently be reduced or eliminated by transcriptional or post-transcriptional silencing of the expression of endogenous beta(1,2)-xylosyltransferase encoding genes. To this end a silencing RNA molecule is introduced in the plant cells targeting the endogenous beta(1,2)-xylosyltransferase encoding genes.

As used herein, "silencing RNA" or "silencing RNA molecule" refers to any RNA molecule, which upon introduction into a plant cell, reduces the expression of a target gene. Such silencing RNA may e.g. be so-called "antisense RNA", whereby the RNA molecule comprises a sequence of at least 20 consecutive nucleotides having 95% sequence identity to the complement of the sequence of the target nucleic acid, preferably the coding sequence of the target gene. However, antisense RNA may also be directed to regulatory sequences of target genes, including the promoter sequences and transcription termination and polyadenylation signals. Silencing RNA further includes so-called "sense RNA" whereby the RNA molecule comprises a sequence of at least 20 consecutive nucleotides having 95% sequence identity to the sequence of the target nucleic acid. Other silencing RNA may be "unpolyadenylated RNA" comprising at least 20 consecutive nucleotides having 95% sequence identity to the complement of the sequence of the target nucleic acid, such as described in WO01/12824 or US6423885 (both documents herein incorporated by reference). Yet another type of silencing RNA is an RNA molecule as described in WO03/076619 (herein incorporated by reference) comprising at least 20 consecutive nucleotides having 95% sequence identity to the sequence of the target nucleic acid or the complement thereof, and further comprising a largely-double stranded region as described in WO03/076619 (including largely double stranded regions comprising a nuclear localization signal from a viroid of the Potato spindle tuber viroid-type or comprising CUG trinucleotide repeats). Silencing RNA may also be double stranded RNA comprising a sense and antisense strand as herein defined, wherein the sense and antisense strand are capable of base-pairing with each other to form a double stranded RNA region (preferably the said at least 20 consecutive nucleotides of the sense and antisense RNA are complementary to each other). The sense and antisense region may also be present within one RNA molecule such that a hairpin RNA (hpRNA) can be formed when the sense and antisense region form a double stranded RNA region. hpRNA is well-known within the art (see e.g WO99/53050, herein incorporated by reference). The hpRNA

may be classified as long hpRNA, having long, sense and antisense regions which can be largely complementary, but need not be entirely complementary (typically larger than about 200 bp, ranging between 200-1000 bp). hpRNA can also be rather small ranging in size from about 30 to about 42 bp, but not much longer than 94 bp (see WO04/073390, herein incorporated by reference). Silencing RNA may also be artificial micro-RNA molecules as described e.g. in WO2005/052170, WO2005/047505 or US 2005/0144667, or ta-siRNAs as described in WO2006/074400 (all documents incorporated herein by reference).

A suitable method for silencing the beta(1,2)-xylosyltransferase is the method as described in WO2009056155.

In a particular embodiment of the invention, the reduced level of beta(1,2)-xylosyltransferase is activity is the result of a knock-out mutation in endogenous beta(1,2)-xylosyltransferase genes.

"A knock-out mutation in endogenous beta(1,2)-xylosyltransferase genes" as used herein is a mutation that renders the beta(1,2)-xylosyltransferase gene inactive, wherein the inactive gene is characterized in that the gene does not encode a functional alfa(1,3)-fucosyltransferase protein. Said gene, also referred to as "knock-out gene" or "knock-out allele" can either be a gene that is not transcribed into a functional mRNA, or a gene of which the encoded RNA is not spliced correctly, or a gene not encoding a functional protein. Mutations that render the beta(1,2)-xylosyltransferase gene inactive thus comprise, for example, mutations in the promoter regions, mutations in the splice-sites, or mutations in the coding sequences resulting in amino acid substitutions or premature translation termination.

Suitable knock-out mutations in endogenous beta(1,2)-xylosyltransferase genes of *Nicotiana benthamiana* are the knock-outs as described in WO2010145846.

The alfa(1,3)-fucosyltransferase and the beta(1,2)-xylosyltransferase activity can be evaluated by determining the level of alfa(1,3)-fucose and the level of beta(1,2)-xylose residues on protein-bound N-glycans from a plant, respectively. The level of alfa(1,3)-fucose and the level of beta(1,2)-xylose residues on protein-bound N-glycans from a plant can be measured e.g. by Western blot analysis using fucose- or xylose specific antibodies, as described e.g. by Faye et al. (Analytical Biochemistry (1993) 209: 104-108) or by mass

spectrometry on glycans isolated from the plant's glycoproteins using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) as described e.g. by Kolarich and Altmann (Anal. Biochem. (2000) 285: 64-75), or using Liquid-Chromatography-ElectroSpray Ionization-Mass Spectrometry (LC/ESI/MS) as described by Pabst et al. (Analytical Chemistry (2007) 79: 5051-5057) or using Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) as described e.g. by Henriksson et al. (Biochem. J. (2003) 375: 61-73).

In yet another embodiment of the method of the invention, said plant or plant cell comprises at least five knock-out alfa(1,3)-fucosyltransferase genes.

At least five knock-out alfa(1,3)-fucosyltransferase genes can be five knock-out alfa(1,3)-fucosyltransferase genes, or six alfa(1,3)-fucosyltransferase genes, or seven alfa(1,3)-fucosyltransferase genes, or more than seven alfa(1,3)-fucosyltransferase genes.

Suitable knock-out alfa(1,3)-fucosyltransferase genes can be mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of nucleic acids encoding the amino acid sequence of SEQ ID No. 3, SEQ ID No. 6, SEQ ID No. 9, SEQ ID No. 12, SEQ ID No. 14, or of nucleic acids encoding amino acid sequences having at least 80%, or at least 85%, or at least 90%, or at least 95%, or at least 97%, or at least 98%, or at least 99% identity to these amino acid sequences.

Suitable knock-out alfa(1,3)-fucosyltransferase genes can further be mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of SEQ ID No. 1, SEQ ID No. 4, SEQ ID No. 7, SEQ ID No. 10, SEQ ID No. 13, or of nucleic acids having at least 80%, or at least 85%, or at least 90%, or at least 95%, or at least 97%, or at least 98%, or at least 99% identity to these sequences.

In yet another embodiment of the method of the invention, said knock-out alfa(1,3)-fucosyltransferase genes are mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of:

- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 3;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 6;

- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 9;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 12;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 14.

In a further embodiment, said knock-out alfa(1,3)-fucosyltransferase genes are mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of:

- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 1;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 4;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 7;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 10;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 13.

Suitable knock-out alfa(1,3)-fucosyltransferase genes for the invention are genes with one or more mutations selected from the group of mutations as depicted in Table 2 and Table 4.

In yet a further embodiment, said knock-out alfa(1,3)-fucosyltransferase gene is selected from the group consisting of:

- FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
- FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
- FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
- FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
- FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

A "mutated version" of a gene as used herein is a version of a gene which contains one or more mutations. A "native alfa(1,3)-fucosyltransferase", also "wild-type alfa(1,3)-fucosyltransferase" as used herein refers to a typical form of an alfa(1,3)-fucosyltransferase gene as it most commonly occurs in nature.

In another specific embodiment, said knock-out alfa(1,3)-fucosyltransferase genes occur in a homozygous state in the genome.

In another embodiment according to the invention, the method according to the invention is further characterized in that the expression of at least five endogenous alfa(1,3)-fucosyltransferase encoding genes is reduced through transcriptional or post-transcriptional silencing. Transcriptional and post-transcriptional silencing can suitably be achieved by introducing a silencing RNA molecule in the plant cells targeting the endogenous alfa(1,3)-fucosyltransferase encoding genes.

For silencing at least five endogenous alfa(1,3)-fucosyltransferase encoding genes, it is suitable to introduce more than one chimeric gene into the plant cells, characterized in that each of the chimeric genes encodes a silencing RNA molecule, each of which is suitable to silence at least one of the alfa(1,3)-fucosyltransferase genes. Alternatively, one chimeric gene can be introduced in the plant cells which encodes a silencing RNA molecule capable of silencing at least five alfa(1,3)-fucosyltransferase genes. Said one chimeric gene can comprise several regions of 21 consecutive nucleotides, each of which having at least 85% sequence identity to a region of 21 nucleotides occurring in at least one of the alfa(1,3)-fucosyltransferase genes. Alternatively, said one chimeric gene can comprise a region of 21 consecutive nucleotides characterized that at least five alfa(1,3)-fucosyltransferase genes comprise a sequence of 21 nucleotides having 85% identity to said region of 21 consecutive nucleotides.

A suitable methods for silencing the alfa(1,3)-fucosyltransferase genes of *Nicotiana benthamiana* are the methods as described in WO2009056155.

In yet a further embodiment, the plant cell according to the invention comprises at least one chimeric gene comprising the following operably linked DNA fragments: a plant-expressible promoter, a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene, a DNA region comprising a transcription termination and polyadenylation signal functional in plants. In a further embodiment, said DNA region yields an RNA molecule capable of forming a double-stranded RNA region at least between an RNA region transcribed from a first sense DNA region comprising a nucleotide sequence of at least 18 out of 21 nucleotides selected from SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 13, or the complement thereof, and an RNA region transcribed from a second antisense DNA region comprising a nucleotide sequence of at least 18 consecutive nucleotides which have at least 95% sequence identity to the complement of said first sense DNA region.

"An RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene" as used herein refers to a silencing RNA molecule which reduces the expression of at least one alfa(1,3)-fucosyltransferase encoding gene.

As used herein, the term "plant-expressible promoter" means a DNA sequence that is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S (Harpster *et al.* (1988) *Mol Gen Genet.* 212(1):182-90, the subterranean clover virus promoter No 4 or No 7 (WO9606932), or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An *et al.* (1996) *Plant Cell* 8(1):15-30), stem-specific promoters (Keller *et al.*, (1988) *EMBO J.* 7(12): 3625-3633), leaf specific promoters (Hudspeth *et al.* (1989) *Plant Mol Biol.* 12: 579-589), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller *et al.* (1989) *Genes Dev.* 3: 1639-1646), tuber-specific promoters (Keil *et al.* (1989) *EMBO J.* 8(5): 1323-1330), vascular tissue specific promoters (Peleman *et al.* (1989) *Gene* 84: 359-369), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

A "transcription termination and polyadenylation region" as used herein is a sequence that drives the cleavage of the nascent RNA, whereafter a poly(A) tail is added at the resulting RNA 3' end, functional in plants. Transcription termination and polyadenylation signals functional in plants include, but are not limited to, 3'nos, 3'35S, 3'his and 3'g7.

In yet a further embodiment, the plant cell according to the invention comprises a chimeric gene comprising a plant-expressible promoter, a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene, and a DNA region comprising a transcription termination and polyadenylation signal functional in plants, characterized in that said DNA region comprises the sequence of SEQ ID No. 19.

In another embodiment of the invention, the glycoproteins produced according to the methods of the invention are heterologous glycoproteins. In yet another embodiment, said heterologous proteins are expressed from a chimeric gene comprising the following

operably linked nucleic acid molecules: a plant-expressible promoter, a DNA region encoding said heterologous glycoprotein, a DNA region involved in transcription termination and polyadenylation. In yet another embodiment, the methods according to the invention further comprise the step of purification of said heterologous proteins.

The word "expression" as used herein shall be taken in its widest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

Heterologous glycoproteins, i.e. glycoproteins which are not normally expressed in such plant cells in nature, may include mammalian or human proteins, which can be used as therapeutics such as e.g. monoclonal antibodies. Conveniently, the foreign glycoproteins may be expressed from chimeric genes comprising a plant-expressible promoter and the coding region of the glycoprotein of interest, whereby the chimeric gene is stably integrated in the genome of the plant cell. Methods to express foreign proteins in plant cells are well known in the art. Alternatively, the foreign glycoproteins may also be expressed in a transient manner, e.g. using the viral vectors and methods described in WO02/088369, WO2006/079546 and WO2006/012906 or using the viral vectors described in WO89/08145, WO93/03161 and WO96/40867 or WO96/12028.

By “heterologous protein” it is understood a protein (i.e. a polypeptide) that is not expressed by the plant or plant cells in nature. This is in contrast with a homologous protein which is a protein naturally expressed by a plant or plant cell. Heterologous and homologous polypeptides that undergo post-translational N-glycosylation are referred to herein as heterologous or homologous glycoproteins.

Examples of heterologous proteins of interest that can be advantageously produced by the methods of this invention include, without limitation, cytokines, cytokine receptors, growth factors (e.g. EGF, HER-2, FGF-alpha, FGF-beta, TGF-alpha, TGF-beta, PDGF, IGF-I, IGF-2, NGF), growth factor receptors. Other examples include growth hormones (e.g. human growth hormone, bovine growth hormone); insulin (e.g., insulin A chain and insulin

B chain), pro-insulin, erythropoietin (EPO), colony stimulating factors (e.g. G-CSF, GM-CSF, M-CSF); interleukins; vascular endothelial growth factor (VEGF) and its receptor (VEGF-R), interferons, tumor necrosis factor and its receptors, thrombopoietin (TPO), thrombin, brain natriuretic peptide (BNP); clotting factors (e.g. Factor VIII, Factor IX, von Willebrands factor and the like), anti-clotting factors; tissue plasminogen activator (TPA), urokinase, follicle stimulating hormone (FSH), luteinizing hormone (LH), calcitonin, CD proteins (e. g., CD2, CD3, CD4, CD5, CD7, CD8, CDI Ia, CDI Ib, CD18, CD19, CD20, CD25, CD33, CD44, CD45, CD71, etc.), CTLA proteins (e.g.CTLA4); T-cell and B-cell receptor proteins, bone morphogenic proteins (BNPs, e.g. BMP-I, BMP-2, BMP-3, etc.), neurotrophic factors, e.g. bone derived neurotrophic factor (BDNF), neurotrophins, e.g. rennin, rheumatoid factor, RANTES, albumin, relaxin, macrophage inhibitory protein (e.g. MIP-I, MIP-2), viral proteins or antigens, surface membrane proteins, ion channel proteins, enzymes, regulatory proteins, immunomodulatory proteins, (e.g. HLA, MHC, the B7 family), homing receptors, transport proteins, superoxide dismutase (SOD), G-protein coupled receptor proteins (GPCRs), neuromodulatory proteins, Alzheimer's Disease associated proteins and peptides. Fusion proteins and polypeptides, chimeric proteins and polypeptides, as well as fragments or portions, or mutants, variants, or analogs of any of the aforementioned proteins and polypeptides are also included among the suitable proteins, polypeptides and peptides that can be produced by the methods of the present invention. The protein of interest can be a glycoprotein. One class of glycoproteins are viral glycoproteins, in particular subunits, than can be used to produce for example a vaccine. Some examples of viral proteins comprise proteins from rhinovirus, poliomyelitis virus, herpes virus, bovine herpes virus, influenza virus, newcastle disease virus, respiratory syncitio virus, measles virus, retrovirus, such as human immunodeficiency virus or a parvovirus or a papovavirus, rotavirus or a coronavirus, such as transmissable gastroenteritisvirus or a flavivirus, such as tick-borne encephalitis virus or yellow fever virus, a togavirus, such as rubella virus or eastern-, western-, or venezuelean equine encephalomyelitis virus, a hepatitis causing virus, such as hepatitis A or hepatitis B virus, a pestivirus, such as hog cholera virus or a rhabdovirus, such as rabies virus.

The heterologous glycoprotein can be an antibody or a fragment thereof. The term "antibody" refers to recombinant antibodies (for example of the classes IgD, IgG, IgA, IgM, IgE) and recombinant antibodies such as single-chain antibodies, chimeric and humanized antibodies and multi-specific antibodies. The term "antibody" also refers to fragments and derivatives of all of the foregoing, and may further comprise any modified or derivatised variants thereof that retain the ability to specifically bind an epitope. Antibody derivatives may comprise a protein or chemical moiety conjugated to an antibody. A monoclonal

antibody is capable of selectively binding to a target antigen or epitope. Antibodies include, monoclonal antibodies (mAbs), humanized or chimeric antibodies, camelized antibodies, camelid antibodies (nanobodies®), single chain antibodies (scFvs), Fab fragments, $F(ab')_2$ fragments, disulfide- linked Fvs (sdFv) fragments, anti-idiotypic (anti-Id) antibodies, intra-bodies, synthetic antibodies, and epitope-binding fragments of any of the above. The term "antibody" also refers to fusion protein that includes a region equivalent to the Fc region of an immunoglobulin. Also envisaged is the production in the plant or plant cells of the invention of so called dual-specificity antibodies (Bostrom J *et al* (2009) *Science* 323, 1610-1614).

Antibodies within the scope of the present invention include those comprising the amino acid sequences of the following antibodies: anti-HER2 antibodies including antibodies comprising the heavy and light chain variable regions (see US5,725,856) or Trastuzumab such as HERCEPTIN™; anti-CD20 antibodies such as chimeric anti-CD20 as in US5,736,137, a chimeric or humanized variant of the 2H7 antibody as in US5,721,108; anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti- VEGF antibody huA4.6.1 AVASTIN™ (WO 96/30046 and WO 98/45331); anti-EGFR (chimerized or humanized antibody as in WO 96/40210); anti-CD3 antibodies such as OKT3 (US4,515,893); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT) and (ZENAPAX) (US5,693,762). The present invention provides a method for the production of an antibody which comprises culturing a transformed plant cell or growing a transformed plant of the present invention. The produced antibody may be purified and formulated in accordance with standard procedures.

The DNA region encoding the heterologous glycoproteins may be codon optimized to increase the level of expression within the plant. By codon optimization it is meant the selection of appropriate DNA nucleotides for the synthesis of oligonucleotide building blocks, and their subsequent enzymatic assembly, of a structural gene or fragment thereof in order to approach codon usage in plants.

"Purification" as used herein is to isolate the heterologous protein from the mixture of total plant proteins. The level of purification can be to at least 50% purity, or to at least 60% purity, or to at least 70% purity, or to at least 80% purity, or to at least 85% purity, or to at least 90% purity, or to at least 95% purity, or to at least 98% purity, or to at least 99% purity. Methods for protein purification are well-known in the art and may consist of, but are not limited to, differential precipitation, ultracentrifugation, chromatography, or affinity purification.

Another embodiment of the invention provides a glycoprotein obtained by the methods according to the invention. In yet another embodiment, said glycoprotein has reduced levels of alfa(1,3)-fucose residues. In yet a further embodiment, said glycoprotein has reduced levels of alfa(1,3)-fucose residues and reduced levels of beta(1,2)-xylose residues.

Another embodiment according to the invention provides a *Nicotiana benthamiana* plant, or a cell, part, seed or progeny thereof, comprising at least three knock-out alfa(1,3)-fucosyltransferase genes. In yet another embodiment, said plant comprises at least five knock-out alfa(1,3)-fucosyltransferase genes.

At least five knock-out alfa(1,3)-fucosyltransferase genes can be five knock-out alfa(1,3)-fucosyltransferase genes, or six knock-out alfa(1,3)-fucosyltransferase genes, or seven knock-out alfa(1,3)-fucosyltransferase genes, or at least seven knock-out alfa(1,3)-fucosyltransferase genes.

Suitable knock-out alfa(1,3)-fucosyltransferase genes can be mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of nucleic acids encoding the amino acid sequence of SEQ ID No. 3, SEQ ID No. 6, SEQ ID No. 9, SEQ ID No. 12, SEQ ID No. 14, or of nucleic acids encoding amino acid sequences having at least 80%, or at least 85%, or at least 90%, or at least 95%, or at least 97%, or at least 98%, or at least 99% identity to these amino acid sequences.

Suitable knock-out alfa(1,3)-fucosyltransferase genes can further be mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of SEQ ID No. 1, SEQ ID No. 4, SEQ ID No. 7, SEQ ID No. 10, SEQ ID No. 13, or of nucleic acids having at least 80%, or at least 85%, or at least 90%, or at least 95%, or at least 97%, or at least 98%, or at least 99% identity to these sequences.

Another embodiment provides plants according to invention, wherein one or more of the knock-out alfa(1,3)-fucosyltransferase genes is a mutated version of the native alfa(1,3)-fucosyltransferase gene selected from the group consisting of:

- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 3;

- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 6;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 9;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 12;
- a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 14.

Yet another embodiment provides plants according to the invention, wherein one or more of the knock-out alfa(1,3)-fucosyltransferase genes is a mutated version of the native alfa(1,3)-fucosyltransferase gene selected from the group consisting of:

- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 1;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 4;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 7;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 10;
- a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 13.

Yet another embodiment provides plants according to the invention wherein the knock-out alfa(1,3)-fucosyltransferase gene is selected from the group consisting of:

- FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
- FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
- FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
- FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
- FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

In a further embodiment, the plant or plant cell according to the invention is homozygous for the knock-out alfa(1,3)-fucosyltransferase genes.

In yet another embodiment, the plant or plant cell according to the invention further comprises at least one knock-out beta(1,2)-xylosyltransferase gene, wherein said knock-out beta(1,2)-xylosyltransferase gene comprises a mutated DNA region consisting of one or more inserted, deleted or substituted nucleotides compared to a corresponding wild-type DNA region in the beta(1,2)-xylosyltransferase gene and wherein said knock-out beta(1,2)-xylosyltransferase gene does not encode a functional beta(1,2)-xylosyltransferase protein.

In yet another embodiment, the said plant or plant cell further comprises at least one chimeric gene comprising the following operably linked DNA fragments: a plant-expressible promoter; a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene; and a DNA region comprising a transcription termination and polyadenylation signal functional in plants.
Suitably, said DNA region yields an RNA molecule capable of forming a double-stranded RNA region at least between an RNA region transcribed from a first sense DNA region comprising a nucleotide sequence of at least 18 out of 21 nucleotides selected from SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 13, or the complement thereof, and an RNA region transcribed from a second antisense DNA region comprising a nucleotide sequence of at least 18 consecutive nucleotides which have at least 95% sequence identity to the complement of said first sense DNA region.
In a further embodiment, said DNA region comprises the sequence of SEQ ID No. 19.

In a further embodiment, the plant or plant cell according to the invention further comprises a glycoprotein foreign to said plant or plant cell. In yet another embodiment, said glycoprotein is expressed from a chimeric gene comprising the following operably linked nucleic acid molecules: a plant-expressible promoter, a DNA region encoding said heterologous glycoprotein, a DNA region involved in transcription termination and polyadenylation.

Another embodiment according to the invention provides a knock-out allele of an alfa(1,3)-fucosyltransferase gene selected from the group consisting of:

- FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
- FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
- FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
- FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
- FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

Yet another embodiment provides the use of the methods according to the invention to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues. A further embodiment provides the use of the methods according to the invention to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues and with a reduced level of beta(1,2)-xylose residues.

Plants according to the invention can be further crossed by traditional breeding techniques and can be used to produce seeds to obtain progeny plants comprising glycoproteins with reduced levels of alfa(1,3)-fucosylation and/or reduced levels of beta(1,2)-xylosylation.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

Unless stated otherwise in the Examples, all recombinant techniques are carried out according to standard protocols as described in "Sambrook J and Russell DW (eds.) (2001) Molecular Cloning: A Laboratory Manual, 3rd Edition, Cold Spring Harbor Laboratory Press, New York" and in "Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K (eds.) (2006) Current Protocols in Molecular Biology. John Wiley & Sons, New York". Standard materials and references are described in "Croy RDD (ed.) (1993) Plant Molecular Biology LabFax, BIOS Scientific Publishers Ltd., Oxford and Blackwell Scientific Publications, Oxford" and in "Brown TA, (1998) Molecular Biology LabFax, 2nd Edition, Academic Press, San Diego". Standard materials and methods for polymerase chain reactions (PCR) can be found in "McPherson MJ and Møller SG (2000) PCR (The Basics), BIOS Scientific Publishers Ltd., Oxford" and in "PCR Applications Manual, 3rd Edition (2006), Roche Diagnostics GmbH, Mannheim or www.roche-applied-science.com ".

All patents, patent applications, and publications or public disclosures (including publications on internet) referred to or cited herein are incorporated by reference in their entirety.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1:	FucTA genomic DNA
SEQ ID No 2:	FucTA coding sequence
SEQ ID No 3:	FucTA protein
SEQ ID No 4:	FucTB genomic DNA

SEQ ID No 5: FucTB coding sequence

SEQ ID No 6: FucTB protein

SEQ ID No 7: FucTC genomic DNA

SEQ ID No 8: FucTC coding sequence

SEQ ID No 9: FucTC protein

SEQ ID No 10: FucTD genomic DNA

SEQ ID No 11: FucTD coding sequence

SEQ ID No 12: FucTD protein

SEQ ID No 13: FucTE genomic DNA

SEQ ID No 14: FucTE protein

SEQ ID No 15: Primer VH031

SEQ ID No 16: Primer VH032

SEQ ID No 17: Primer VH033

SEQ ID No 18: Primer VH034

SEQ ID No 19: Sequence encoding FucT silencing RNA

SEQ ID No 20: Sequence encoding FucT silencing RNA: part of the Nicotiana benthamiana FucTB coding sequence from 1183 to 1265: gaaactgtctatcatgtatatgtacgtgaaagagggaggtttgagatggattccattttcttaaggtcgagtgatttgtcttt

SEQ ID No 21: Sequence encoding FucT silencing RNA:

```
FH   Key             Location/Qualifiers
FH
FT   intron          84..307
FT                   /vntifkey="15"
FT                   /label=intron\2
FT                   /note="Arabidopsis XylT gene intron 2"
FT   misc_feature    1..83
FT                   /vntifkey="21"
FT                   /label=Nb\FucTB
FT                   /note="Part of N. benthamiana FucTB coding sequence
from 1183 - 1265"
FT   misc_feature    complement(308..390)
FT                   /vntifkey="21"
FT                   /label=Nb\FucTB
FT                   /note="Inverse complement of part of N. benthamiana
FucTB coding sequence from 1183 - 1265 "
SQ   Sequence 390 BP; 100 A; 71 C; 79 G; 140 t;

     gaaactgtct atcatgtata tgtacgtgaa agagggaggt ttgagatgga ttccattttc  60
     ttaaggtcga gtgatttgtc tttgatccac tgcacggtat gctcctcttc ttgttcatgg 120
     tcatgatcct tatatgagca gggaaagtcc agtttagact tgtagttagt tactcttcgt 180
     tataggattt ggatttcttg cgtgtttatg gttttagttt ccctcctttg atgaataaaa 240
     ttgaatcttg tatgagtttc atatccatgt tgtgaatctt tttgcagacg cagctaggta 300
     ccggatcaaa gacaaatcac tcgaccttaa gaaaatggaa tccatctcaa acctccctct 360
     ttcacgtaca tatacatgat agacagtttc
```

Examples

1. Isolating the FucT genes from *Nicotiana benthamiana*.

To produce a FucT KO plant, it was needed to identify and isolate all members of the FucT gene family. Therefore, we first determined the gene family size by Southern blot analysis. Genomic DNA from *N. benthamiana* was digested with EcoRI, EcoRV, PstI, HindIII, NsiI, or AseI, run on 1% agarose gel and blotted on nylon membrane. The blots were hybridized with a cDNA clone of FucTA from *N. benthamiana* (Strasser et al. (2008) Plant Biotech J. 6:392). After exposure, the autoradiogram showed up to seven hybridizing bands per lane indicating a family of maximum seven genes (Figure 1).

To isolate all members of this FucT gene family, 2 BAC libraries were constructed by Amplicon Express. Each covered the genome 2.5 fold using MboI and HindIII as cloning enzymes, respectively. The libraries were screened with the FucTA cDNA probe. In total, 32 BAC clones were found. These clones were classified into different families based on Southern blot analyses comparing the hybridization pattern of each individual clone with the hybridization pattern of *N. benthamiana* genomic DNA (Figure 2). Of the 32 clones, 8 did not hybridize. The remaining clones could be classified into 8 families. Five of these families displayed hybridization patterns that overlapped with bands in the *N. benthamiana* genomic Southern blot hybridization.

One representative of each BAC clone family was sequenced using 454 sequencing technology and analyzed for the presence of a FucT gene by BLAST homology search using the FucTA cDNA sequence. Of the 8 families tested in this way, five contained FucT sequences that were all full length with respect to the FucTA coding sequence. These five genes were named FucTA, -B, -C, -D, and –E. The sequences of these five FucT genes are represented in SEQ ID No 1, SEQ ID No 4, SEQ ID No 7, SEQ ID No 10, and SEQ ID No 13, respectively.

EST2Genome (Mott (1997) Comput. Applic. 13:477) analysis using these contigs and the published FucTA cDNA sequence, showed that all genes except FucTE have the same number of introns as compared to the *A. thaliana* FucT-A and –B genes and that the intron-exon boundaries are also preserved between these two species. Surprisingly, no introns were found in the *N. benthamiana* FucTE gene. The FucT-D gene was found to contain an unusually large intron 1 of 7833 bp.

Analysis of the upstream sequences for promoter elements using TSSP (Shahmuradov et al. (2005) Nucl. Acids Res. 33:1069) showed that all genes except FucTE had TATA regions predicted with high confidence levels. In addition, analysis of the amino acid sequence of FucTE gene showed that it contains a Tyrosine to Aspartic Acid substitution at position 288 (Y288D). This position is part of the highly conserved donor substrate binding site ("MOTIFII") and mutation of this Tyrosine residue has been shown to completely inactivate the enzyme activity of human FucT VI (Jost et al. 2005 Glycobiology 15:165). By contrast, all other *N. benthamiana* FucT genes contain the conserved Tyrosine residue at this position. Together, this indicates that FucTE is likely an inactive gene coding for an inactive FucT enzyme.

Finally, to determine the homology between the genes, we aligned the derived coding sequences of the genes on the nucleotide level using the Clonemanager program, resulting in a FucT gene family divided in two groups: FucTA and FucTB form one group, FucTA has 100% identity to the previously published *N. benthamiana* FucTA cDNA (Strasser et al. (2008) Plant Biotech J. 6:392). The coding regions of FucTA and –B have 96% identity. The main striking difference between the two genes is that FucTB has a shorter coding sequence due to a premature stop codon. FucTC, FucTD and FucTE form the second group. All three genes have 96% identity in the coding regions. Genes from the two groups share 80% relative identity.

2. EMS mutagenesis

We used EMS mutagenesis to come to a selection of null mutations for each FucT gene. Ethyl MethaneSulfonate (EMS) causes G -> A and C -> T point mutations by alkylating Guanine (G). These point mutations can knock out genes if they generate null mutations by inducing stop codons or splice site mutations. Using this method we can screen for knock outs for all FucT genes. A total knock out will be achieved after crossing these mutants.

Determination of the optimal EMS dosage for M2 seed production.

Different EMS dosages and the effect on seed set, germination and plant phenotype were tested. This was needed to find out the optimal EMS dose to find EMS induced FucT knock outs in *N. benthamiana*.

The optimum dose for EMS mutagenesis was determined by treating seeds with 0, 50, 75, 100, 150, and 200 mM EMS. Briefly, seeds were imbibed for 2 hours at room temperature, treated with EMS for 4 hours at room temperature and washed 5 times for 15 minutes at

room temperature. Seeds were dried overnight and sown immediately. The effects on germination, seedling lethality and plant fertility were recorded. As *N. benthamiana* most probably is an amphidiploid species from a combination of *N. debneyi* and *N. suaveolens* (Goodspeed, T. H. 1954 Pages 485-487 in: The Genus *Nicotiana*: Origins, Relationships and Evolution of its Species in the Light of Their Distribution, Morphology and Cytogenetics. Chronica Botanica, Waltham, MA, U.S.A.) they initially were also included in the tests. However, as they showed to be less sensitive to EMS as compared to *N. bethamiana* (data not shown) they were not used for the fertility tests. Although EMS treatment caused a delay in germination (Figure 3A), no lethality was detected up to 75 mM EMS. At higher EMS doses, lethality rose quickly and at 150 mM no seeds survived the treatment (Figure 3B). Fertility already was affected at 50 mM. By treating the seeds with 75 mM approximately 60 % of the M1 plants were infertile (Figure 3C). Based on these results, the optimum EMS dose was set at 75 mM.

Production of EMS-mutagenized plants and DNA samples of M2 populations to screen for FucT mutants.

To have a good chance finding our mutants, we needed to screen about 10000 plants. To obtain more than 10000 M2 plants by using the EMS dosage of 75mM, we needed to grow at least 20000 M1 plants. At the determined density and generation time, 7000 M1 plants could be grown in 4 months. Therefore, at least 3 M1 populations needed to be grown.

M2 seed was sown and a DNA extraction on leaf samples of the M2 *N. benthamiana* plants was done. The DNA extraction was done in-house, extracting 4 leaf discs per plant following the in-house Edwards and Kingfisher method. DNA plates coming from 1 EMS treatment were defined as EMS batch.

In total we made 6 EMS batches. Two batches failed: batch 2 due to a bad mutation frequency, batch 4 due to the plant death unrelated to EMS mutagenesis. Together, four batches were left, comprising 99 plates of 95 DNA samples each extracted from M2 *N. benthamiana* leaf samples. On position H12 of each plate we included an internal control DNA sample of *N. benthamiana* accession NBNPGS2 from the USDA National Germplasm System (accession code PI555684). This accession contained several known SNPs compared to the *benthamiana* accession used for EMS mutagenesis (i.e. Cultivar "BENTHAMIANA" supplied by Icon Genetics GmbH). The positions of these SNPs are summarized in Table 1. Plates were stored at -70°C.

Table 1: SNP's in the sequences of the FucT genes between Bayer's "BENTHAMIANA" and NBNPGS2 accessions (USDA National Germplasm System accession PI555684).

	exon 3(target 1)		exon 1 (target2)	
	position	SNP	position	SNP
FucTA	3080	T/C	32	A/T
			63	C/G
			76	A/G
FucTB			218	T/A
			296	A/C
			307	G/T
FucTC	2809	C/T		
FucTD	9653	G/A	34	A/C
	9656	C/A	56	T/C
	9710	G/A	107	T/C
	9833	T/C	192	T/C
FucTE	582	T/A	353	G/A
	708	T/C	427	A/T
	723	A/G		
	725	C/A		
	783	C/T		
	912	G/T		

Detecting EMS-induced point mutations by direct sequencing and Single Nucleotide Polymorphism (SNP) detection.

For high throughput detection of the EMS-induced point mutations by direct sequence analysis, we used the method described by Smits et al. (2006), Pharmacogenet. Genomics 16:159. The method was adapted for us by Agowa GmbH (currently part of LGC laboratory services). Briefly, specific gene fragments were amplified by PCR from DNA of leaf tissue of individual plants using gene specific primers. Each primer carried an additional sequence at its 5' end that would allow the sequence of both strands of the resulting PCR fragment to be analyzed.

The chromatograms of sequences were analyzed for Single Nucleotide Polymorphisms (SNPs) by comparing them to the FucTA, FucTB, FucTC, FucTD and FucTE sequences in NovoSNP (Weckx, S. et al. 2005 Genome Research 15:436).

Defining the target area for mutagenesis detection.

Because the SNP detection by direct sequencing was limited to sequence fragments of 500 bp, it was necessary to identify a 500 bp region in the FucTA- E genes that had the highest chance to produce a null mutation when mutagenized with EMS. Therefore we needed to identify a region that (1) had the highest density of codons that can change into stop codons by one G to A or C to T mutation and/or splice donor and acceptor sites and (2) was placed in or upstream of a catalytic or conserved domain.

In order to find the highest density of candidate stop or splice mutations, we used an algorithm that identifies all codons in a coding sequence that can be mutated to a stop codon or a splice mutant by one EMS mutation.

Two general targets were defined for mutagenesis detection within the FucT genes:

For our first target our choice was based on a shared conserved amino acid sequence for the α1,3- FucT's "MOTIF II" and 2 other motifs, "Mn binding" and "SSD motif", upstream of "MOTIFII" (Jost et al. 2005 Glycobiology 15:165; Wilson et al. 2001 Biochim Biophys Acta. 1527:88). Therefore as target we took an exon between "MOTIFII" and the "Mn binding, SSD motif" described above. For the FucTA- D genes this was exon3 (nt 2833-3074 of SEQ ID No 1 for FucTA; nt 2813-3054 of SEQ ID No 4 for FucTB, nt 2565-2806 of SEQ ID No 7 for FucTC, and nt 9685-9926 of SEQ ID No 10 for FucTD), all having a length of 241bp; for FucTE (consisting of only one exon) we took a fragment of 320bp (nt 592-912 of SEQ ID No 13).

We screened a second target to have more chance in finding mutations. We took exon1, having the highest density of codons that can change into stop codons (nt 1-354 of SEQ ID No 1 for FucTA, nt 1-354 of SEQ ID No 4 for FucTB, nt 1-396 of SEQ ID No 7 for FucTC, nt 1-396 of SEQ ID No 10 for FucTD), and a fragment of 396 bp for FucTE (nt 1-396 of SEQ ID No 13).

As screening for mutants delivered stop codon mutants for all genes except FucTE and FucTA, of which the latter only delivered splice site mutants, it was decided to include a third target for the FucTA gene. This target was located in exon 2 (nt 1098-1258 of SEQ ID No 1).

For each gene, the possible SNP's causing a stop codon or splice site mutation are listed per target in Tables 2 and 3. It is clear that using exon1 as target should give a lot more possible stop codon- or splice site mutation positions. However these mutations had a lower confidence level to produce an effective knock out mutant, because it is possible that an ATG downstream of the mutation might function as a new start codon. This then could produce a protein devoid of a transmembrane domain which still could have an active glycosyltransferase activity (Jost et al., 2005, Glycobiology 15:165).

Table 2: Exon3, splice-site/stopcodon mutation prediction list of FucT genes. Nucleotides that, when mutated with EMS, would result in the mutation of a splice-site or the introduction of a stopcodon are indicated gray. Dashed lines indicate the actual splice site. The positions of the nucleotides are given in the gene sequences and in the coding sequences.

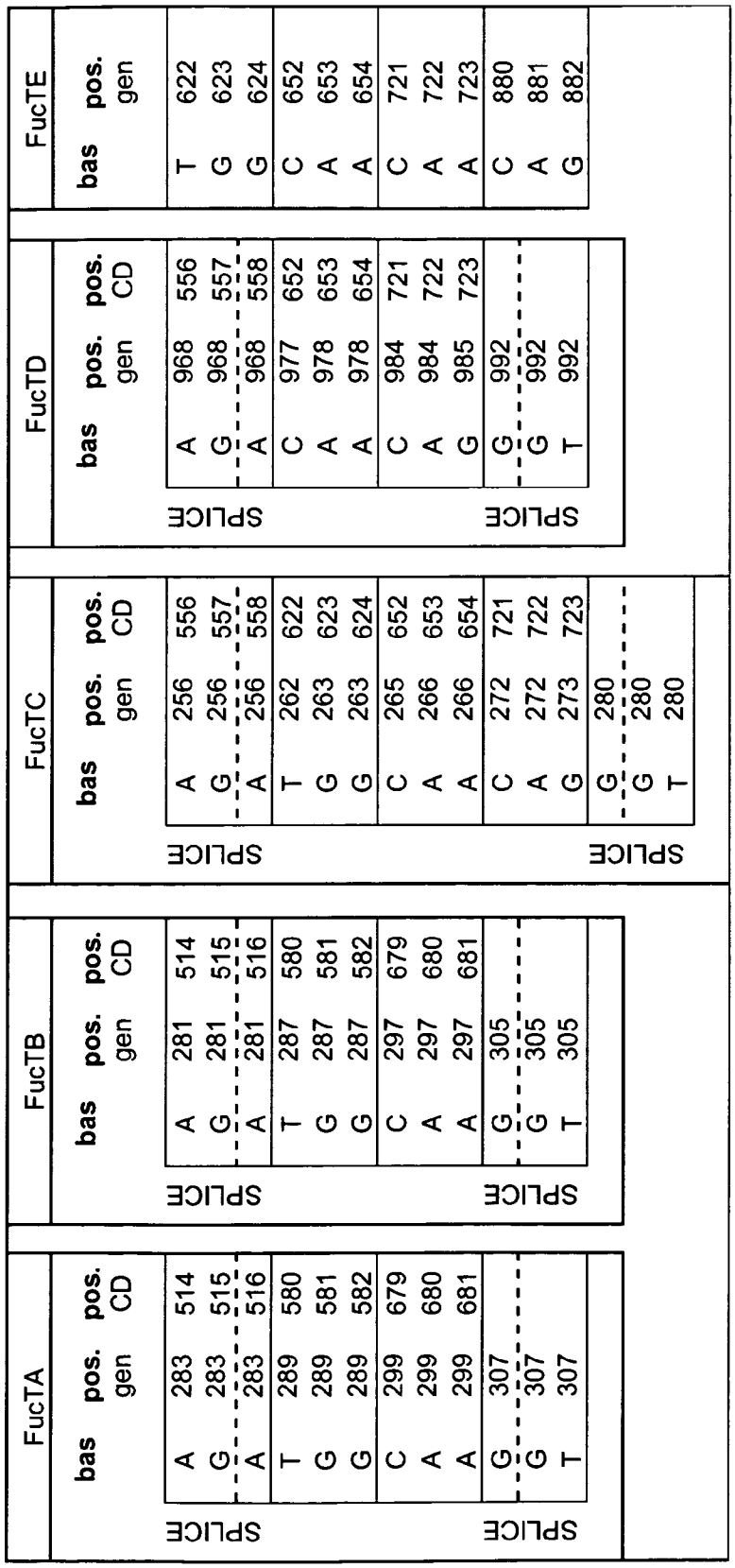
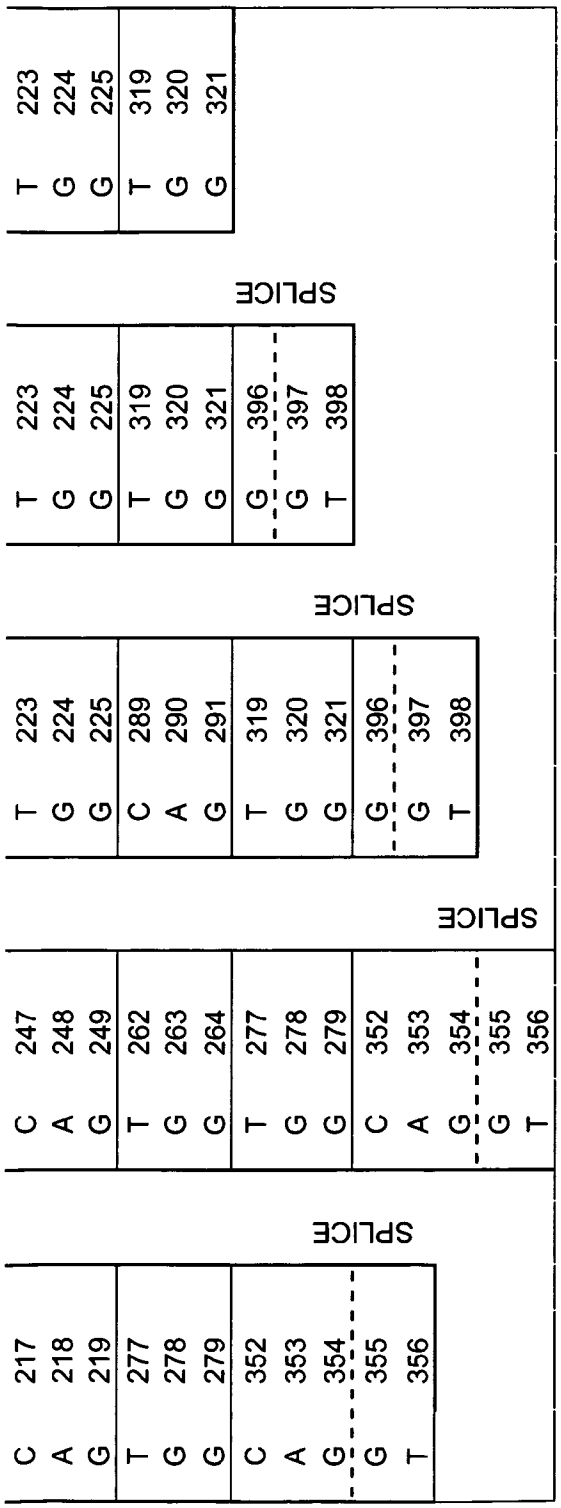


Table 3: Exon1, splice site/stopcodon mutation prediction lists FucT genes. Nucleotides that, when mutated with EMS, would result in the mutation of a splice site or the introduction of a stopcodon are indicated gray. Dashed lines indicate the actual splice site.

FucTA			FucTB			FucTC			FucTD			FucTE		
base	pos.	gene	base	pos.	gene	base	pos.	gene	base	pos.	gene	base	pos.	gene
C	37		C	37		C	22		C	22		C	22	
A	38		A	38		A	23		A	23		A	23	
A	39		A	39		A	24		A	24		A	24	
T	40		T	40		C	76		C	76		C	76	
G	41		G	41		A	77		A	77		A	77	
G	42		G	42		A	78		A	78		A	78	
T	49		T	49		T	85		T	85		T	85	
G	50		G	50		G	86		G	86		G	86	
G	51		G	51		G	87		G	87		G	87	
C	103		C	103		T	94		T	94		T	94	
G	104		G	104		G	95		G	95		G	95	
A	105		A	105		G	96		G	96		G	96	
T	133		T	133		C	148		C	148		C	148	
G	134		G	134		G	149		G	149		G	149	
G	135		G	135		A	150		A	150		A	150	
C	151		C	151		T	187		T	187		T	187	
A	152		A	152		G	188		G	188		G	188	
G	153		G	153		G	189		G	189		G	189	
T	169		T	169		C	205		C	205		C	205	
G	170		G	170		A	206		A	206		A	206	
G	171		G	171		G	207		G	207		G	207	


C A G
217 218 219
T G G
277 278 279
C A G
352 353 354
G T
355 356
SPLICE
C A G
247 248 249
T G G
262 263 264
T G G
277 278 279
C A G
352 353 354
G T
355 356
SPLICE
T G G
223 224 225
C A G
289 290 291
T G G
319 320 321
G G T
396 397 398
SPLICE
T G G
223 224 225
T G G
319 320 321
G G T
396 397 398
SPLICE
T G G
223 224 225
T G G
319 320 321

Results from screening the different EMS-mutagenized populations for possible knock-out mutations in the different FucT genes

For the FucT genes, the following number of EMS lines were screened: 4275 M2 individuals were screened for mutations in FucTA, 8075 for FucTB, 6555 for FucTC, 6270 for FucTD and 4370 for FucTE. The following number of putative null alleles were identified: three in FucTA, two splice site mutations and one stop codon mutation, respectively labeled FucT001, FucT004, and FucT013. Two putative null alleles, respectively one splice site mutation and one stop codon mutation, were identified for FucTB, labeled FucT006 and FucT008. For FucTC, 4 putative null alleles were identified, respectively 1 splice site mutation and three stop codon postitions, labeled FucT007, FucT010, FucT011 and FucT012. For FucTD, one splice site mutation and one stop codon mutation, were identified, labeled FucT005 and FucT009. Finally for FucTE, no stop codon mutations were identified. Instead, two alleles with substitution mutations were identified, labeled FucT002 and FucT003. The FucT003 substitution was located in the conserved "MOTIFII".

Table 4 summarizes the results of the screening for FucT genes: mutation position, mutation sequence and mutant type.

Table 4: Overview of possible EMS mutants for the FucT genes. Seeds comprising the mutants FucT004, FucT006, FucT007, FucT009 and FucT003 have been deposited at the National Collection of Industrial, Marine and Food Bacteria (NCIMB), NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB219YA, Scotland, on 12 September 2011, under accession number NCIMB 41860.

EMS mutants for FucTA					
Mutant Name	Position	WT Sequence	MUT sequence	Allele	Type
FucT001	3074	**G**GT	**A**GT	FucTA-1	SPL
FucT004	355	G**G**T	G**A**T	FucTA-2	SPL
FucT013	1176	**C**AA	**T**AA	FucTA-3	STOP
EMS mutants for FucTB					
Mutant Name	Position	WT Sequence	MUT sequence	Allele	Type
FucT006	3054	**G**GT	**A**GT	FucTB-1	SPL
FucT008	135	TG**G**	TG**A**	FucTB-2	STOP
EMS mutants for FucTC					
Mutant Name	Position	WT Sequence	MUT sequence	Allele	Type
FucT007	2807	G**G**T	G**A**T	FucTC-1	SPL
FucT010	188	T**G**G	T**A**G	FucTC-2	STOP
FucT011	86	T**G**G	T**A**G	FucTC-3	STOP
FucT012	87	T**G**G	T**G**A	FucTC-4	STOP
EMS mutants for FucTD					
Mutant Name	Position	WT Sequence	MUT sequence	Allele	Type
FucT005	397	G**G**T	G**A**T	FucTD-1	SPL
FucT009	224	T**G**G	T**A**G	FucTD-2	STOP
EMS mutants for FucTE					
Mutant Name	Position	WT Sequence	MUT sequence	Allele	Type
FucT002	811	**G**AA (Glu)	**A**AA (Lys)	FucTE-1	SUBST
FucT003	910	**G**TG (Val)	**A**TG (Met)	FucTE-2	SUBST

3. Crossing scheme to produce *N. benthamiana* plants homozygous for knock out mutants of all XylT and FucT genes: the seven-fold knock out plant.

We retrieved homozygous mutants for all lines, listed in Table 4, by sowing and screening 24 plants from the original M2 seed lot in which the mutation had been identified. DNA samples from each of these plants were screened using the direct sequencing technique described above. We were unable to retrieve mutant FucT013.

The homozygous mutants that were selected this way, were allowed to self-fertilize to create a stable mutant seedlot. In addition, a selected number of mutants were entered into a 5-fold backcrossing scheme with the "BENTHAMIANA" accession to eliminate most if not all of the mutation drag. Finally, a selected number of mutants were entered in a crossing scheme to produce the 7-fold knock out plants. The crossing scheme is shown in Figure 4. The final set of mutants that were used to generate the 7-fold knock out plant was: XYL001 (XylTg14-1 as described in WO2010145846), XYL002 (XylTg19-1 as described in WO2010145846), FucT003, FucT004, FucT006, FucT007, FucT009. The selection of the final set of FucT mutants was based on a gene transcription- and a complementation assay. Both are described below.

In order to be able to quickly and more economically identify zygosity of the mutant alleles in the back-crossing and crossing schemes describes above, an End Point TaqMan assay was designed by Applied Bioscience. The RT-PCR analyses for this were run in-house. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluorescent and the quench molecules prevents the detection of a fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This uncouples the fluorescent and quenching dyes. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage which, in turn, is related to the zygosity level of the target. When compared to an internal standard, the level of fluorescence can thus be translated into the zygosity levels: "wt", "heterozygous" and "homozygous".

4. Linkage analysis of the FucT genes.

To determine whether any of the FucT genes were genetically linked, we performed a linkage analysis making use of the SNPs in all FucT genes in accessions "BENTHAMIANA" and NBNPGS2 (USDA National Germplasm System accession PI555684; see also Table 1). To this end, BENTHAMIANA and NBNPGS2 were crossed, the F1 was crossed with BENTHAMIANA, and the FucT genotypes of 576 individuals from the next BC1 generation were analyzed.

If no linkage exists between any of the FucT genes, alleles would be seemingly randomly spread over the different individual's genotypes. If linkage exits between two or more FucT genes, this would show up as approximately 50% of the individuals being homozygous for two or more specific FucT genes. As the latter was not observed in the

population of 96 that was analyzed, we concluded that the five FucT genes are unlinked.

5. Determining whether the different FucT genes are being transcribed.

As the crossing scheme for the full knock out plant would run over 5 generations, we looked for opportunities to shorten this timeline. One possibility was to check whether any of the five FucT genes was not expressed. To determine this, we amplified FucT transcripts from leaf mRNA using primer sets with broad specificity. We then cloned and sequenced individual cDNAs resulting from this amplification. Sequence analysis of this set of clones should thus reveal if and which FucT genes were expressed. In addition, as we used primers that hybridized to regions that were conserved between FucT genes, we could pick up additional genes that we might have missed in the BAC screening.

cDNA was prepared from mRNA extracted from *N. benthamiana* leaves, following the protocol of the superscript II (Invitrogen) kit.

We performed a PCR on these cDNA samples, using primers designed on the FucTA CDS, taking the SNP's between genes into account. Using primers VH031 (SEQ ID No. 15) and VH032 (SEQ ID No. 16), described as primer combination 1 (PC1), a fragment of 570bp will be amplified. Using primer combination 2 (PC2), formed by primers VH033 (SEQ ID No. 17) and VH034 (SEQ ID No. 18), a fragment of 348bp will be amplified. The PCR's were run with annealing temperatures of 56°C (PC2) and 62°C (PC1), using a standard PCR mix [10 µl Go Taq buffer 5x; 1 µl dNTM 10 mM; 1 µl forward primer 10 µM; 1 µl reverse primer 10 µM; 0.4 µl Taq polymerase 5 U/µl; 2 µl purified PCR product in 50 µl total volume] and standard protocol [2 min 94 °C; 30x[30 sec 94°C , 30 sec 56°C /62°C , 30 sec 72°C], 10 min 72°C].

The resulting PCR products were purified with the Qiagen PCR purification kit, cloned in the PGemT Easy vector (Promega) and transformed into commercial thermo competent TOP10 cells (Invitrogen). 100µl was plated out on LB plates containing 100µg/ml triacelline. 192 clones resulting from primer combination PC1 and 96 from PC2 were sequenced by AGOWA. Based on SNPs in the five FucT sequences, it was possible to distinguish which of the different FucT genes was expressed.

For PC1, 148 clones gave usable sequence information resulting in 61 clones homologous to FucTA, 58 to FucTB, 2 to FucTC, 27 to FucTD and none for FucTE, 44 samples failed by sequencing. Checking the 96 clones of PC2, we found 15 clones homologous to FucTA, 39 to FucTB, none to FucTC, 12 to FucTD and none to FucTE, 30 samples failed by sequencing. In addition, none of the two primer combinations produced any new FucT sequences.

Together, this indicated that likely all FucT genes except for FucTE are expressed in *N. benthamiana* leaves. These findings corroborate the TSSP prediction data presented in example 1. In addition, these results indicated that likely no other FucT genes are present besides the five that were identified by BAC screening.

As FucTE appeared not to be expressed in *N. benthamiana* leaves, we decided to keep the FucTE gene as last one to cross into to the crossing scheme for the 7-fold knock out plant (see "generation 4" in Figure 4).

6. Complementation assay shows which FucT genes are likely active and which mutations are likely null mutations.

In order to determine the functionality of the individual FucT genes and also to determine whether the putative null mutations, that were isolated from our EMS screen, are true null or knock-out mutants, we devised a complementation assay. In this assay, the mutant to be complemented was an *Arabidopsis thaliana* line in which the FucT and XylT genes were knocked out by T-DNA insertion ("triple knock-out mutant"). This line has been described by Kang et al. (2008) Proc Natl Acad Sci USA and was also created in our laboratory by crossing three different T-DNA knock out lines available from SALK (see also WO2010121818).

To set up the system, we first tested whether the *Arabidopsis* triple mutant could be complemented with any one of the *N. benthamiana* FucT genes. We transformed the *Arabidopsis* triple mutant, using the *Agrobacterium* dipping method, with a T-DNA containing the cDNA sequence of one of the FucT genes driven by the CaMV 35S promoter. The cDNA sequence was produced synthetically based on the predicted coding sequence and intron-exon boundaries of the genes. After selection of the transformants using basta (glufosinate), protein samples from leaf tissue were analyzed for the presence of glycans containing core α1,3 fucose using a western blot probed with an anti-core α1,3 fucose antibody. This antibody was prepared as described by Faye et al. (1993) Anal Biochem 209:104. In Figure 5 (left panel) the results show that the *A. thaliana* triple mutant can be complemented by the *N. benthamiana* FucTA cDNA. The wt control lane shows a clear chemoluminescence signal, produced by binding of the antibody to core α1,3 fucoses. No chemoluminescence signal was detected in the lane containing protein sample from *A. thaliana* triple mutant. This was caused by absence of core α1,3 fucoses as a result of inactivation of the endogenous FucT genes. By contrast, a clear signal could be detected in the lanes containing protein from several different individual triple mutants transformed with the FucTA cDNA. Together, this shows that the complementation assay can be used to determine whether the *N. benthamiana* FucT genes are active.

Using this assay, we have shown that all genes except for FucTB and FucTE are able to complement and, therefore, represent active genes (data not shown). The fact that FucTB was unable to complement and therefore probably represents an inactive gene was unexpected because FucTB is 100% homologous to the FucTA gene except for a premature stop codon removing 41 amino acids from the C-terminal end of the FucT protein. The fact that FucTE probably represents an inactive gene, based on the complementation assay, is in line with the finding that this gene also does not seem to be transcribed in *N. benthamiana* leaves and contains an inactivating Y288D substitution in MOTIFII.

Next, we used this complementation assay to determine whether the putative null mutations, that were isolated from the EMS-mutagenized populations, indeed rendered the respective FucT genes inactive. The right panel of Figure 5 shows the results of a complementation assay with a FucTA in which an EMS mutation was simulated at the 8th possible stop codon (position 217; see table 3 FucTA gene). From the absence of a chemoluminescence signal in lanes 1 to 5 in the section labeled "At3KO + mut FucTA (stop in Exon1)", it is clear that this mutated version of FucTA cannot complement the triple knock-out mutant. Absence of chemoluminescence was not caused by the fact that the plants were not transformed (see "copy nr" below each of the lanes) nor by the fact the mutated gene was not expressed as determined by real time RT-PCR (data not shown). Therefore, we can conclude that this mutation can be considered a null mutation.

We subsequently applied this complementation analysis to all putative null mutations for the FucTA, -C, and -D genes that we had found in the EMS population. FucTB and –E mutations were not analyzed as their wt genes were not able to complement.

Complementation was investigated first for the splice site mutants that were identified for FucTA (introns 3 and 1; FucT001, -and -004, respectively) and FucTC (intron 2; FucT007) (Table 4). The splice site mutation for FucTD was not analyzed because of the size of the intron (7833 bp). To analyze the FucTA and -C mutations, we transformed the triple knock-out mutants with FucTA or FucTC CDS containing their own intron 3, 1, or 2 and compared the complementation obtained with these genes with the genes containing the splice site mutation. The results showed that, for FucTA, mutant FucT001 does not represent a null mutation, whereas FucT004 very likely represents a null mutation (data not shown). For FucTC, the intron splice site mutation could not be assessed because the triple knock-out plants transformed with the FucTC CDS containing intron 3 did not complement the mutant phenotype. The gene prediction program FGENESH did predict a strongly disruptive effect for the FucTC splice site mutation however.

Based on a next complementation assay, we confirmed that mutant FucT004 (FucTA), FucT010, -011, and -012 (FucTC), and FucT009 (FucTD) were null mutants (data not shown). Because by the time we had all the data from the complementation assay at hand we were already advanced with crossing FucT004, -007, and -009, we continued with those and used the other mutants as back-up mutant FucT. Our crossing strategy was aimed at first achieving a 5-fold knock-out mutant (XYL001, XYL002, FucT004, FucT007, and FucT009) as the most likely strategy to create a full knock out plant. Our second stategy was aimed at creating a 7-fold knock-out by further introducing FucT006 and FucT003 (see generations 4 and 5 in Figure 4, respectively).

7. Glycan analysis of the seven-fold knock out plant: *N. benthamiana* plants homozygous for null mutations in all FucT and XylT genes.

While producing seven-fold knock out plant, we also generated three- four, and five-fold knock-out plants as by-products of the crossing scheme. We used these plants to assess whether knocking out consecutive FucT genes had an additive effect and thus whether the FucT-B and -E genes indeed are inactive as was suggested from the complementation assay.

Figure 6 clearly shows that knocking out more FucT genes progressively removes core α1,3 Fucosyltransferase activity from the mutant plants as indicated by the decreasing chemoluminescence signal from the bound anti-α1,3 fucose antibody. This result indicates that probably the FucTB and –E genes still have some fucosyltransferase activity although this was not detected (i.e. compare lanes "aBcdE" versus "abcdE" and compare lanes "abcdE" versus "abcde").

Seeds of the plants in which the 5 FucT genes FucTA, FucTB, FucTC, FucTD and FucTE are knocked out, containing knock-out alleles FucT004, FucT006, FucT007, FucT009, and FucT003, have been deposited at the National Collection of Industrial, Marine and Food Bacteria (NCIMB), NCIMB Ltd, Ferguson Building, Craibstone Estate, Bucksburn, Aberdeen AB219YA, Scotland, on 12 September 2011, under accession number NCIMB 41860 by Bayer BioScience NV, Technologiepark 38, BE-9052 Gent, Belgium. The depositor Bayer BioScience NV, assignor of this invention to the applicant, has merged with and into Bayer CropScience NV having its registered office at J.E. Mommaertslaan 14, 1831 Diegem, Belgium.

In order to determine which specific glycan levels were reduced and also to determine what types of glycans were present in the four-fold ("abcdE") and five-fold plants ("abcde"), we performed a MALDI-TOF analysis on glycans isolated from total soluble

endogenous proteins from leaves of above-mentioned plants. Results are summarized in Table 5 and shown in Figure 7.

When comparing the glycans in WT and 4- and 5-fold KO plants it is clear that the levels of the fucose-containing glycans are sharply reduced albeit not completely eliminated. By contrast the levels of glycans carrying xylose only (i.e not carrying fucose) are sharply increased. Similar results have been reported by Strasser et al. for FucT knock outs in *A. thaliana* (Strasser et al. 2004, FEBS Lett 561:132).

Finally, we have analyzed the glycan quantity and quality in the full knock-out plants (7KO) in which all FucT and XylT genes were mutated and knocked out. Results are summarized in Table 5 and Figure 8.

Comparing the WT plants with the 5KO and 7KO plants, a strong reduction in all glycans that contain either fucose, xylose or both is observed. When comparing the 5KO and 7KO plants, it is clear that all xylose containing glycans have disappeared from the 7KO spectrum as was to be expected from our previous results on the double XylT knock-out plants (WO2010145846). Also, it seems that the bars representing glycans that contained both xylose and fucose in the 5KO plants had shifted to glycans carrying only fucoses (for instance, compare MMXF and MMF; GnMXF and GnMF; GnGnXF and GnGnF). Finally, when comparing the glycans obtained from 7KO plants with the glycans obtained from plants expressing the XylT- and FucT RNAi genes (Strasser et al. 2008, Plant Biotech J 6:392), the spectra are almost identical. Notable differences are a strong presence of MM glycans in the 7KO plants which are absent in the RNAi plants similar, albeit to a lesser extent, for the Man4Gn glycan. Also, the 7KO plants have a higher level of GnGnF glycans as compared to RNAi and, vice versa, the RNAi plants have a higher level of GnM and GnGn glycans.

Table 5: Relative glycan levels on endogenous soluble leaf proteins from *N. benthamiana* plants in which Xylosyl- and/or Fucosyltransferase activity has been reduced by gene mutation or RNAi. Total protein was isolated from leaves of plants in which different XylT and/or FucT genes were mutated or in which XylT and FucT RNAi genes were expressed. Glycans were isolated and analyzed by MALDI-TOF. Relative levels are expressed as percentage of the total peak area as determined from the MALDI-TOF spectra. 4KO-: FucTA (FucT004), -B (FucT006), -C (FucT007), and –D (FucT009) knocked out; 5KO-: all FucT genes knocked out (FucT004, -006, -007, -009, and -003); 7KO-: all FucT and XylT genes knocked out (FucT004, -006, -007, -009, and -003, and XylTg14-1 and XylTg19-1 as described in WO2010145846); WT: Wild Type; RNAi: plants expressing XylT and FucT RNAi genes (Strasser et al. 2008, Plant Biotech J 6:392).

	4KO			5KO			7KO			WT	RNAi
	4KO-0447	4KO-0660	4KO-0772	5KO-0023	5KO-0044	5KO-0046	7KO-0095	7KO-0910	7KO-0925	WT	RNAi
MM	0.0	0.0	1.4	0.0	0.0	0.0	16.3	13.4	12.2	0.0	0.0
MMX	27.9	21.2	21.4	0.0	41.5	49.5	0.0	0.0	0.0	3.5	0.0
MMF	1.4	0.9	1.3	0.0	0.0	0.0	5.8	5.1	6.5	0.0	7.0
Man4	0.0	0.0	0.0	0.0	0.0	0.0	2.3	2.0	1.7	0.0	2.2
GnM / MGn	0.0	0.0	0.8	0.0	0.0	0.0	13.3	11.6	11.4	0.0	21.6
MMXF	13.6	18.5	15.0	14.7	10.7	13.4	0.0	0.0	0.0	34.8	0.0
Man4X	0.0	1.0	0.0	3.9	2.0	3.2	0.0	0.0	0.0	1.8	0.0
Man5	0.0	2.0	2.0	1.9	1.8	1.6	4.0	4.3	3.2	2.4	4.3
GnMX*	15.4	10.6	14.1	25.0	15.9	13.0	0.0	0.0	0.0	3.2	0.0
GnMF*	0.0	0.0	0.0	0.0	0.0	0.0	3.5	3.4	4.7	0.0	3.9
Man4Gn / MA / Man4Gn*	0.0	0.0	0.0	0.0	0.0	0.0	1.3	1.5	1.5	0.0	0.0
GnGn	0.0	0.0	0.0	0.0	0.0	0.0	25.0	25.2	23.0	0.0	30.8
GnMXF	3.6	4.0	4.3	4.8	2.6	2.0	0.0	0.0	0.0	14.3	0.0

Man6	1.5	2.6	1.9	0.0	0.0	0.0	2.9	2.8	2.9	2.1	3.6
Man4GnX / MAX	0.0	0.0	0.9	3.0	1.2	0.0	0.0	0.0	0.0	0.9	0.0
GnGnX	19.1	12.5	16.5	21.8	12.7	10.0	0.0	0.0	0.0	1.7	0.0
GnGnF	0.0	0.0	0.0	0.0	0.0	0.0	12.1	12.7	16.7	0.0	9.7
GnA	0.0	0.0	0.0	0.0	0.0	0.0	2.4	3.2	3.3	0.0	2.1
Man7	2.0	3.2	1.8	3.1	1.5	1.3	3.3	3.5	3.6	2.3	4.5
GnGnXF	12.7	18.2	15.9	14.8	6.3	6.0	0.0	0.0	0.0	27.8	0.0
Man5A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
GnAX	0.0	0.0	0.0	3.6	2.0	0.0	0.0	0.0	0.0	0.0	0.0
LeaGn / GnLea *	0.0	0.0	0.0	0.0	0.0	0.0	1.1	1.3	1.3	0.0	1.8
AA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0
Man8	1.9	3.2	1.6	3.3	1.8	0.0	3.6	4.8	4.5	2.5	5.7
AAX	0.0	0.0	0.0	0.0	0.0	0.0	0.8	1.0	1.3	0.0	0.0
Man9	1.0	1.2	1.1	0.0	0.0	0.0	1.6	2.2	2.2	1.0	2.8
LeaGnXF / GnLeaXF	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.2	0.0
LeaLea	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.6	0.0	0.0	0.0
Man9 + Glc	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.5	0.0	0.0	0.0

8. Glycan analysis of an IgG1 expressed in the N. benthamiana full knock-out plant using magnICON®.

Since the glycan quality and quantity on the endogenous proteins of the 7KO plants were comparable those of the plants expressing the XylT- and FucT RNAi genes and since it has been described that IgG1 proteins expressed in the latter plants do not contain glycans carrying xylose or fucoses (i.e. despite the fact that their endogenous proteins do carry fucoses; Nagels et al. 2011, Plant Physiol 155:1103), we decided to test whether glycans on an IgG1 molecule expressed in the full knock plants would similarly be free of fucose and xylose.

IgG1 was isolated from leaf extract nine days after infiltration using protein G. The heavy chain of the purified antibody was isolated by cutting the corresponding band from a reducing SDS-PAGE. The heavy chain protein in this band was used for glycan analysis by LC-MS as described by Kolarich et al. 2006, Proteomics 6:3369.

Figure 9 shows the resulting spectrum from this analysis. The upper panel shows a wider mass spectrum to illustrate the presence of non-glycosylated peptides. Peptide 1 (EEQYNSTY) and peptide 2 (TKPREEQYNSTYR) are two variants from the same trypsin digestion. They differ in length caused by steric hindrance of the trypsin by the presence of N-glycans. As a result, all peptide-glycans produce two peaks in this LC-MS spectrum: indicated on the lower panel in black for glycopeptide 1 and orange for glycopeptide 2. In the lower panel of Figure 9, only one major glycan peak can be found for GnGn. In addition, some minor peaks for high mannose glycans are also visible (Man7, 8, and -9). However, in the full summary of all glyco-peptides that were identified by LC-MS, listed in Table 6, a small fraction of GnGnF glycans representing 2.6% of the total fraction of glycosylated and non-glycosylated glyco-peptides was identified.

Table 6: Relative glycan levels on heavy chain of IgG1 expressed in a *N. benthamiana* full knock out plant. In the full knock-out plant, all FucT and XylT genes are knocked out (FucT004, -006, -007, -009, and -003, and XylTg14-1 and XylTg19-1 as described in WO2010145846). Relative levels are expressed as percentage of the total peak area as determined from the LC-MS spectrum in Figure 9.

	Relative glycan level
non-glyc peptide	19.8
MGn	2.3
GnGn	51.7
GnGnF	2.6
GnA	0.9
AA	0.2
Man5	0.7
Man7	6.0
Man8	8.6
Man9	7.2

Combining the seven-fold knock out plant with a FucT RNAi gene further reduces the Fucose levels on N-glycans

In an attempt to further decrease the amount of residual Fucose residues on the N-glycans in the seven-fold knock out plants, we introduced a FucT RNAi gene in these plants by crossing these plants with plants containing the FucT RNAi gene from pGAX3 (WO 2009/056155). Homozygosity of the seven knock-out genes as well as the FucT RNAi gene was confirmed by End Point Taqman assays. Endogenous proteins from these plants (i.e. 7KO/FucT RNAi) were analyzed by Western blot and by MALDI-TOF analysis.

Results from the Western blot analysis in Figure 11 clearly show that adding the FucT RNAi gene to the seven-fold knock out plants further removes core α1,3 Fucose residues from the N-glycans as indicated by the complete absence of chemoluminescence signal from the lanes containing proteins from the 7KO/FucT RNAi plants as compared to lanes containing proteins from plants in which 6 or 7 genes have been knocked out. Even after a prolonged exposure of 1hour, no signal could be detected in 7KO/FucT RNAi lanes.

In order to determine specific glycan levels, MALDI-TOF analysis on glycans isolated from total soluble endogenous proteins from leaves of 7KO/FucT RNAi plants was performed. When comparing the glycans of the 7KO/FucT RNAi plants with WT, 4-, 5- and 7-fold KO plants, it is clear that the levels of the fucose-containing glycans are further reduced to only trace amounts of MMF, GnGnF and GnAF (LeaGn) glycans. As was the case for the 7KO plants, xylosylated N-glycans have completely disappeared in the 7KO/FucT RNAi plants (as shown in table 7)

Table 7: Relative glycan levels on endogenous soluble leaf proteins from *N. benthamiana* 7KO/FucT RNAi plants. Total protein was isolated from leaves of plants in which all XylT and FucT genes were mutated and in which a FucT RNAi gene was expressed. Glycans were isolated and analyzed by MALDI-TOF. Relative levels are expressed as percentage of the total peak area as determined from the MALDI-TOF spectra. Fucosylated N-glycans in shadow.

	7KO/FucT RNAi			
	7KO-1679	7KO-2125	7KO-2264	7KO-2512
MM	24.93	41.72	31.98	26.95
MMX	0.00	0.00	0.00	0.00
MMF	0.00	0.00	0.77	0.00
Man4	0.00	0.00	0.55	0.00
GnM / MGn	13.58	14.64	14.59	16.16
MMXF	0.00	0.00	0.00	0.00
Man4X	0.00	0.00	0.00	0.00
Man4F	0.00	0.00	0.00	0.00
Man5	1.27	2.81	2.68	1.73
GnMX	0.00	0.00	0.00	0.00
GnMF	0.00	0.00	0.00	0.00
Man4Gn / MA / Man4Gn	0.00	0.00	0.00	0.00
GnGn	44.03	33.60	36.05	40.06
Man4XF	0.00	0.00	0.00	0.00
Man5X	0.00	0.00	0.00	0.00
Man5F	0.00	0.00	0.00	0.00
GnMXF	0.00	0.00	0.00	0.00
Man6	1.34	1.60	2.15	1.63
Man4GnX / MAX	0.00	0.00	0.00	0.00
Man4GnF / MAF	0.00	0.00	0.00	0.00
Man5Gn / Man4A	0.00	0.00	0.00	0.00
GnGnX	0.00	0.00	0.00	0.00
GnGnF	0.83	0.60	0.91	0.72
GnA	0.00	0.00	0.00	0.00
Man5XF	0.00	0.00	0.00	0.00
GnGnGn	0.00	0.00	0.00	0.00
Man4GnXF / MAXF	0.00	0.00	0.00	0.00

	7KO/FucT RNAi			
	7KO-1679	7KO-2125	7KO-2264	7KO-2512
Man7	2.33	1.79	2.99	2.17
Man5GnX / Man4AX	0.00	0.00	0.00	0.00
Man5GnF / Man4AF	0.00	0.00	0.00	0.00
GnGnXF	0.00	0.00	0.00	0.00
Man5A	0.00	0.00	0.00	0.00
GnAX	0.00	0.00	0.00	0.00
GnAF/(LeaGn)	0.83	0.50	0.94	0.60
AA	0.00	0.00	0.00	0.00
GnGnGnX	0.00	0.00	0.00	0.00
GnGnGnF	0.00	0.00	0.00	0.00
GnGnA	0.00	0.00	0.00	0.00
Man5GnXF / Man4AXF	0.00	0.00	0.00	0.00
Man8	3.62	2.65	2.90	2.79
GnGnGnGn	0.00	0.00	0.00	0.00
Man5AX	0.00	0.00	0.00	0.00
Man5AF	0.00	0.00	0.00	0.00
GnAXF	0.00	0.00	0.00	0.00
(AF)GnF	0.00	0.00	0.00	0.00
AAX	0.00	0.00	0.00	0.00
AAF	0.00	0.00	0.00	0.00
GnGnGnXF	0.00	0.00	0.00	0.00
AA + Hex	0.00	0.00	0.00	0.00
GnGnAX	0.00	0.00	0.00	0.00
GnGnAF	0.00	0.00	0.00	0.00
GnAA	0.00	0.00	0.00	0.00
GnGnGnGnX	0.00	0.00	0.00	0.00
GnGnGnGnF	0.00	0.00	0.00	0.00
Man5AXF	0.00	0.00	0.00	0.00
Man9	6.68	0.70	3.49	6.37
GnGnGnA	0.00	0.00	0.00	0.00
LeaGnXF / GnLeaXF	0.00	0.00	0.00	0.00
AAXF	0.00	0.00	0.00	0.00
(AAF)F/LeaLea	0.00	0.00	0.00	0.00
AAX+Hex	0.00	0.00	0.00	0.00
AAF+Hex	0.00	0.00	0.00	0.00
GnGnAXF	0.00	0.00	0.00	0.00
AA + 2 Hex	0.00	0.00	0.00	0.00
GnAAX	0.00	0.00	0.00	0.00
GnAAF	0.00	0.00	0.00	0.00
GnGnGnGnXF	0.00	0.00	0.00	0.00
GnGnGnAX	0.00	0.00	0.00	0.00
GnGnGnAF	0.00	0.00	0.00	0.00
Man9 + Glc	0.55	0.00	0.00	0.00
GnGnAA	0.00	0.00	0.00	0.80
A(AF)XF	0.00	0.00	0.00	0.00
(AF)(AF)F	0.00	0.00	0.00	0.00
AAXF+Hex	0.00	0.00	0.00	0.00
GnAAXF	0.00	0.00	0.00	0.00

	7KO/FucT RNAi			
	7KO-1679	7KO-2125	7KO-2264	7KO-2512
GnGnGnAXF	0.00	0.00	0.00	0.00
GnGnAAX	0.00	0.00	0.00	0.00
GnGnAAF	0.00	0.00	0.00	0.00
Man9+2Glc	0.00	0.00	0.00	0.00
GnAAA	0.00	0.00	0.00	0.00
LeaLeaXF	0.00	0.00	0.00	0.00
GnGnAAXF	0.00	0.00	0.00	0.00
GnAAAX	0.00	0.00	0.00	0.00
GnAAAF	0.00	0.00	0.00	0.00
AAAA	0.00	0.00	0.00	0.00
GnAAAXF	0.00	0.00	0.00	0.00
AAAAX	0.00	0.00	0.00	0.00
AAAAF	0.00	0.00	0.00	0.00
AAAXF	0.00	0.00	0.00	0.00

Figure 12 shows a quantitative overview of fucosylated resp. xylosylated N-glycans present on the endogenous proteins of WT, 4-, 5-, 7-fold KO, RNAi and 7KO/FucT RNAi plants.

Introducing a FucT RNAi gene into the seven-fold knock out plants to further reduce fucose levels on N-glycans.

In order to further reduce the fucose levels on N-glycans in seven-fold knock-out plants, RNAi genes are constructed that target silencing of all FucT genes by including multiple stretches of 25 or more nucleotides that are 100% homologous to two or more FucT genes and, combined, target all FucT genes. For example, a fragment of the FucTB coding sequence (Seq ID No 5) from nucleotide 1183 to 1265 (Seq ID No 20) contains a stretch of 44 nucleotides, from 1183 to 1226, that is 100 % homologous to FucT-B, -C, -D, and -E and a fragment of 47 nucleotides, from 1219 to 1265, that is 100% homologous to FucT-A, and -B. This fragment (Seq ID No 20) is assembled into an RNAi gene as shown in Seq ID No 21. Expression of the RNAi gene is driven by the 35S promoter by cloning it into a T-DNA vector similar to pGAX3 (WO 2009/056155). The seven-fold knock-out N. benthamiana plants are transformed with this construct and analyzed for N-glycan composition on endogenous proteins and on heterologously magnICON®-expressed proteins like, for instance, an IgG1 molecule.

In addition, the FucT RNAi gene is cloned in a promoterless T-DNA vector similar to pICH3781 and pICH3831 (WO 02/101060) where the existing BAR gene is replaced by the FucT RNAi gene fragment. The seven-fold knock-out N. benthamiana plants are transformed with these constructs. Use of promoterless vectors will provide a broader choice of primary

transformants in comparison to vectors with strong constitutive promoter. In such case the RNAi becomes part of a transcriptional fusion with a residential gene (the promoterless vector contains splice acceptor sites in front of the RNAi gene). This can be an advantage, as the RNAi usually targets multigene family and this might compromise plant phenotype - growth, development, abiotic or biotic stress resistance, etc. The resulting stably transformed plants are screened for absence of fucoses on the N-glycans of their endogenous proteins and of heterologously magnICON®-expressed proteins like, for instance, an IgG1 molecule. Those selected can be additionally screened for their performance in glasshouses, e.g. vegetative growth efficiency in comparison with wild type plants.

The content of US patent application 61/542,965 filed on October 4, 2011 and European patent application No. 11 075 218.5 filed on October 6, 2011 the priorities of which are claimed by the present patent application are herewith incorporated by reference in their entirety including descriptions, all claims, all figures and SEQ ID NOs 1 to 19 of the sequence listing.

Claims

1. A method to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues in Nicotiana benthamiana, said method comprising the steps of:
   a. providing a plant or plant cell comprising at least three knock-out alfa(1,3)-fucosyltransferase genes; and
   b. cultivating said cell and isolating glycoproteins from said cell.

2. A method to produce glycoproteins with reduced levels of core alfa(1,3)-fucose residues and reduced levels of beta(1,2)-xylose residues in Nicotiana benthamiana, said method comprising the steps of:
   a. providing a plant cell characterized in that said plant cell
      i. comprises at least three knock-out alpha(1,3)-fucosyltransferase genes; and
      ii. has a reduced level of beta(1,2)-xylosyltransferase activity; and
   b. cultivating said cell and isolating glycoproteins from said cell.

3. The method according to claim 2, wherein said reduced level of beta(1,2)-xylosyltransferase activity is the result of a knock-out mutation in endogenous beta(1,2)-xylosyltransferase genes.

4. The method according to any one of claims 1 to 3, in which said plant or plant cell comprises at least five knock-out alfa(1,3)-fucosyltransferase genes.

5. The method according to any one of claims 1 to 4, wherein said knock-out alfa(1,3)-fucosyltransferase genes are mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of:
   a. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 3;
   b. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 6;
   c. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 9;

d. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 12;
e. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 14.

6. The method according to claim 5, wherein said knock-out alfa(1,3)-fucosyltransferase genes are mutated versions of the native alfa(1,3)-fucosyltransferase genes selected from the group consisting of:
a. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 1;
b. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 4;
c. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 7;
d. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 10;
e. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 13.

7. The method according to claim 6, wherein said knock-out alfa(1,3)-fucosyltransferase gene is selected from the group consisting of:
a. FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
b. FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
c. FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
d. FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
e. FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

8. The method according to any one of claims 1 to 7, wherein said knock-out alfa(1,3)-fucosyltransferase genes occur in a homozygous state in the genome.

9. The method according to any one of claims 1 to 8, further characterized in that the expression of at least five endogenous alfa(1,3)-fucosyltransferase encoding genes is reduced through transcriptional or post-transcriptional silencing.

10. The method according to claim 9, wherein said plant or plant cell further comprises at least one chimeric gene comprising the following operably linked DNA fragments:
    a. a plant-expressible promoter;
    b. a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene;
    c. a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

11. The method according to claim 10, further characterized in that said DNA region yields an RNA molecule capable of forming a double-stranded RNA region at least between:
    a. an RNA region transcribed from a first sense DNA region comprising a nucleotide sequence of at least 18 out of 21 nucleotides selected from SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 11, SEQ ID NO: 13, or the complement thereof;
    b. an RNA region transcribed from a second antisense DNA region comprising a nucleotide sequence of at least 18 consecutive nucleotides which have at least 95% sequence identity to the complement of said first sense DNA region.

12. The method according to claim 11, wherein said DNA region comprises the sequence of SEQ ID No. 19.

13. The method according to any one of claims 1 to 12, characterized in that said glycoproteins are heterologous glycoproteins.

14. The method according to claim 13, characterized in that said heterologous glycoproteins are expressed from a chimeric gene comprising the following operably linked nucleic acid molecules:
    a. a plant-expressible promoter,
    b. a DNA region encoding said heterologous glycoprotein,
    c. a DNA region involved in transcription termination and polyadenylation.

15. The method according to claim 13 or 14, further comprising the step of purification of said heterologous glycoproteins.

16. A glycoprotein obtained by the methods of any one of claims 1 to 15.

17. A glycoprotein with reduced levels of core alfa(1,3)-fucose residues obtained by the methods of any one of claims 1 to 15.

18. A glycoprotein with reduced levels of core alfa(1,3)-fucose residues and with reduced levels of beta(1,2)-xylose residues obtained by the methods of any one of claims 2 to 15.

19. A Nicotiana benthamiana plant, or a cell, part, seed or progeny thereof, comprising at least three knock-out alfa(1,3)-fucosyltransferase genes.

20. The plant according to claim 19, comprising at least five knock-out alfa(1,3)-fucosyltransferase genes.

21. The plant according to claim 19 or 20, wherein one or more of the knock-out alfa(1,3)-fucosyltransferase genes is a mutated version of the native alfa(1,3)-fucosyltransferase gene selected from the group consisting of:

a. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 3;
b. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 6;
c. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 9;
d. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 12;
e. a nucleic acid molecule encoding an amino acid sequence comprising at least 90% sequence identity to SEQ ID NO: 14.

22. The plant according to claim 21, wherein one or more of the knock-out alfa(1,3)-fucosyltransferase genes is a mutated version of the native alfa(1,3)-fucosyltransferase gene selected from the group consisting of:

a. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 1;

b. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 4;
c. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 7;
d. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 10;
e. a nucleic acid molecule comprising at least 90% sequence identity to SEQ ID NO: 13.

23. A plant according to claim 21 or 22, wherein the knock-out alfa(1,3)-fucosyltransferase gene is selected from the group consisting of:
a. FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
b. FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
c. FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
d. FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
e. FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

24. The plant or plant cell according to any one of claims 19 to 23 which is homozygous for the knock-out alfa(1,3)-fucosyltransferase genes.

25. The plant or plant cell according to any one of claims 19 to 24, further comprising at least one knock-out beta(1,2)-xylosyltransferase gene, wherein said knock-out beta(1,2)-xylosyltransferase gene comprises a mutated DNA region consisting of one or more inserted, deleted or substituted nucleotides compared to a corresponding wild-type DNA region in the beta(1,2)-xylosyltransferase gene and wherein said knock-out beta(1,2)-xylosyltransferase gene does not encode a functional beta(1,2)-xylosyltransferase protein.

26. The plant or plant cell according to any one of claims 19 to 25, further comprising at least one chimeric gene comprising the following operably linked DNA fragments:
a. a plant-expressible promoter;
b. a DNA region, which when transcribed yields an RNA molecule inhibitory to at least one alfa(1,3)-fucosyltransferase encoding gene;

c. a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

27. The plant or plant cell according to claim 26, wherein said DNA region comprises the sequence of SEQ ID No. 19.

28. The plant or plant cell according to any one of claims 19 to 27, further comprising a glycoprotein foreign to said plant or plant cell.

29. The plant or plant cell according to claim 28, wherein said glycoprotein is expressed from a chimeric gene comprising the following operably linked nucleic acid molecules:
a. a plant-expressible promoter,
b. a DNA region encoding said heterologous glycoprotein,
c. a DNA region involved in transcription termination and polyadenylation.

30. A knock-out allele of an alfa(1,3)-fucosyltransferase gene selected from the group consisting of:
a. FucTA gene containing a G to A substitution at position 355 of SEQ ID NO: 1;
b. FucTB gene containing a G to A substitution at position 3054 of SEQ ID NO: 4;
c. FucTC gene containing a G to A substitution at position 2807 of SEQ ID NO: 7;
d. FucTD gene containing a G to A substitution at position 224 of SEQ ID NO: 10;
e. FucTE gene containing a G to A substitution at position 910 of SEQ ID NO: 13.

31. Use of the method according to any one of claims 1 to 15 to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues.

32. Use of the method according to any one of claims 2 to 15 to obtain glycoproteins with a reduced level of core alfa(1,3)-fucose residues and with a reduced level of beta(1,2)-xylose residues.

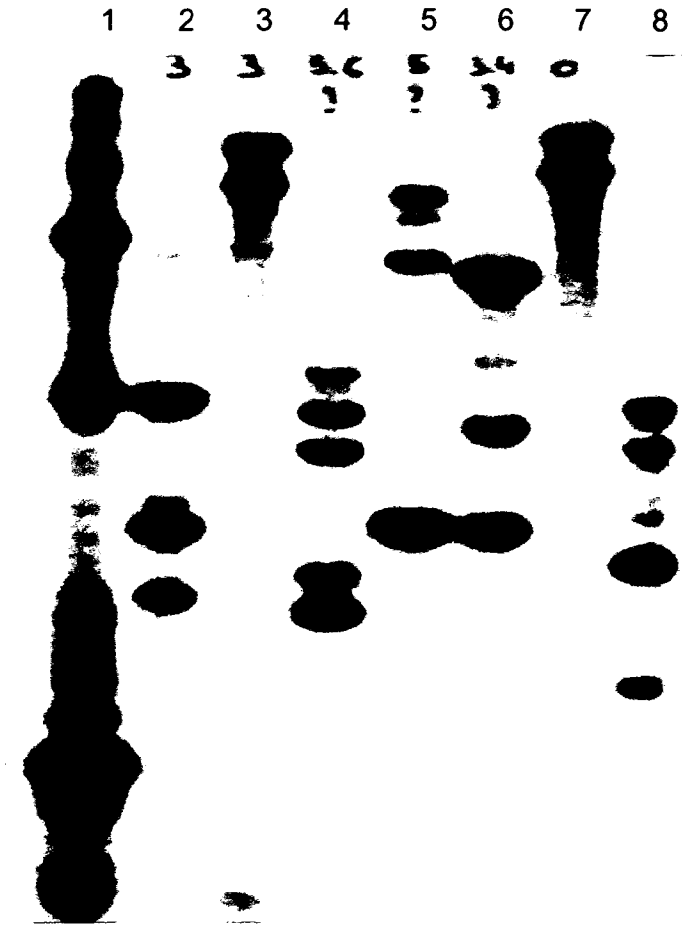


Fig. 1

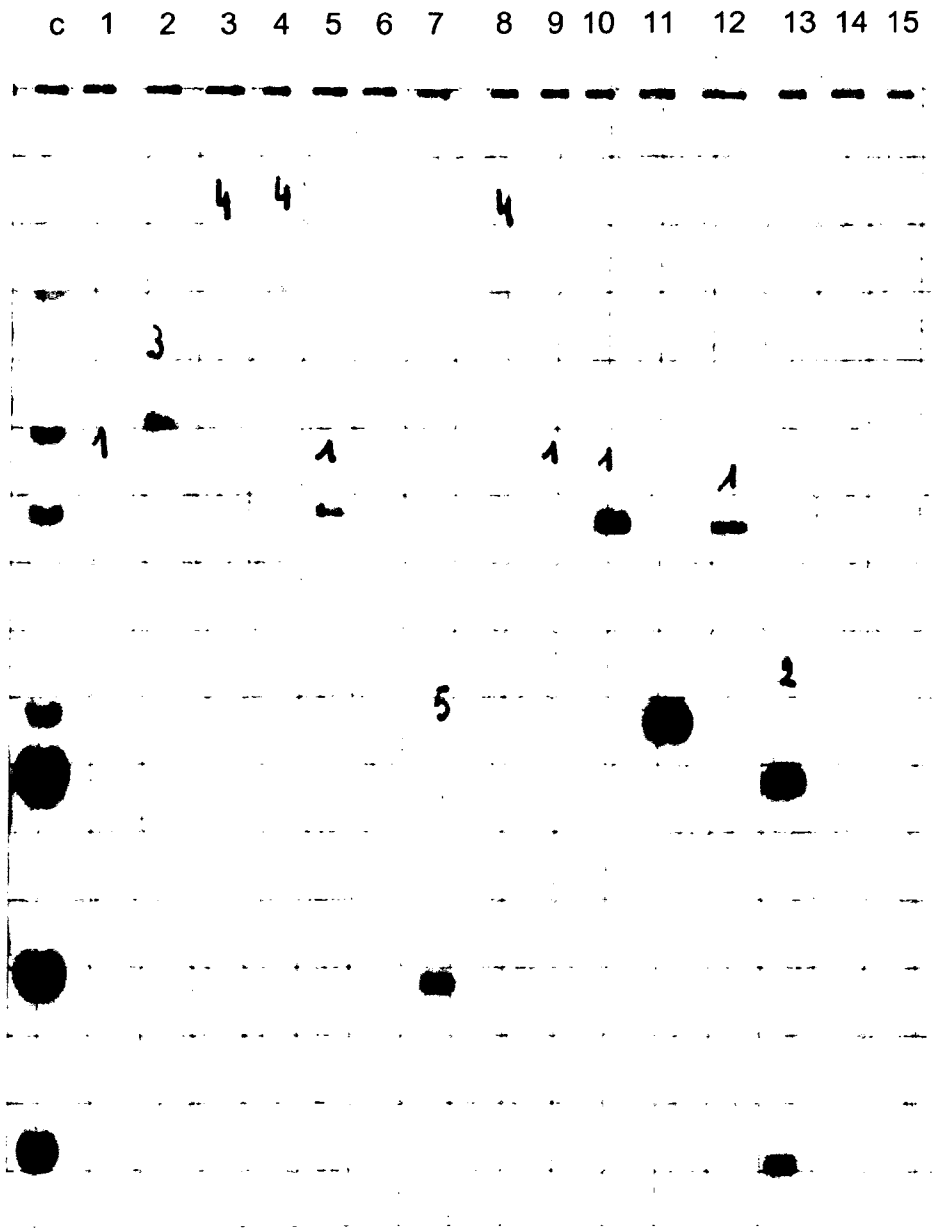


Fig. 2

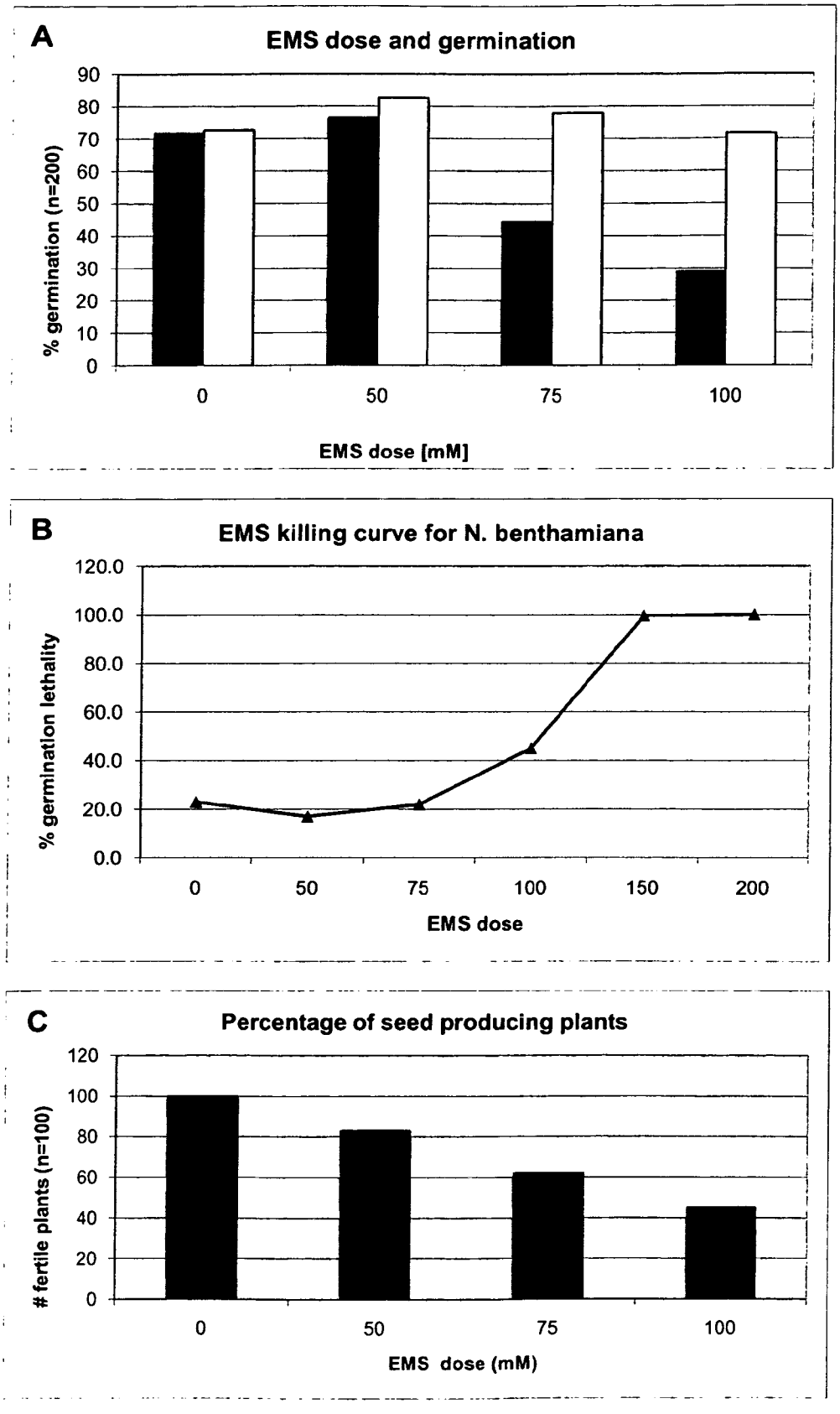

A
EMS dose and germination
% germination (n=200)
90
80
70
60
50
40
30
20
10
0
0
50
75
100
EMS dose [mM]
B
EMS killing curve for N. benthamiana
% germination lethality
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
50
75
100
150
200
EMS dose
C
Percentage of seed producing plants
fertile plants (n=100)
120
100
80
60
40
20
0
0
50
75
100
EMS dose (mM)

Fig. 3

generation	selected genotypes	crosses
1	x14/x14 x19/x19	x14/x14 x19/x19 ✖ a/a
	a/a	b/b ✖ c/c
	b/b	c/c ✖ d/d
	c/c	d/d ✖ e/e
	d/d	a/a ✖ b/b
	e/e	
2	selected genotypes	crosses
	X14/x14 X19/x19 A/a	X14/x14 X19/x19 A/a ✖ B/b C/c
	B/b C/c	X14/x14 X19/x19 A/a ✖ C/c D/d
	C/c D/d	X14/x14 X19/x19 A/a ✖ D/d E/e
	D/d E/e	X14/x14 X19/x19 A/a ✖ A/a B/b
3	selected genotypes	crosses
	X14/x14 X19/x19 A/a B/b C/c	X14/x14 X19/x19 A/a B/b C/c ✖ X14/x14 X19/x19 A/a D/d E/e
	X14/x14 X19/x19 A/a C/c D/d	X14/x14 X19/x19 A/a C/c D/d ✖ X14/x14 X19/x19 A/a C/c D/d
	X14/x14 X19/x19 A/a D/d E/e	
4	selected genotypes	crosses
	x14/x14 x19/x19 a/a c/c d/d	
	x14/x14 x19/x19 a/a B/b C/c D/d E/e	x14/x14 x19/x19 a/a B/b C/c D/d E/e ✖ x14/x14 x19/x19 a/a B/b C/c D/d E/e
5	selected genotypes	
	x14/x14 x19/x19 a/a b/b c/c d/d e/e	

Fig. 4

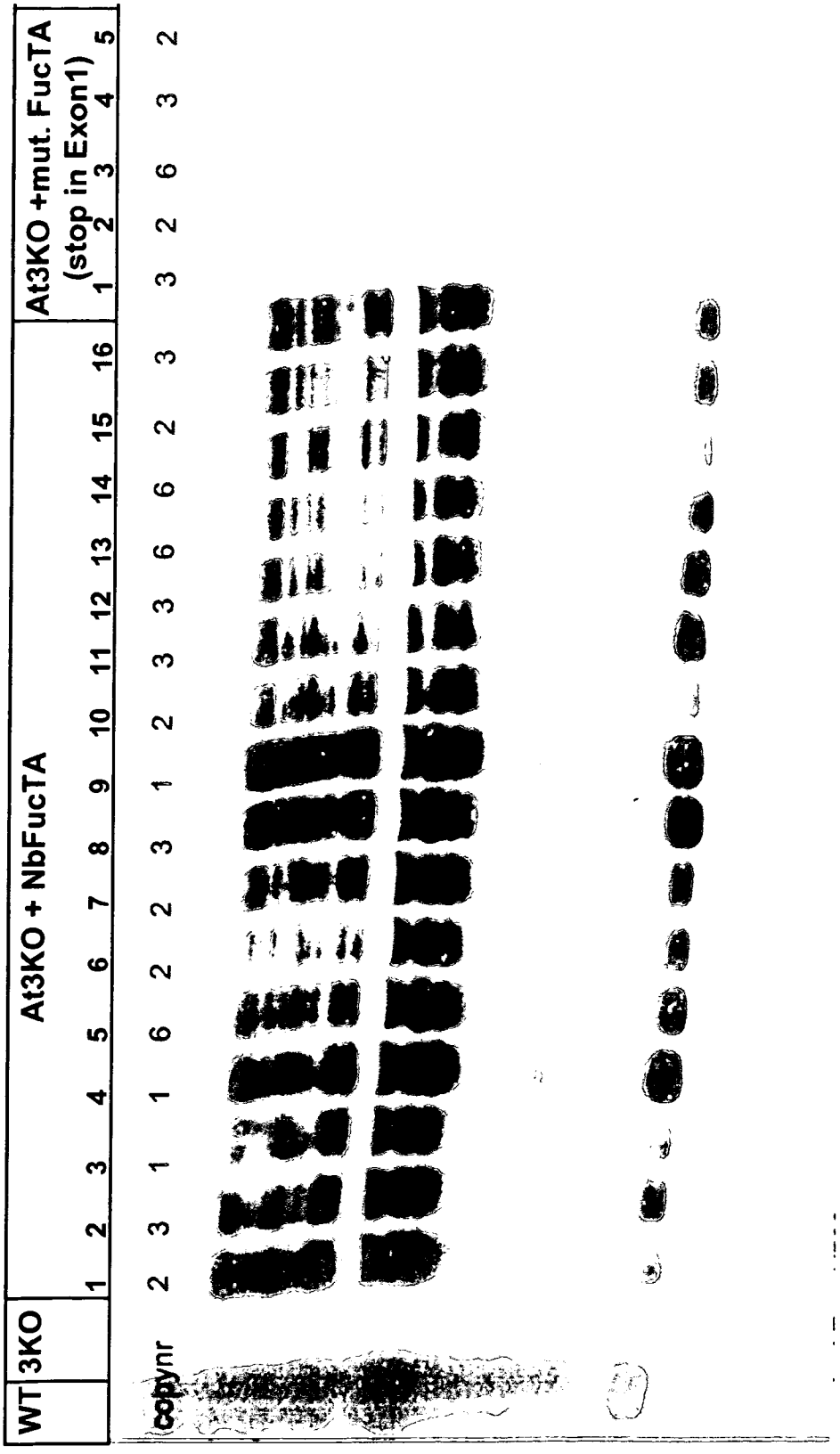
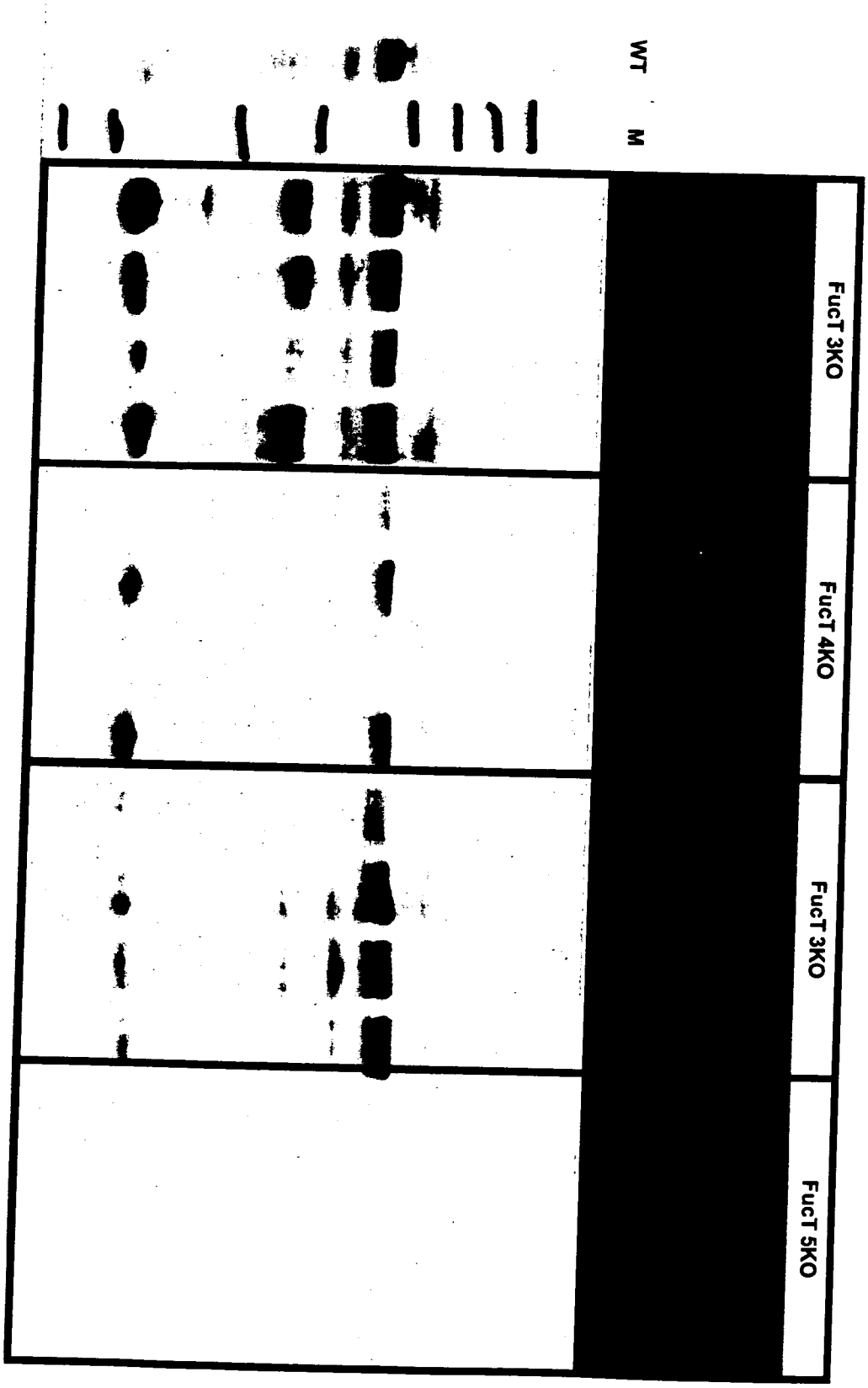

WT
3KO
At3KO + NbFucTA
At3KO +mut. FucTA (stop in Exon1)
copynr
1 2 3 1 2 3 4 1 2 3 5 6 2 6 7 2 2 3 8 1 9 2 10 3 3 11 3 12 6 13 6 14 2 15 3 16 1 2 3 2 6 3 4 5 2

Fig. 5

Fig. 6



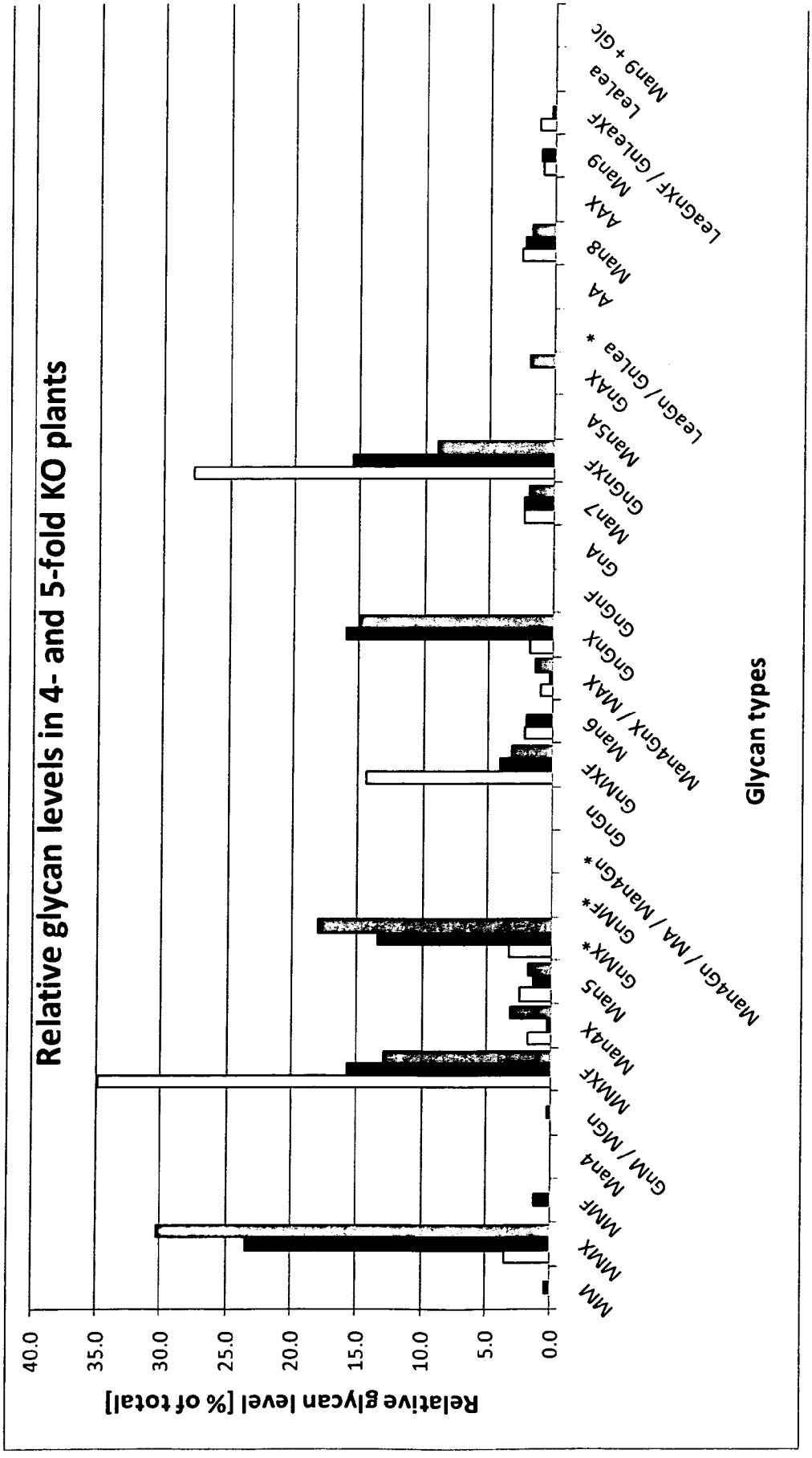

Relative glycan levels in 4- and 5-fold KO plants
Relative glycan level [% of total]
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
MM
MMX
MMF
Man4
GnM / MGn
MMXF
Man4X
Man5
GnMX*
GnMF*
Gn4Gn / Man4Gn / MA / Man4Gn*
GnGn
GnMXF
Man6
Man4GnX / MAX
GnGnX
GnGnF
GnA
Man7
GnGnXF
Man5A
GnAX
Lea2Gn / GnLea *
AA
Man8
AAX
Man9
Lea2GnXF / GnLeaXF
Lea2Lea
Man9 + Glc
Glycan types

Fig. 7

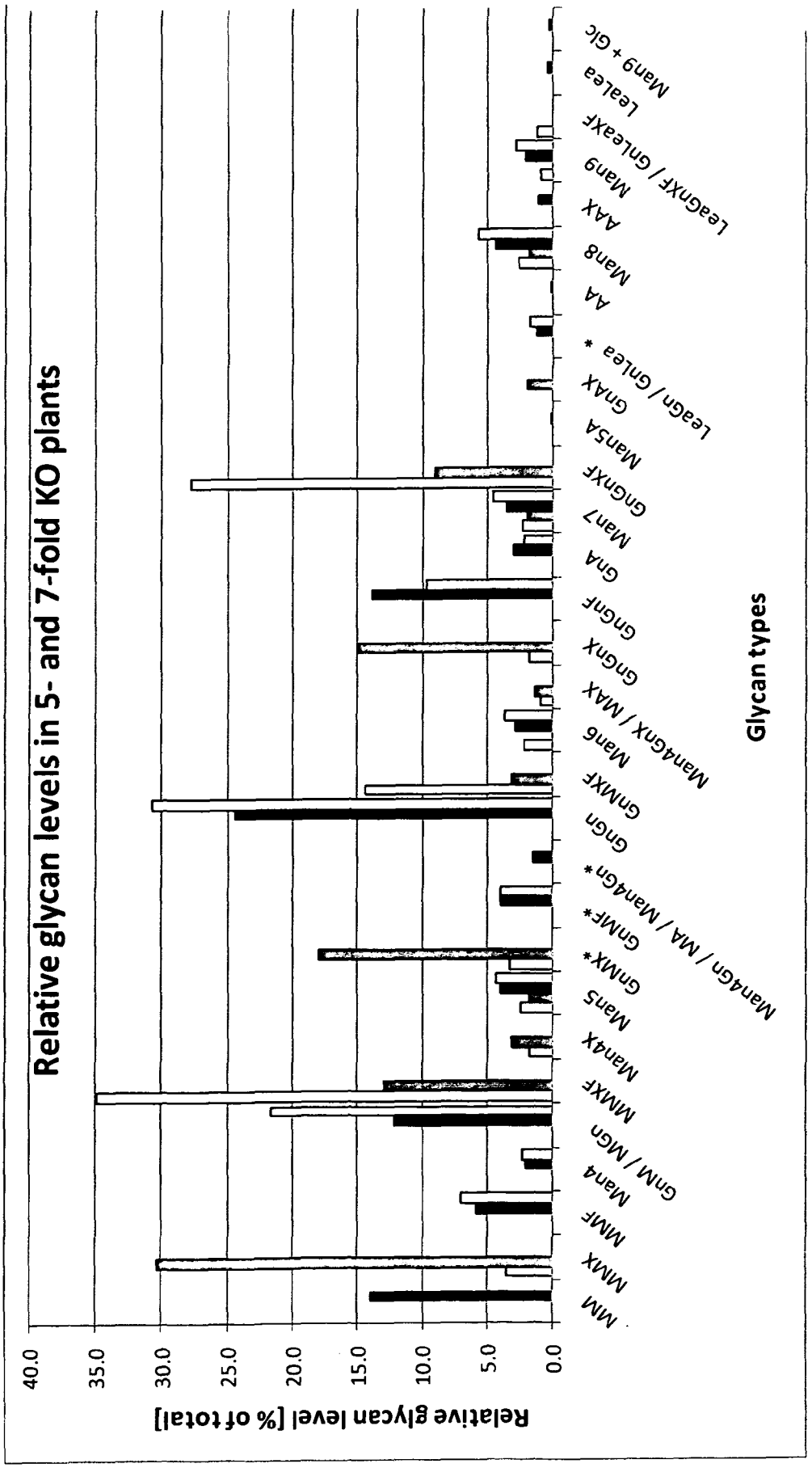

Relative glycan levels in 5- and 7-fold KO plants
Relative glycan level [% of total]
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Glycan types
MM
MMX
MMF
Man4
GnM / MGn
MMXF
Man4X
Mans
GnMX*
GnMF*
Man4Gn / MA / Man4Gn*
GnGn
GnMXF
Man6
Man4GnX / MAX
GnGnX
GnGnF
GnA
Man7
GnGnXF
Man5A
GnAX
Leagn / Gnlea *
AA
Man8
AAX
Man9
LeaGnXF / GnLeaXF
Lealea
Man9 + Glc

Fig. 8

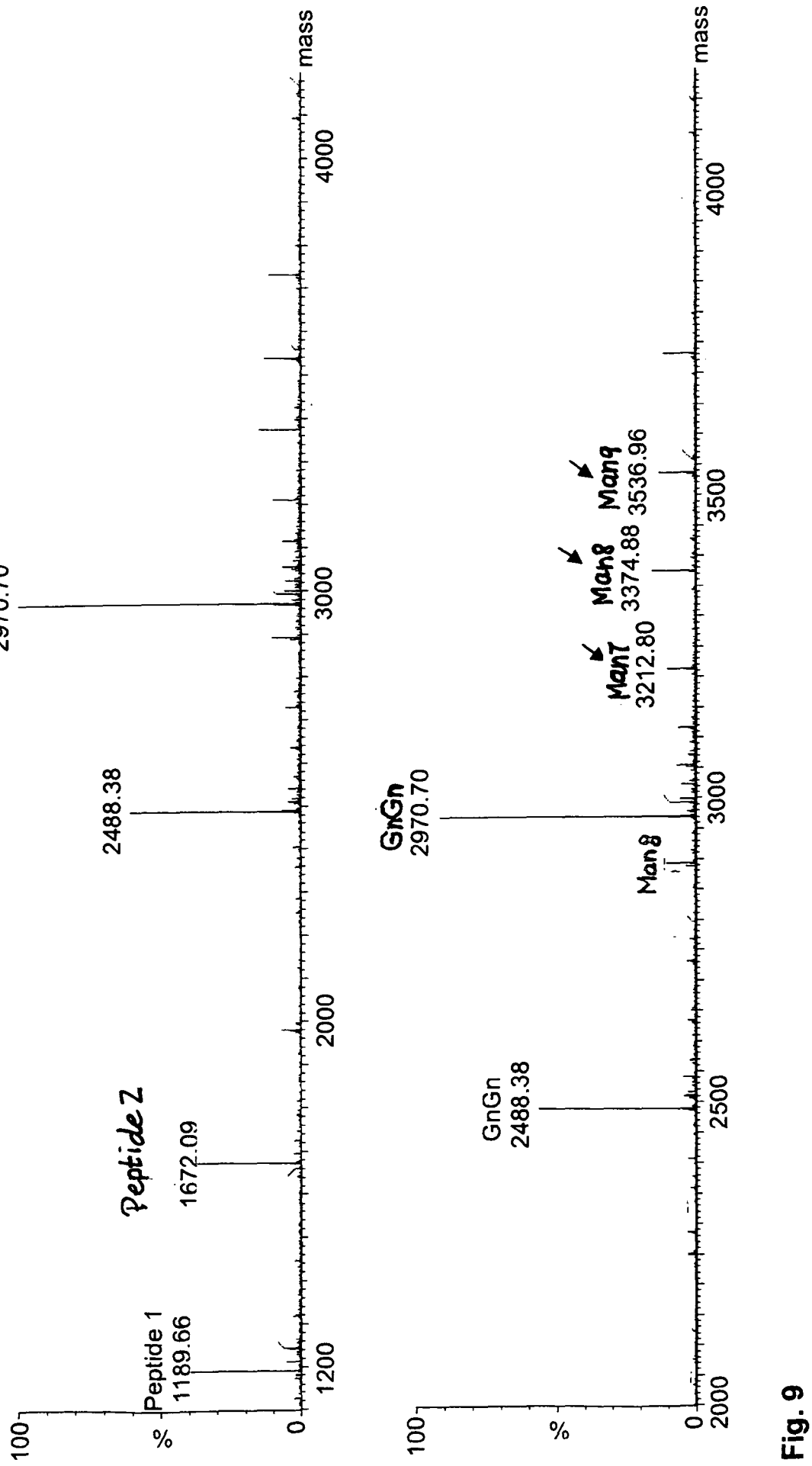

Peptide 2
2970.70
2488.38
1672.09
Peptide 1
1189.66
100
%
0
1200
2000
3000
4000
mass
GnGn
2970.70
Man8
Man7
3212.80
Man8
3374.88
Man9
3536.96
GnGn
2488.38
100
%
0
2000
2500
3000
3500
4000
mass

Fig. 9

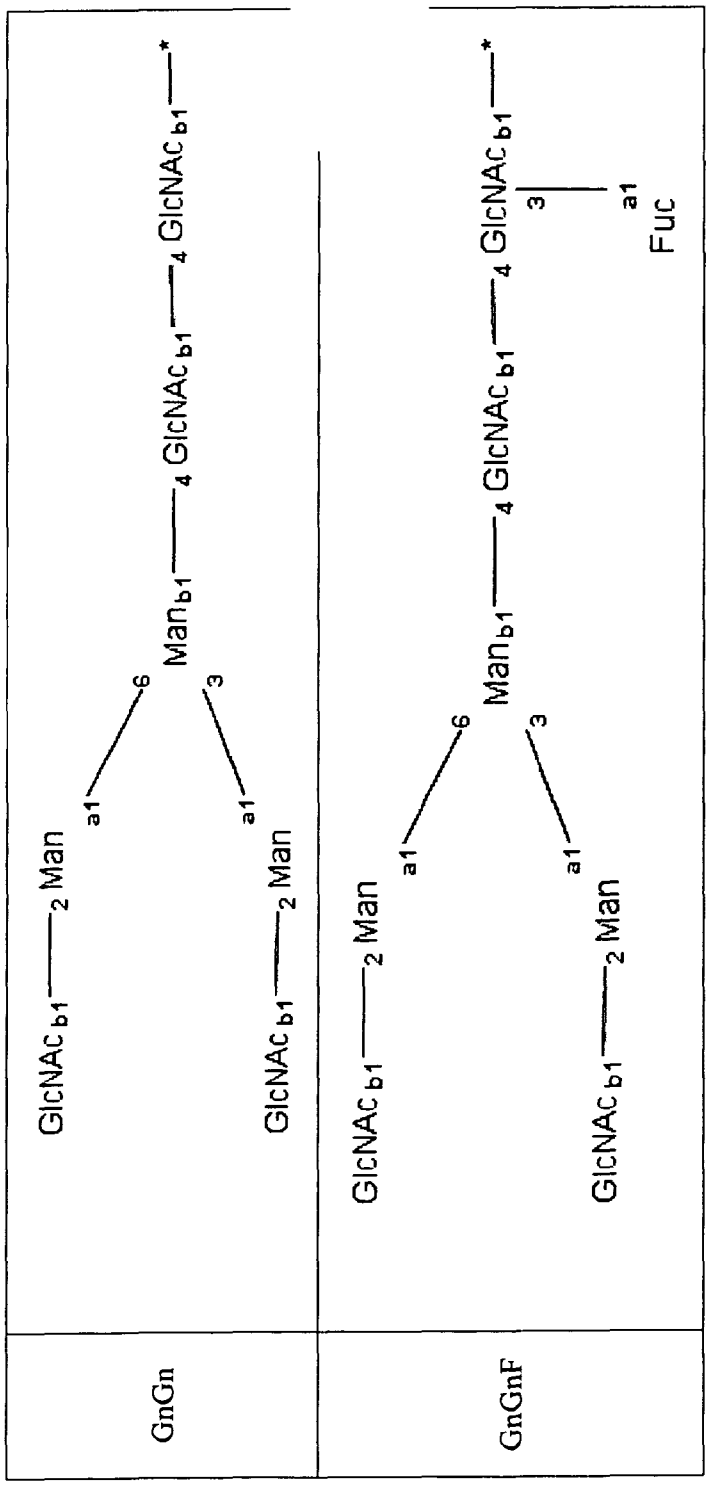

GnGn
GnGnF
GlcNAc b1 — 2 Man
a1
6
3
Man b1 — 4 GlcNAc b1 — 4 GlcNAc b1 — *
GlcNAc b1 — 2 Man
a1
a1
Fuc

Fig. 10

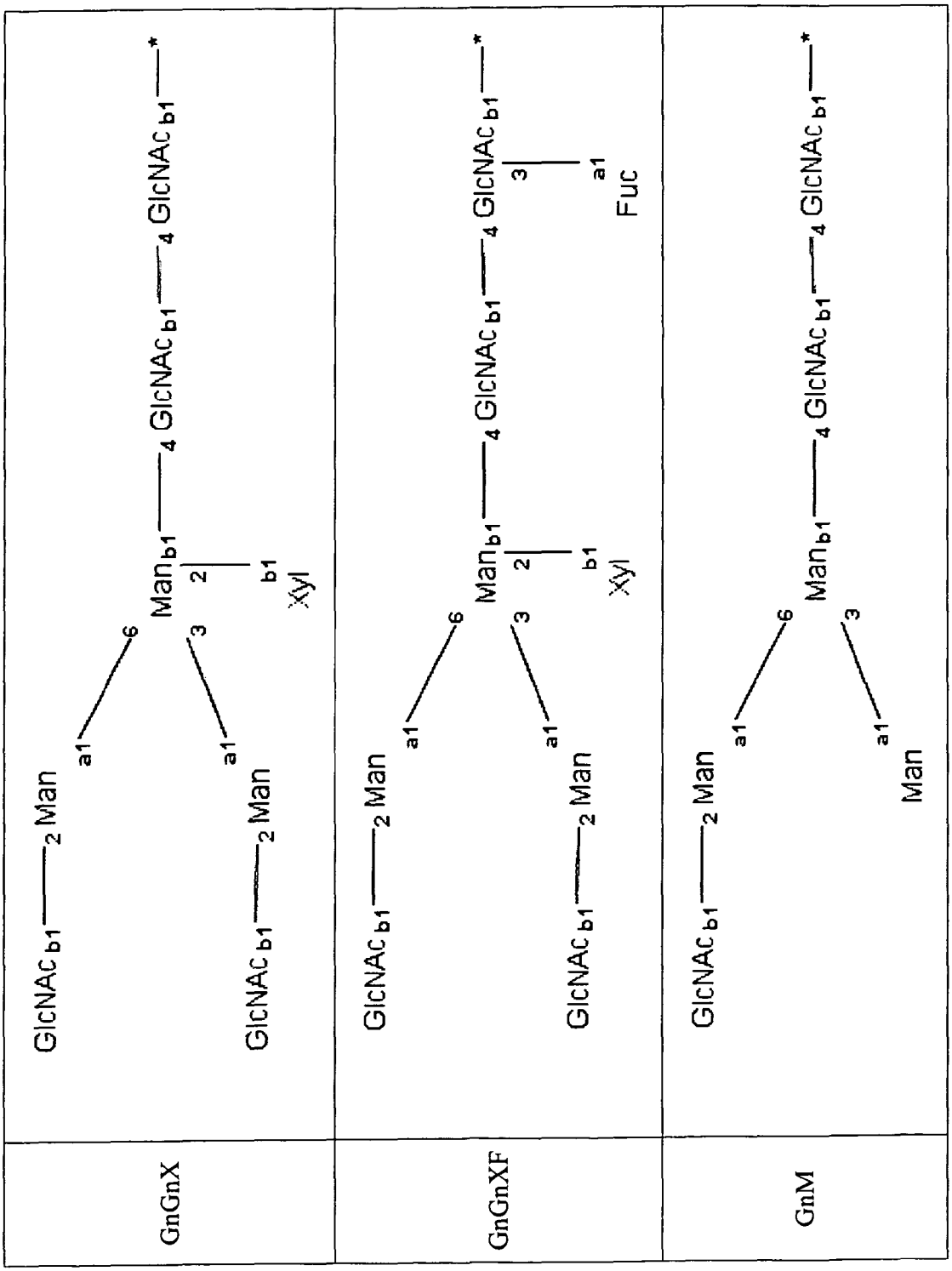
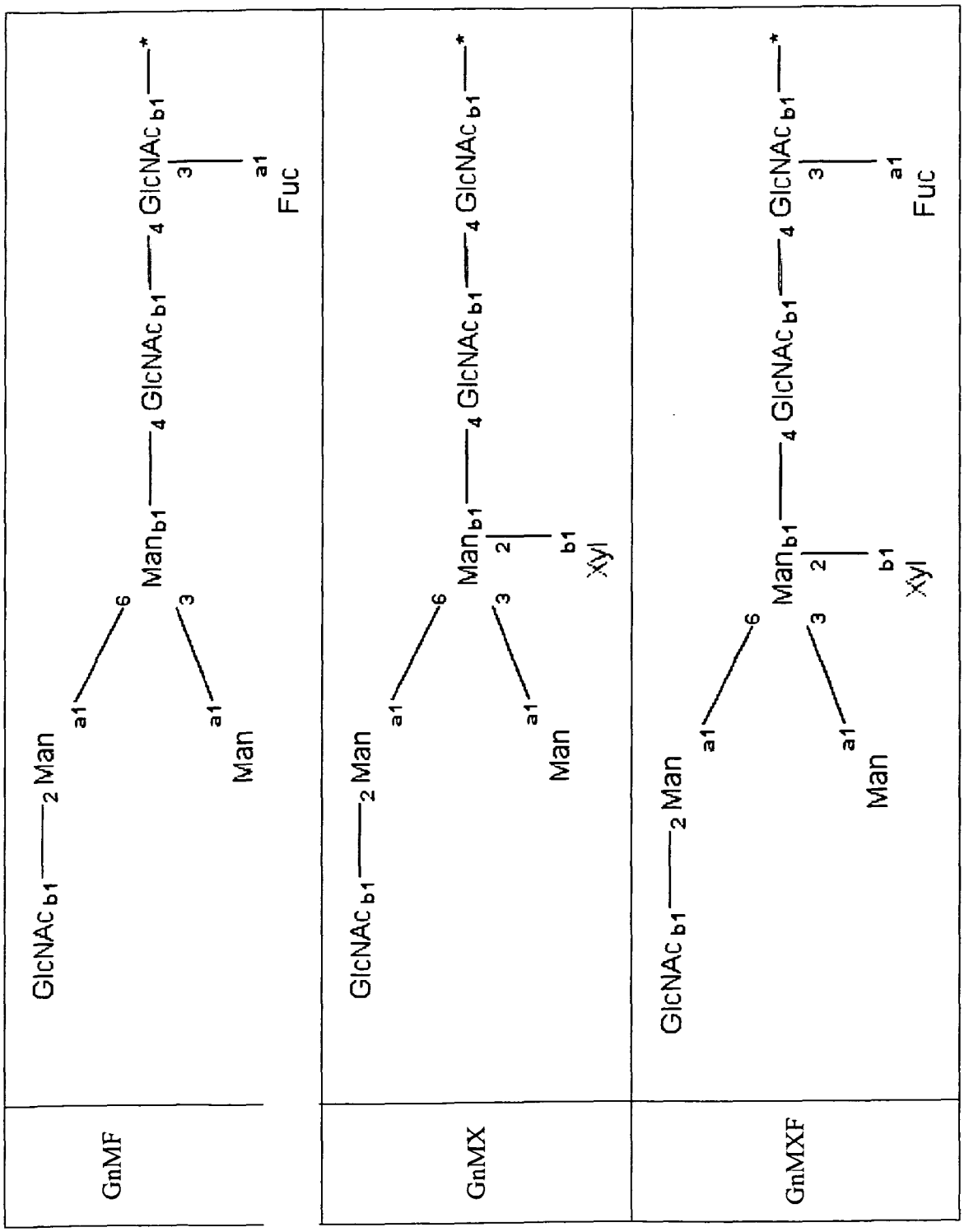


Fig. 10, continued



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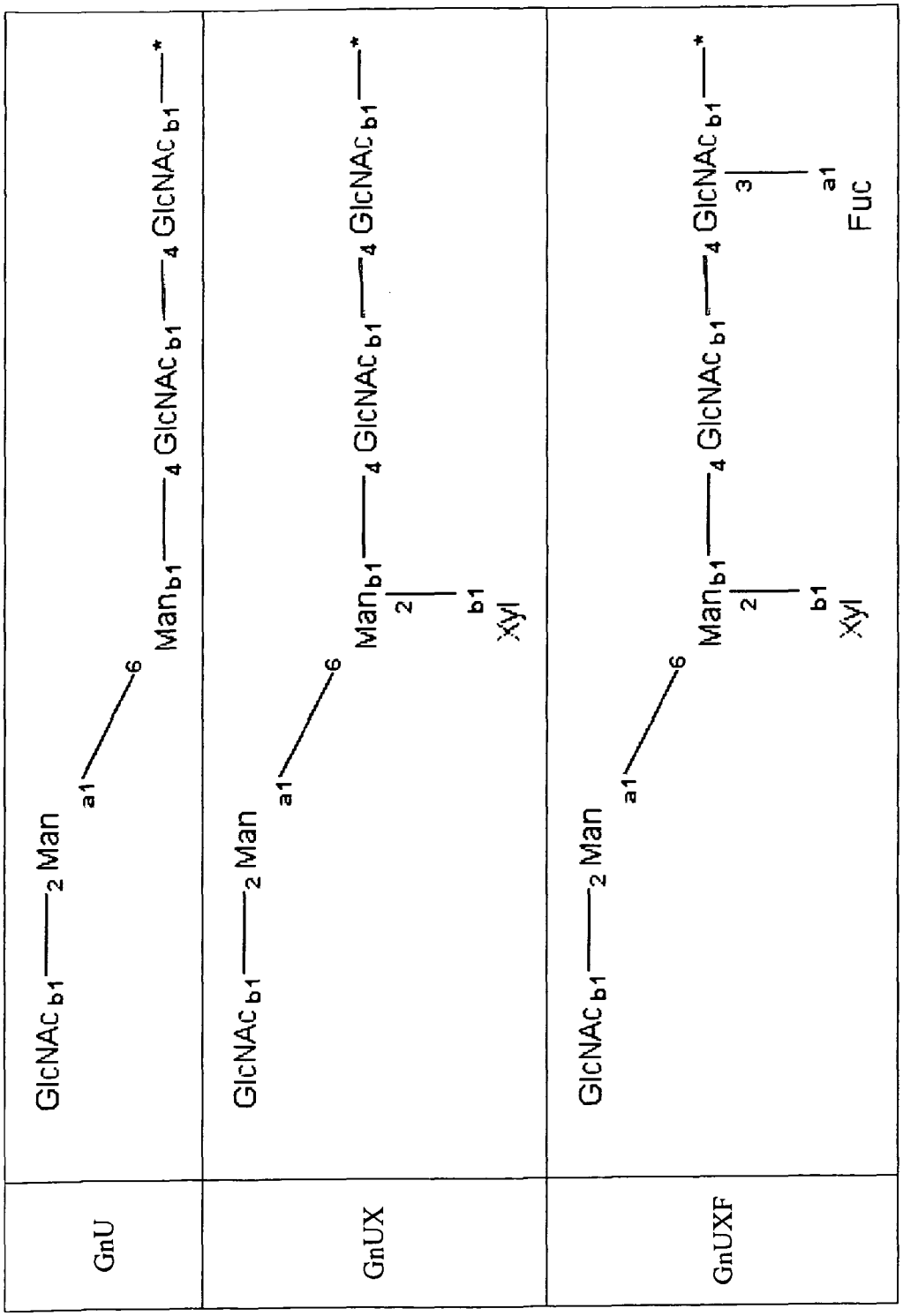


Fig. 10, continued

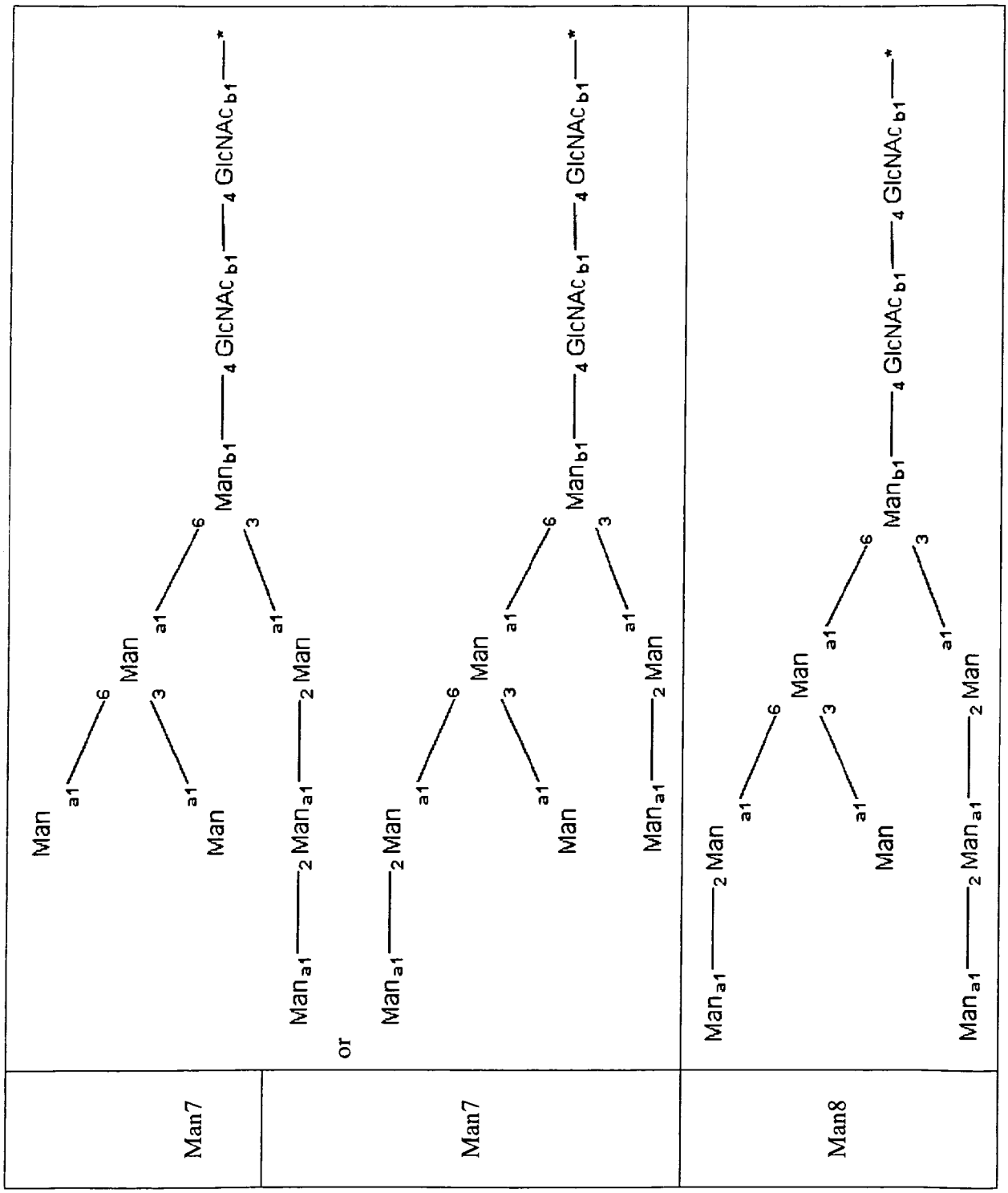


Fig. 10, continued

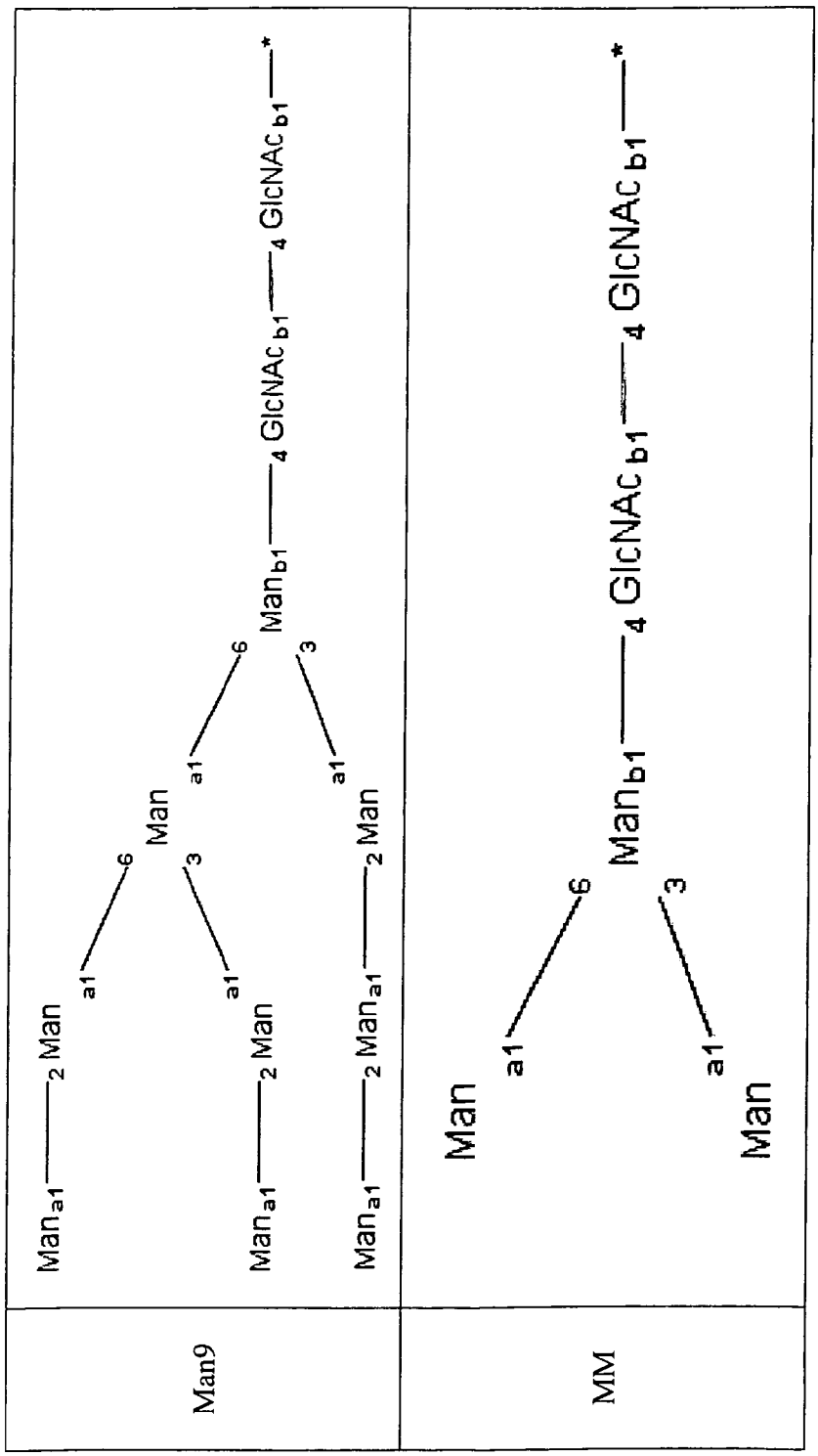


Fig. 10, continued

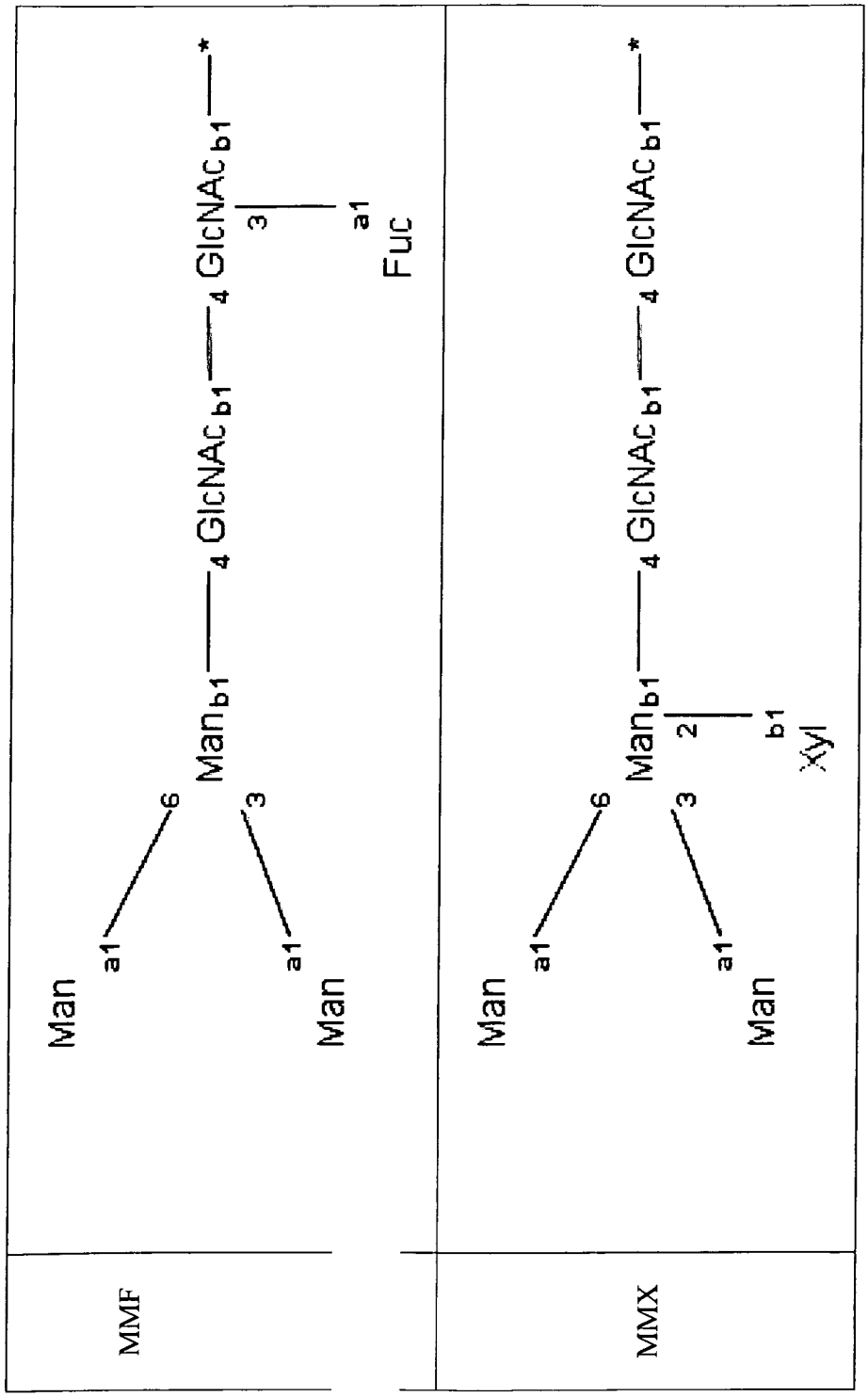


Fig. 10, continued

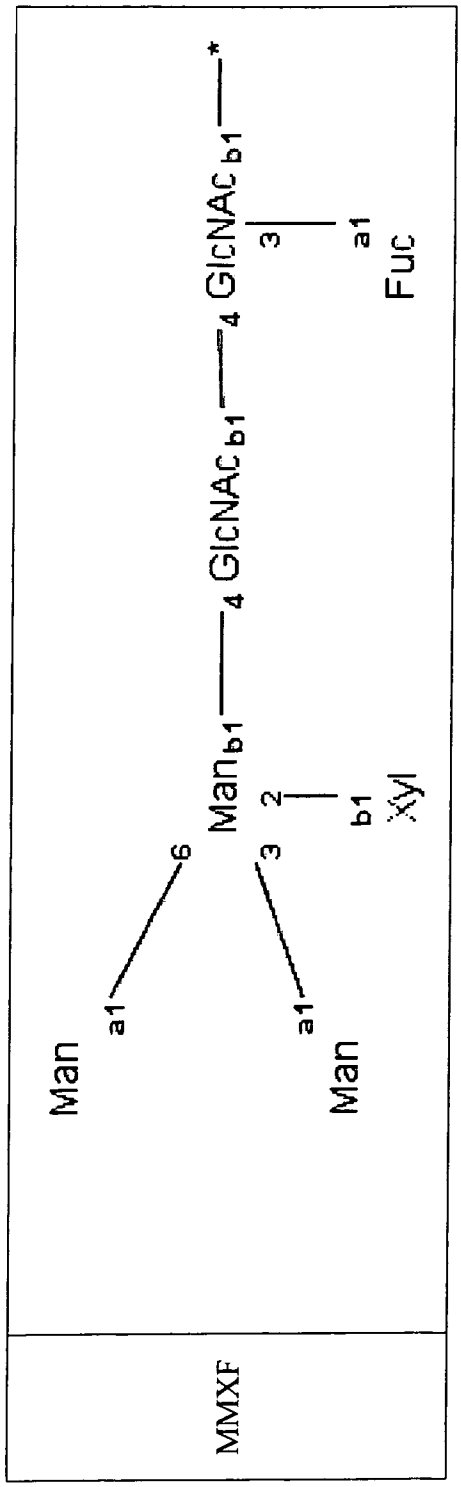
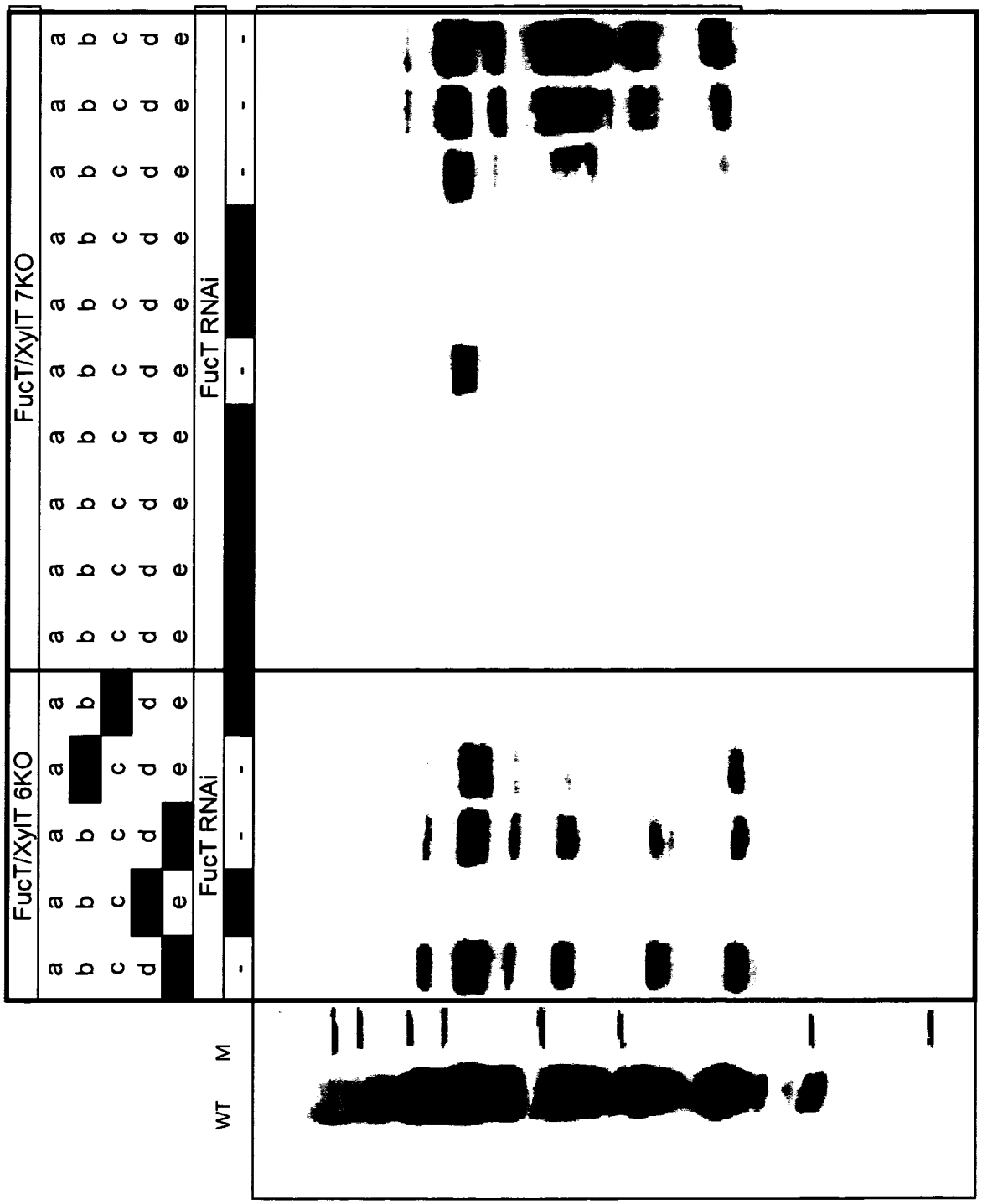


Fig. 10, continued

Fig. 11



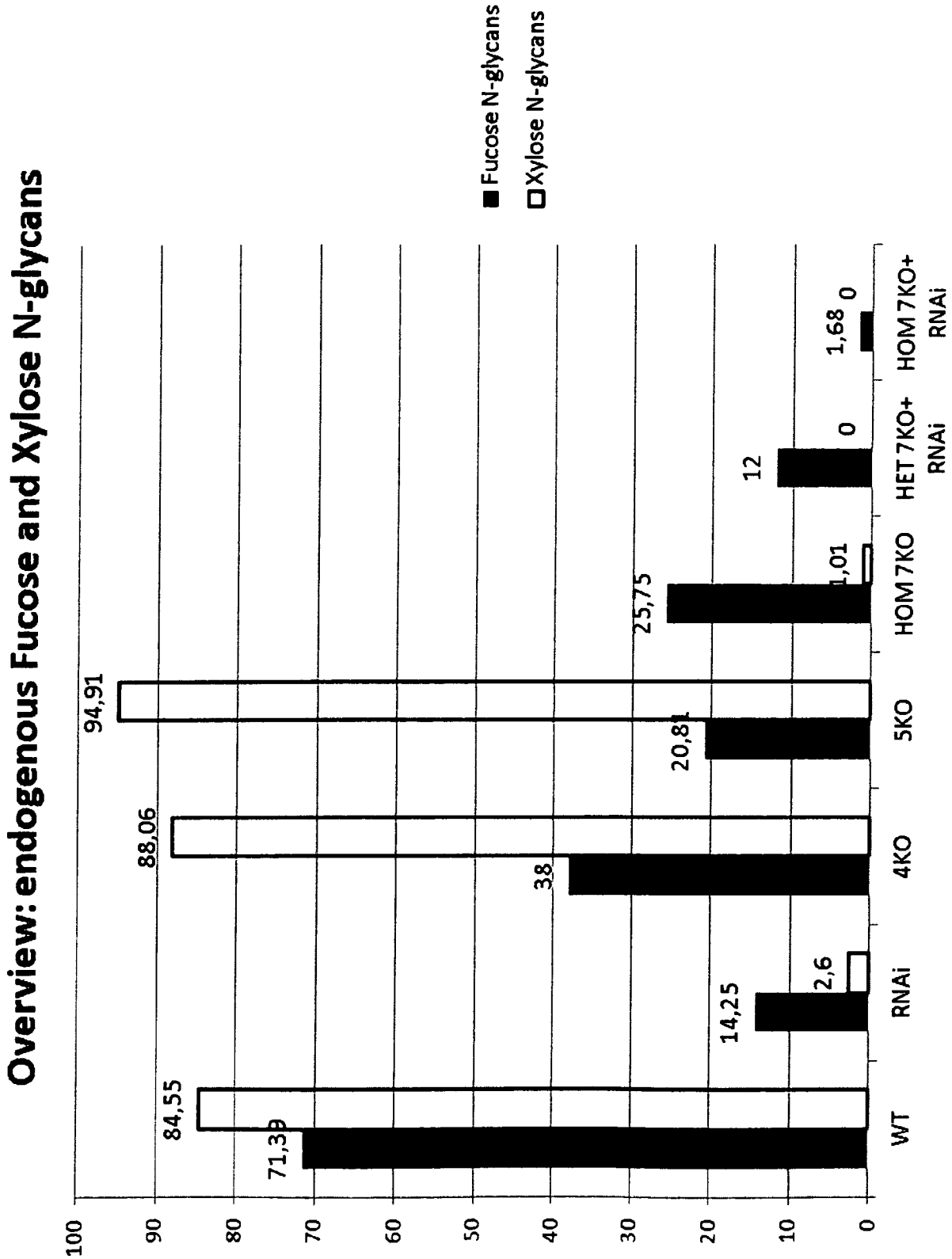

Overview: endogenous Fucose and Xylose N-glycans
Fucose N-glycans
Xylose N-glycans
100
90
80
70
60
50
40
30
20
10
0
94,91
84,55
71,39
88,06
38
20,81
25,75
14,25
2,6
12
0
1,01
1,68
0
WT
RNAi
4KO
5KO
HOM 7KO
HET 7KO+ RNAi
HOM 7KO+ RNAi

Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/004160

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/004160

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 C12N9/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/141806 A1 (BAYER BIOSCIENCE NV [BE]; WETERINGS KOEN [BE]; VAN ELDIK GERBEN [BE];) 27 November 2008 (2008-11-27) cited in the application paragraph [0036] - paragraph [0048] paragraph [0055] claims 6,16-18 ----- -/--	1-32

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2013	17/01/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Chakravarty, Ashok

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/004160

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. S. KANG ET AL: "Salt tolerance of Arabidopsis thaliana requires maturation of N-glycosylated proteins in the Golgi apparatus", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 105, no. 15, 15 April 2008 (2008-04-15), pages 5933-5938, XP055047633, ISSN: 0027-8424, DOI: 10.1073/pnas.0800237105 cited in the application page 5933, last paragraph - page 5934, paragraph 1	1-32
A	WO 2010/145846 A1 (BAYER BIOSCIENCE NV [DE]; WETERINGS KOEN [US]; VAN ELDIK GERBEN [BE]) 23 December 2010 (2010-12-23) cited in the application the whole document	1-32
A	WO 2009/056155 A1 (BAYER BIOSCIENCE NV [BE]; STEINKELLNER HERTA [AT]; STRASSER RICHARD [A) 7 May 2009 (2009-05-07) cited in the application the whole document	1-32

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/004160

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