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DESCRIPTION

Method for Secretory Production of Glycoprotein Having Human-Type Sugar Chain Using Plant Cell

TECHNICAL FIELD

The present invention relates to a method for the secretory production of a heterologous glycoprotein having a human-type sugar chain using a plant cell, a plant cell capable of secreting this glycoprotein, and a glycoprotein having a human-type sugar chain secreted by this plant cell.

BACKGROUND ART

Production of extraneous proteins using plant cultured cells is proceeding. For example, attempts are being made to produce the following proteins useful for humans using tobacco cultured cell:

GM-CSF (see, E.A. James, C. Wang, Z. Wang, R. Reeves, J.H. Shin, N.S. Magnuson and J.M. Lee, "Production and Characterization of Biologically Active Human GM-CSF Secreted by Genetically Modified Plant Cells", Protein Expr. Purif., 19, 131-138 (2000)), IL-2 and IL-4 (see, N.S. Magnuson, P.M. Linzmaier, R. Reeves, G. An, K. HayGlass and J.M. Lee, "Secretion of Biologically Active Human Interleukin-2 and Interleukin-4 from Genetically Modified Tobacco Cells in Suspension Culture", Protein Expr. Purif., 13, 45-52 (1998)), immunoglobulin (see, N.S. Magnuson, P.M. Linzmaier, J.W. Gao, R. Reeves, G. An and J.M. Lee, "Enhanced Recovery of a Secreted Mammalian Protein from Suspension Culture of Genetically Modified Tobacco Cells", Protein Expr. Purif., 7, 220-228 (1996)), erythropoietin

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(see, S. Matsumoto, A. Ishii, K. Ikura, M. Ueda and R. Sasaki, "Expression of Human Erythropoietin in Cultured Tobacco Cells", Biosci. Biotechnol. Biochem., 57, 1249-1252 (1993)), and α1-antitrypsin (see, M. Terashima, Y. Murai, M. Kawamura, S. Nakanishi, T. Stoltz, L. Chen, W. Drohan, R.L. Rodriguez and S. Katoh, "Production of Functional Human α1-Antitrypsin by Plant Cell Culture", Appl. Microbiol. Biotechnol., 52, 516-523 (1999)).

On other hand, it is reported that plant cultured cells secrete many proteins or glycoproteins (see, A. Sturm, "Heterogeneity of the Complex N-Linked Oligosaccharides at Specific Glycosylation Sites of Two Secreted Carrot Glycoproteins", Eur. J. Biochem., 199, 169-179 (1991); Y. Okushima, N. Koizumi, T. Kusano and H. Sano, "Secreted Proteins of Tobacco Cultured BY2 Cells: Identification of A New Member of Pathogenesis-Related Proteins", Plant Mol. Biol., 42, 479-488 (2000); and Y. Okushima, N. Koizumi, T. Kusano and H. Sano, "Glycosylation and Its Adequate Processing is Critical for Protein Secretion in Tobacco BY2 Cells", J. Plant Physiolo., 154, 623-627 (1999)). Of these, in the case of tobacco BY2 cultured cells, two kinds of peroxidases are purified and their genes have been cloned (see, H. Narita, Y. Asaka, K. Ikura, S. Matsumoto and R. Sasaki, "Isolation, Characterization and Expression of Cationic Peroxidase Isozymes Released into the Medium of Cultured Tobacco Cells", Eur. J. Biochem., 228, 855-862 (1995)). It is also reported that by adding polyvinylpyrrolidone (PVP) to the medium, the concentration of protein secreted in the medium could be increased (see, N.S. Magnuson, P.M. Linzmaier, J.W. Gao, R. Reeves, G. An and J.M. Lee, "Enhanced Recovery of A Secreted Mammalian Protein from Suspension Culture of

Generically Modified Tobacco Cells", Protein Expr. Purif., 7, 220-228 (1996)) and by Y. Okushima et al., supra. (1999) that from tobacco BY2 strain cultured cells, hundreds of proteins are extracellularly secreted. Among these, extracellular secretion of many glycoproteins are confirmed because of their reaction with lectin (concanavalin A) which recognizes high mannose-type sugar chains (see, Y. Okushima, supra. (1999)).

With respect to these glycoproteins, in particular, immunoglobulin, interleukin and GM-CSF, produced within plant cells, a signal peptide of each glycoprotein itself is also recognized in the secretion mechanism within the plant cell, and is secreted in the culture solution (see, E.A. James et al., supra.; N.S. Magnuson et al., supra. (1998); and N.S. Magnuson et al., supra. (1996)). In any of these glycoproteins, it is suggested, the sugar chain participates in the determination of half-life in blood, sensitivity to protease and stability. However, the sugar chain structures of recombinant proteins actually produced within plant cells and purified have not been examined and these proteins are presumed to have a plant-type sugar chain structure.

In the analysis of sugar chain structure, the secretion-type antibody molecule sIgA, produced from tobacco plants is revealed to have a plant-type sugar chain (see, M. Cabanes-Macheteau, A.C. Fitchette-Laine, C. Loutelier-Bourhis, C. Lange, N.D. Vine, J.K. Ma, P. Lerouge and L. Faye, "N-Glycosylation of a Mouse IgG Expressed in Transgenic Tobacco Plants", Glycobiology, 9, 365-372 (1999)). Furthermore, in the case where another antibody molecule is produced from the same tobacco plant body, the

antibody protein produced within the cell is decomposed by the protease and is unstable (see, L.H. Stevens, G.M. Stoopen, I.J. Elbert, J.W. Molthoff, H.A. Bakker, A. Lommen, D. Bosch and W. Jordi, "Effect of Climate Conditions and Plant Developmental Stage on the Stability of Antibodies Expressed in Transgenic Tobacco", Plant Physiol., 124, 173-182 (2000)). By the Western method using an antiplant-type sugar chain antibody, addition of a plant-type sugar chain to this antibody is confirmed. Although it is reported that the β1, 4-linked galactose residue present in the sugar chain of an antibody molecule produced by human or mouse contributes to the stabilization of antibody protein, this sugar residue is absent in the antibody molecule produced by plant cells. Because of this, the antibodies produced by tobacco plants are considered to be prone to decomposition by the protease.

In the case where erythropoietin is produced by tobacco cultured cells, the biological activity is recognized in vitro, but the activity in vivo is not detected (see, S. Matsumoto, K. Ikura, M. Ueda and R. Sasaki, "Characterization of a Human Glycoprotein (Erythropoietin) Produced in Cultured Tobacco Cells", Plant Mol. Biol., 27 1163-1172 (1995). This is concluded to occur because erythropoietin of which sugar chain is considered to greatly participate in the biological activity, has a largely different sugar chain structure when produced by plant cells.

On the other hand, it is suggested that the plant-type sugar chain may be an allergen in mammals including humans. That is, the sugar chain structure peculiar to plants, such as β1,2-xylose and α1,3-fucose

which are not seen in glycoproteins of mammals, are reported to act as an allergen (see, K. Fotisch, F. Altmann, D. Haustein and S. Vieths, Involvement of Carbohydrate Epitopes in the IgE Response of Celery-Allergic Patients, Int. Arch. Allergy Immunol., 120, 30-42 (1999); I.B. Wilson, J.E. Harthill, N.P. Mullin, D.A. Ashford and F. Altmann, "Core α1,3-Fucose is a Key Part of the Epitope Recognized by Antibodies Reacting Against Plant N-Linked Oligosaccharides and is Present in a wide Variety of Plant Extracts", Glycobiology, 8, 651-661 (1998); and R. van Ree, M. Cabanes-Macheteau, J. Akkerdaas, J.P. Milazzo, C. Loutelier-Bourhis, C. Rayon, M. Villalba, S. Koppelman, R. Aalberse, R. Rodriguez, L. Faye and P. Lerouge, "β(1,2)-Xylose and α(1,3)-Fucose Residues Have a Strong Contribution in IgE Binding to Plant Glycoallergens", J. Biol.Chem., 275(15), 11451-11458 (April 14, 2000). Accordingly, proteins for medical uses must have a sugar chain structure free of β1,2-xylose or α1,3-fucose.

DISCLOSURE OF THE INVENTION

The object of the present invention is to solve the above-described problems in conventional techniques and provide a method for the secretory production, in plant cells, of a glycoprotein which is stable, maintains its original physiological activity and is not an allergen, a plant cell capable of secreting such glycoprotein, and a glycoprotein having a human-type sugar chain secreted by this plant cell.

As a result of extensive investigations, the present inventors have found that when a human-derived galactose transferred enzyme gene cDNA is expressed in a

tobacco cultured cell BY2 strain, galactose is added to sugar chains of most glycoproteins secreted to the exterior medium and the glycoproteins have a sugar chain structure free of β1,2-xylose or α1,3-fucose. The present invention has been accomplished based on this finding. Accordingly, when a human-derived useful protein is produced using this genetic recombinant tobacco cultured cell, the objective protein having a sugar structure which is not an allergen, can be secreted in the extracellular fluid.

The present invention relates to a method for the secretory production of a glycoprotein having a human-type sugar chain. This method comprises a step of introducing a gene of an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and a gene of a heterologous glycoprotein, into a plant cell to obtain a transformed plant cell, and a step of culturing the obtained plant cell.

In the method above, the glycoprotein having a human-type sugar chain may comprise a core sugar chain and an outer sugar chain, the core sugar chain may substantially comprise a plurality of mannoses and acetylglucosamines, and the outer sugar chain may have a terminal sugar chain moiety containing a non-reducing terminal galactose.

In the method above, the outer sugar chain may have a linear or branched structure.

In the method above, the branched sugar chain moiety may be a mono-, bi-, tri- or tetra-structure

In the method above, the glycoprotein may be free

of fucose or xylose.

The method above for the secretory production may preferably further comprise a step of recovering the medium of the plant cells.

In one embodiment, the method above for the secretory production may further comprise a step of adding sugar or sugar chain in vitro.

In one aspect, the present invention relates to a plant cell which comprises a sugar chain-adding mechanism capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and can secrete a protein with an added sugar chain added by the sugar chain-adding mechanism, wherein the sugar chain-adding mechanism adds a sugar chain comprising a core sugar chain and an outer sugar chain, the core sugar chain substantially comprises a plurality of mannoses and acetylglucosamines, and the outer sugar chain has a terminal sugar chain moiety containing a non-reducing terminal galactose.

The present invention further relates to a glycoprotein having a human-type sugar chain obtained by the method described above.

The present invention still further relates to a method for the secretory production of a glycoprotein having a human-type sugar chain, comprising a step of introducing a gene for an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and a gene for a

heterologous glycoprotein, to obtain a transformed plant cell, and a step of expressing the enzyme within an intracellular organelle.

The present invention still further relates to a plant cell transformed with a gene for an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, wherein the enzyme is localized in the plant cell such that the plant cell is capable of synthesizing a glycoprotein having a human-type sugar chain structure.

In one aspect, in the plant cell above, the sugar chain structure includes an added galactose residue.

In one aspect, in the plant cell above, the sugar chain structure is free of β1,2-xylose or α1,3-fucose.

In one aspect, in the plant cell above, the sugar chain structure includes an galactose residue added to a N-linked type sugar chain of (N-acetylglucosamine)$_{1-2}$(Mannose)$_{2-5}$(N-acetylglucosamine)$_2$ selected from the group consisting of $GlcNAc_1Man_3GlcNAc_2$, $GlcNAc_1Man_5GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$ and $GlcNAc_1Man_4GlcNAc_2$.

In one aspect, in the plant cell above, the enzyme is localized within an intracellular organelle in the plant cell.

The present invention still further relates to a plant regenerated from the plant cell above.

The present invention still further relates to a

seed produced from the plant above.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic view showing a process for cloning hGT.

Fig. 2 is a schematic view showing a process for constructing vector pGAhGT for expressing hGT.

Fig. 3 is a photograph showing the Southern analysis of genome of transformant tobacco cultured cell. Fig. 3(A) shows the results when genomic DNA (40 μg) was digested by EcoRI and HindIII and then electrophoresed. The numerals in the left side show the sites of the DNA molecular weight marker. Fig. 3(B) shows a schematic view of fragment 2.2 kb containing promoter, hGT and terminator integrated into each transformant.

Fig. 4A is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium by high-performance liquid chromatography.

Fig. 4B is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium by high-performance liquid chromatography.

Fig. 5 is a view showing the structures and analysis results of sugar chains in the glycoprotein secreted in the GT6 strain culture medium. The numerals in parentheses in the Figure show the molar ratio of sugar chain having each structure shown in the Figure.

Fig. 6 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. 7 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. 8 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. 9 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. 10 is a view showing the IS-MS/MS analysis of PA-sugar chain prepared from GT6 strain culture medium. B is a partial enlarged view of A.

Fig. 11 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. 12 is a view showing the analysis of PA-sugar chain prepared from GT6 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. **13** is a photograph showing the results of sialic acid transferase reaction in vitro using GT6 stain culture medium-derived glycoprotein, BY2 strain culture medium-derived glycoprotein or asialo fetuin as the substrate.

Fig. **14A** is a view showing the analysis of PA-sugar salt prepared from BY2 strain culture medium by high-performance liquid chromatography.

Fig. **14B** is a view showing the analysis of PA-sugar salt prepared from BY2 strain culture medium by high-performance liquid chromatography.

Fig. **15A** is a view showing the structures and analysis results of sugar chains in the glycoprotein secreted in the GT6 strain culture medium. The numerals in parentheses in the Figure show the molar ratio of sugar chain having each structure shown in the Figure.

Fig. **15B** is a view showing the structures and analysis results of sugar chains in the glycoprotein secreted in the GT6 strain culture medium. The numerals in parentheses in the Figure show the molar ratio of sugar chain having each structure shown in the Figure.

Fig. **16** is a view showing the analysis of PA-sugar chain prepared from BY2 strain culture medium.

Fig. **17** is a view showing the analysis of PA-sugar chain prepared from BY2 strain culture medium and the exoglycosidase digestion product thereof by high-

performance liquid chromatography.

Fig. **18A** is a view showing the analysis of PA-sugar chain prepared from BY2 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. **18B** is a view showing the analysis of PA-sugar chain prepared from BY2 strain culture medium and the exoglycosidase digestion product thereof by high-performance liquid chromatography.

Fig. **19** is a photograph showing the results of isoelectric focusing electrophoresis. Proteins in the spent media of cultured tobacco cells were analyzed by isoelectric focusing and stained for peroxidase activity. Wild Type denotes BY2 strain. WT-HRP denotes transformant of BY2 strain with HRP. GT-HRP denotes the transformant of GT6 strain with HRP gene.

Fig. **20** is a photograph showing the results of lectin staining of proteins in the spent media of transgenic cultured tobacco cells. Proteins were fractionated by SDS-PAGE and stained with Coomassie brilliant blue (A), or transfered to nitrocellulose membrane and treated with ConA(B), and RCA120(C). Wild Type denotes BY2 strain. WT-HRP-2 denotes one of the transformants of BY2 strain with HRP gene. GT-HRP-5 denotes one of the transformants of GT6 strain with HRP gene.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is described in detail below.

Herein, unless otherwise specified, protein separating and analyzing methods and immunological techniques known in the art can be employed. These techniques can be performed using commercially available kits, antibodies, marker substances and the like.

The method of the present invention is a method for producing a glycoprotein having a human type sugar chain. In the present invention, the "human-type sugar chain" means a sugar chain having a galactose residue bonded to an N-acetylglucosamine residue. The galactose residue in the human-type sugar chain may be a terminal of the sugar chain or a sialic acid residue may further be bonded to the outer side of the galactose residue. In the glycoprotein having a human-type sugar chain comprising a core sugar chain moiety, a branched sugar chain moiety and a terminal sugar chain moiety of the present invention, at least one of xylose and fucose is preferably not bonded in one or more of the moieties, more preferably in any of the moieties. Most preferably, the human-type sugar chain contains neither xylose nor fucose.

The plant cell may be any plant cell. The plant cell may have any form of cultured cell, cultured tissue, cultured organ and plant body. Among these, preferred are cultured cell, cultured tissue and cultured organ, more preferred is cultured cell. The plant species which can be used in the production method of the present invention may be any plant species which can perform the genetic transduction. Examples of the plant species which can be used in the production method of the present invention include plants belonging to *Solanaceae*, *Gramineae*,

Cruciferae, *Rosaceae*, *Leguminosae*, *Cucurbitaceae*, *Labiatae*, *Liliaceae*, *Chenopodiaceae* and *Umbelliferae*.

Examples of the *Solanaceae* plant include plants belonging to *Nicotiana*, *Solanum*, *Datura*, *Lycopersion* or *Petunia*, such as tobacco, egg-plant, potato, tomato, red pepper and petunia.

Examples of the *Gramineae* plant include plants belonging to *Oryza*, *Hordenum*, *Secale*, *Soccharum*, *Echinochloa* or *Zea*, such as rice, barley, rye, barnyard millet, sorghum and corn.

Examples of the *Cruciferae* plant include plants belonging to *Raphanus*, *Brassica*, *Arabidopsis*, *Wasabia* or *Capsella*, such as radish, rape, whitlowgrass, horseradish and shepherd's purse.

Examples of the *Rosaceae* plant include plants belonging to *Orunus*, *Malus*, *Pynus*, *Fragaria* or *Rosa*, such as Japanese apricot, peach, apple, pear, strawberry and rose.

Examples of the *Leguminosae* plant include plants belonging to *Glycine*, *Vigna*, *Phaseolus*, *Pisum*, *Vicia*, *Arachis*, *Trifolium*, *Alphalfa* or *Medicago*, such as soybean, red bean, kidney bean, green pea, horsebean, peanut, clover and bur clover.

Examples of the *Cucurbitaceae* plant include plants belonging to *Luffa*, *Cucurbita* or *Cucumis*, such as luffa, cushaw, cucumber and melon.

Examples of the *Labiatae* plant include plants belonging to *Lavandula*, *Mentha* or *Perilla*, such as lavender, mint and perilla.

Examples of the *Liliaceae* plant include plants belonging to *Allium*, *Lilium* or *Tulipa*, such as Welsh onion, garlic, lily and tulip.

Examples of the *Chenopodiaceae* plant include plants belonging to *Spinacia*, such as spinach.

Examples of the *Umbelliferae* plant include plants belonging to *Angelica*, *Daucus*, *Cryptotaenia* or *Apitum*, such as Angelica polyclada, carrot, trefoil and celery.

Among these plants for use in the production method of the present invention, preferred are tobacco, tomato, potato, rice, corn, radish, soybean, green pea, bur clover and spinach, more preferred are tobacco, tomato, potato, corn and soybean.

The "enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" means an enzyme which can transfer a galactose residue to a non-reducing terminal acetylglucosamine residue generated at the addition of a sugar chain after the synthesis of the protein moiety of a glycoprotein within a plant cell. Examples of such an enzyme include galactosyl transferase, lactose synthase and β-galactosidase. Such an enzyme may be derived from any animal species but is preferably derived from mammals, more preferably from human.

This enzyme is preferably localized in an intracellular organelle. Although restriction to a specific theory is not intended, the present inventors consider that this enzyme being present in an intracellular organelle, such as endoplasmic reticulum and Golgi body, thereby acting on a protein or sugar chain before a fucose or xylose residue is added, or acting so as to inhibit the addition of a fucose or xylose residue, at the time of expression and secretion of a heterologous glycoprotein in plant cells.

The "enzyme gene capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue" may be isolated from any animal cell using a nucleotide sequence known to code this enzyme, or a commercially available enzyme may be purchased or may be used after modifying it to suit to the expression in plants.

Herein, the "gene" means the structural gene moiety. In order to facilitate the expression in plants, a regulator sequence such as promoter, operator and terminator may be linked to the gene.

The "heterologous glycoprotein" means a glycoprotein which is originally not expressed in plants for use in the present invention. Examples of the heterologous glycoprotein include enzyme, hormone, cytokine, antibody, vaccine, receptor and serum protein. Examples of the enzyme include horseradish peroxidase, kinase, glucocerebrosidase, α-galactosidase, phytase, TPA (tissue-type plasminogen activator) and HMG-CoA reductase. Examples of the hormone and cytokine include enkephalin,

interferon alpha, GM-CSF, G-CSF, chorionic gonadotropic hormone, interleukin-2, interferon beta, interferon gamma, erythropoietin, vascular endothelial growth factor, human chorionic gonadotropin (HCG), luteinizing hormone (LH), thyroid-simulating hormone (TSH), prolactin and follicle-stimulating hormone. Examples of the antibody include IgG, scFv and secretory IgA. Examples of the vaccine include hepatitis B surface antigen, rotavirus antigen, Escherichia coli enterotoxin, malaria antigen, G protein of rabies virus, and HIV virus glycoprotein (e.g., gp120). Examples of the receptor and matrix protein include EGF receptor, fibronectin, α1-antitrypsin and coagulation factor VIII. Examples of the serum protein include albumin, complement system protein, plasminogen, corticosteroid-binding globulin, Thyroxine-binding globulin and protein C.

The "gene of heterologous glycoprotein" may be isolated from any cell using a nucleotide sequence known to code the objective heterologous glycoprotein or a commercially available gene may be purchased or may be used after modifying it to suit to the expression in plants.

The gene of an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and the gene of heterologous glycoprotein are introduced into a plant cell by a method known in the art. These genes may be introduced separately or simultaneously. Those skilled in the art will appreciate that the choice of method might depend on the type of plant targeted for transformation.

Suitable methods of transforming plant cells

include microinjection (Crossway et al., BioTechniques 4:320-334 (1986)), electroporation (Riggs et al., Proc. Natl. Acad. Sci. USA 83:5602-5606 (1986), Agrobacterium-mediated transformation (Hinchee et al., Biotechnology 6:915-921 (1988); See also, Ishida et al., Nature Biotechnology 14:745-750 (June 1996) for maize transformation), direct gene transfer (Paszkowski et al., EMBO J. 3:2717-2722 (1984); Hayashimoto et al., Plant Physiol 93:857-863 (1990)(rice)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wis. and Dupont, Inc., Wilmington, Del. (see, for example, Sanford et al., U.S. Pat. No. 4,945,050; and McCabe et al., Biotechnology 6:923-926 (1988)). See also, Weissinger et al., Annual Rev. Genet. 22:421-477 (1988); Sanford et al., Particulate Science and Technology 5.27-37 91987)(onion); Svab et al., Proc. Natl. Acad. Sci. USA 87:8526-8530 (1990) (tobacco chloroplast); Christou et al., Plant Physiol. 87:671-674 (1988)(soybean); McCabe et al., Bio/Technology 6.923-926 (1988)(soybean); Klein et al., Proc. Natl. Acad. Sci. USA, 85:4305-4309 (1988)(maize); Klein et al., Bio/Technology 6:559-563 (1988) (maize); Klein et al., Plant Physiol. 91:440-444 (1988) (maize); Fromm et al., Bio/Technology 8:833-839 (1990); and Gordon-Kamm et al., Plant Cell 2: 603-618 (1990) (maize); Koziel et al., Biotechnology 11: 194-200 (1993) (maize); Shimamoto et al., Nature 338: 274-277 (1989) (rice); Christou et al., Biotechnology 9: 957-962 (1991) (rice); Datta et al., Biol/Technology 3:736-740 (1990) (rice); European Patent Application EP 0 332 581 (orchardgrass and other Pooideae); Vasil et al., Biotechnology 11: 1553-1558 (1993) (wheat); Weeks et al., Plant Physiol. 102: 1077-1084 (1993) (wheat); Wan et al., Plant Physiol. 104: 37-48 (1994) (barley); Jahne et al., Theor. Appl. Genet.

89:525-533 (1994)(barley); Umbeck et al., Bio/Technology 5: 263-266 (1987) (cotton); Casas et al., Proc. Natl. Acad. Sci. USA 90:11212-11216 (December 1993) (sorghum); Somers et al., Bio/Technology 10:1589-1594 (December 1992) (oat); Torbert et al., Plant Cell Reports 14:635-640 (1995) (oat); Weeks et al., Plant Physiol. 102:1077-1084 (1993) (wheat); Chang et al., WO 94/13822 (wheat) and Nehra et al., The Plant Journal 5:285-297 (1994) (wheat). A particularly preferred set of embodiments for the introduction of recombinant DNA molecules into maize by microprojectile bombardment can be found in Koziel et al., Biotechnology 11: 194-200 (1993), Hill et al., Euphytica 85:119-123 (1995) and Koziel et al., Annals of the New York Academy of Sciences 792:164-171 (1996). An additional preferred embodiment is the protoplast transformation method for maize as disclosed in EP 0 292 435. Transformation of plants can be undertaken with a single DNA species or multiple DNA species (i.e. co-transformation) and both these techniques are suitable for use with the peroxidase coding sequence.

The gene product expressed and secreted by the plant cell having incorporated thereinto the above-described genes can be identified by a method known in the art. Examples of the identification method include silver staining, Western blotting, Northern hybridization and detection of enzymatic activity.

The transformed cell expressing an enzyme capable of performing a transfer reaction of a galactose residue into a non-reducing terminal acetylglucosamine residue and expressing a heterologous glycoprotein, expresses and secretes a heterologous glycoprotein having a human-type sugar chain. In other words, the thus-obtained transformed

plant has a human-type sugar chain-adding mechanism and by culturing this transformed cell, human-type glycoprotein can be expressed and secreted in a large amount in the medium.

This human-type glycoprotein comprises a core sugar chain and an outer sugar chain, and the core sugar chain substantially comprises a plurality of mannoses and acetylglucosamines. The outer sugar chain of the glycoprotein obtained contains a non-reducing terminal sugar chain moiety. The outer sugar chain may have a linear structure or a branched structure. The branched sugar chain moiety may be any of mono-, bi-, tri- and tetra-structures. The glycoprotein produced by the transformed cell preferably is free of fucose or xylose.

The resulting transformed plant cell may be maintained in the state of cultured cell, may be differentiated into a specific tissue or organ, or may be regenerated in a complete plant body or in a part such as seed, fruit, leaf, root, stem or flower obtained from a complete plant body.

For the culture, differentiation or regeneration of the transformed plant cell, means and culture mediums known in the art are used. Examples of the medium include Murashige-Skoog (MS) medium, Gamborg B5 (B) medium, White medium and Nitsch & Nitsch (Nitsch) medium, however, the present invention is not limited thereto. These mediums are usually used after adding thereto an appropriate amount of a plant growth control substance (e.g., plant hormone) and the like.

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (Fujimura et al., Plant Tissue Culture Letters, 2:74, 1985; Toriyama et al., Theor. Appl. Genet., 73:16, 1986; Yamada et al., Plant Cell Rep., 4:85, 1986; . Abdullah et al., Biotechnology, 4:1087, 1986).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, Biotechnology, 6:397, 1988).

Agrobacterium-mediated transfer is also a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of Agrobacterium-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described above.

Fundamentally, as long as the transformed plant cell grows and then expresses and secretes the desired gene product, a culture medium having any composition containing trace nutrients necessary for the growth of plant cells, such as carbon source, nitrogen source, vitamins and salts, may be used for the secretory production of a glycoprotein having a human-type sugar chain. Also, polyvinylpyrrolidone, protease inhibitor and the like may

be added so as to stabilize the secreted heterologous protein and attain efficient secretion of the heterologous protein.

The glycoprotein having a human-type sugar chain expressed and secreted by the transformed plant cell can be typically isolated from the medium of plant cells. The isolation of glycoprotein from the medium of plant cells can be performed using a method well-known to one skilled in the art. For example, the glycoprotein can be purified to isolate it from the medium using, individually or in combination, techniques such as salting out (e. g., ammonium sulfate precipitation, sodium phosphate precipitation), solvent precipitation (e.g., protein fractional precipitation by acetone or ethanol), dialysis, gel filtration, ion exchange, column chromatography such as reverse phase, ultrafiltration, and high-performance chromatography (HPLC).

Alternatively, the glycoprotein of the present invention may also be isolated or extracted from plant cells. Furthermore, the glycoprotein of the present invention, which is contained in transformed cells, can be used as it is for food. The glycoprotein of the present invention has a human-type sugar chain addition and therefore, is free of antigenicity and suitable for the administration to animals including humans.

Further also included within the present invention, the plant cell or the plant body transformed with a gene of an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue, may have inactivated or

suppressed activity of β1,2-xylose transfer enzyme or α 1,3-fucose transfer enzyme.

Strasser et al. has isolated cDNA encoding β 1,2-xylosyltransferase from *Arabidopsis thaliana* (Strasser R, Mucha J, Mach L, Altmann F, Wilson IB, Glossl J, Steinkellner H, Molecular cloning and functional expression of β 1, 2-xylosyltransferase cDNA from *Arabidopsis thaliana*. FEBS Lett. (2000) 472:105-108). Homology searching against databases such as NIH GenBank may show nucleotide sequences corresponding to some EST (expression sequence tag) clones and genome sequence in some plants, are similar to the nucleotide sequence encoding Arabidopsis β1,2-xylosyltransferase. Based on these sequences, nucleic acid encoding β 1,2-xylosyltransferase may be cloned from the host plant and used to establish a plant cell or plant with reduced xylosyltransferase activity by repressing β 1,2-xylosyltransferase gene expression. Repression of β 1,2-xylosyltransferase gene expression may be carried out by an antisense method, co-suppression method, RNAi method or so on.

Alternatively, a plant cell or plant with reduced xylosyltransferase activity may be established by chemical mutagenesis, site-directed mutagenesis using oligonucleotides, tagging methods or so on. In said plant, introduction and expression of one or more glycosyltransferase genes encoding galactose transferase together with a gene encoding a heterologous polypeptide may produce a heterologous polypeptide with humanized glycan structure or excrete a heterologous polypeptide with humanized glycan structure.

Similarly, Wilson et al. isolated cDNA encoding α 1,3-fucosyltransferase from *Arabidopsis thaliana* (Wilson IB, Rendic D, Freilinger A, Dumic J, Altmann F, Mucha J, Muller S, Hauser MT Cloning and expression of cDNAs encoding α 1,3-fucosyltransferase homologues from *Arabidopsis thaliana*. Biochim Biophys Acta (2001) 1527:88-96). Leiter et al. isolated cDNA encoding α1,3-fucosyltransferase from mung bean (Leiter, H., Mucha, J., Staudacher, E., Grimm, R., Glossl, J., Altmann, F. Purification, cDNA cloning, and expression of GDP-L-Fuc:Asn-linked GlcNAc α 1,3-fucosyltransferase from mung beans. J. Biol. Chem. (1999) 274:21830-21839). Homology searching against databases such as NIH GenBank may show nucleotide sequences corresponding to some EST (expression sequence tag) clones and genome sequence in some plants, are similar to the nucleotide sequence encoding *Arabidopsis* α 1,3-fucosyltransferase. Based on these sequences, nucleic acid encoding α1,3-fucosyltransferase may be cloned from the host plant and used to establish a plant cell or plant with reduced fucosyltransferase activity by repressing α1,3-fucosyltransferase gene expression. Repression of α1,3-fucosyltransferase gene expression can be carried out by an antisense method, co-suppression method, RNAi method or so on.

Alternatively, a plant cell or plant with reduced fucosyltransferase activity may be established by chemical mutagenesis, site-directed mutagenesis using oligonucleotides, tagging methods or so on. In said plant, introduction and expression of one or more glycosyltransferase genes encoding galactose transferase together with a gene encoding a heterologous polypeptide

may produce a heterologous polypeptide with humanized glycan structure or excrete a heterologous polypeptide with humanized glycan structure.

(Examples)

The present invention is described below by referring to the Examples. The following Examples are only to illustrate but not to limit the present invention.

1. Cloning of Human β1-4 Galactose Transferase Gene

The β1-4 galactose transferase (hGt) (EC2.4.1.38) has been already cloned and a primary structure comprising 400 amino acids has been revealed (K.A. Masri et al., Biochem. Biophys. Res. Commun., 157, 657-663 (1988)).

(1) Primer Preparation and Template DNA

By referring to the report of Masri et al., the following primer were prepared.

hGT-5Eco:5'-AAAGAATTCGCGATGCCAGGCGCGCGTCCCT-3' (SEQ. ID NO:1)

hGT-2Sal:3'-TCGATCGCAAAACCATGTGCAGCTGATG-5' (SEQ. ID. NO:2)

hGT-7Spe:3'-ACGGGACTCCTCAGGGGCGATGATCATAA-5' (SEQ. ID. NO:3)

hGT6Spe:5'-AAGACTAGTGGGCCCCATGCTGATTGA-3' (SEQ. ID. NO:4)

As the template DNA, human genomic DNA, human placenta cDNA and human kidney cDNA purchased from Clontech were used.

(2) Cloning of hGT gene cDNA

Using two combinations of (i) template of human

genomic DNA with primers of hGT-5Eco and hGT-7Spe and (ii) template of human placenta cDNA with primers of hGT-2Sal and hGT6Spe, a PCR reaction was performed under the following conditions to obtain fragments of 0.4 kb and 0.8 kb containing an hGT-coding region.

(PCR Reaction System)

Water was added to 1 μl of template DNA, 5 μl of 10×PCR buffer, 4 μl of dNTPs (200 μM), primer (10 pmol) and 0.5 μl of Tag polymerase (produced by Takara Shuzo) (in the case of Tub polymerase, 0.2 μl) to make 50 μl.

(PCR Reaction Condition)

First Stage:

cycle number: 1, denaturation (94°C): 5 min., annealing (55°C): 1 min., extension (72°C): 2 min.

Second Stage:

cycle number: 30, denaturation (94°C): 1 min., annealing (55°C): 1 min., extension (72°C): 2 min.

Third Stage:

cycle number: 1, denaturation (94°C): 1 min., annealing (55°C): 2 min., extension (72°C): 5 min.

Two fragments obtained were combined to construct hGT gene cDNA and subcloned into pBluescriptIISK+(SK). The pBluescriptIISK+(SK) was purchased from Stratagene. Fig. 1 shows the construction of plasmid containing hGT gene cDNA. SEQ. ID. NO:5 shows the base sequence of the obtained hGT gene and SEQ. ID. NO:6 shows the presumed amino acid sequence.

The obtained sequence was different from the hGT sequence disclosed in Masri et al. supra. in the following

points: a) A at the position 528, C at the position 562 and A at the position 1047 were changed to G, T and G, respectively, but the amino acids coded were not changed; b) 9 bases at the positions 622 to 630 were deleted; and c) in order to connect the above-described fractions of 0.4 kb and 0.8 kb, G at the position 405 and T at the position 408 were transformed to A and A, respectively, at the preparation of primer.

Incidentally, the hGT gene cDNA which has two initiation codons (ATG), was designed in this experimentation such that the translation starts from the second initiation codon (position 37).

2. Introduction of hGT Gene into Tobacco Cultured Cell

(1) hGT has been reported to be *Escherichia coli* expressed as an active type (see, D. Aoki et al., EMBO J., 9, 3171 (1990) and K. Nakazawa et al., J. Biochem., 113, 747 (1993)).

In order to express hGT in tobacco cultured cells, a vector pGAhGT for expression was constructed as shown in Fig. 2. The promoter used was cauliflower mosaic virus 35S promoter (CaMV 35S promoter) and the selectable marker used was kanamycin resistant gene. The pGAhGT was introduced into tobacco cultured cells through Agrobacterium.

The transformation of Agrobacterium was performed using a triparental mating method by Bevan et al (see, M. Bevan, Nucleic Acid Res., 12, 8711 (1984)). *Escherichia coli* DH 5α strain (suE44, ΔlacU169 (ϕ80lacZΔM15), hsdR17) (Bethesda Research Laboratories Inc., Focus 8(2), 9(1986)) having pGA-type plasmid (see, G. An, Methods Enzymol. 153,

292 (1987)) and *Escherichia coli* HB101 having helper plasmid pRK2013 (see, M. Bevan, Nucleic Acid Res., 12, 8711 (1984)) each was cultured at 37 °C overnight in 2×YT medium containing 12.5 mg/L of tetracycline and 50 mg/L of kanamycin, and *Agrobacterium tumefaciens* EHA101 strain (see, E.H. Elizabeth, J.Bacteriol., 168, 1291 (1986)) was cultured at 28 °C over two nights in 2×YT medium containing 50 mg/L of kanamycin and 25 mg/L of chloramphenicol. From each culture medium, 1. 5 ml was transferred into an Eppendorf tube and cells were collected and washed three times with LB medium. The obtained cells of each species were suspended in 100 µl of 2×YT medium, these three kinds of cells were mixed, the mixture was smeared on 2×YT agar medium and cultured at 28 °C to conjugation-transmit the pGA-type plasmid from *Escherichia coli* to *Agrobacterium*. After 2 days, a part of cells grown throughout the surface of the 2×YT agar medium were scraped by a loop and coated on LB agar medium containing 50 mg/L of kanamycin, 12.5 mg/L of tetracycline and 25 mg/L of chloramphenicol. After culturing at 28 °C for 2 days, a single colony was selected.

The transformation of tobacco cultured cells was performed by the method described in G. An, Plant Mol. Bio. Manual, A3, 1. A suspension of *Agrobacterium* (EHA101 strain having pGA-type plasmid) cultured at 28 °C for 36 hours in LB medium containing 12.5 mg/L of tetracycline and a suspension of tobacco cultured cells (*Nicotiana tabacum* L. cv. bright yellow 2) after culturing for 4 days (purchased under Cell Name of BY-2 in catalogue No. RPC1 from The Institute of Physical and Chemical Research, Riken Gene Bank, Plant Cell Bank) were placed in a petri dish in amounts of 100 µl and 4 ml, respectively, then thoroughly mixed and left standing at 25 °C in a dark place. After 2 days, the

culture medium in the petri dish was transferred into a centrifugation tube and the supernatant was removed by the centrifugal separation (1,000 rpm, 5 minutes). Subsequently, a fresh medium was added and after the centrifugal separation, the cells were smeared on a plate of modified LS agar medium containing from 150 to 200 mg/L of kanamycin and 250 mg/L of carbenicillin and left standing at 25 °C in the dark. After about 2 to 3 weeks, the callused cells were implanted on a new plate and the growing clones were selected. After further 2 to 3 weeks, the clones were transferred to 30 ml of modified LS medium having added thereto kanamycin and carbenicillin and subjected to passage culture. For about 1 month, the selection was repeated. From some resistant strains obtained, 6 resistant strains (GT1, 4, 5, 6, 8 and 9) were randomly selected.

(2) Identification of Introduced hGT Gene

The resistant strains obtained were analyzed by the Southern analysis and it was confirmed that the fragment of 2.2 kb containing CaMV35S promoter-hGT gene cDNA-NOS terminator in T-DNA was integrated into genomic DNA of tobacco cultured cell. From each resistant strain obtained above, genomic DNA was prepared, digested using EcoRI, HindIII, and analyzed by Southern analysis.

The preparation of chromosome DNA from tobacco cultured cells was performed according to the Watabe method (see, K. Watabe, "Cloning to Sequence (Cloning and Sequence)", Shokubutsu Biotechnology Jikken Manual (Plant Biotechnology Experimentation Manual), Noson Bunka Sha). 10 ml of Tobacco cultured cells were frozen by liquid nitrogen and ground into the powder form using mortar and

pestle. Before liquefaction started, about 5 g of the thus-obtained powder was added to 5 ml of 2×CTAB (cetyltrimethylammonium bromide) solution preheated to 60 °C in a centrifugal tube (40 ml) and gradually well-mixed and while occasionally mixing at 60 °C for 10 minutes or more, the temperature was maintained. Thereto, 5 ml of chloroform:isoamyl alcohol (24:1) was added and thoroughly mixed until an emulsion was formed, and the emulsion was then centrifuged (2,800 rpm, 15 minutes, room temperature). The upper layer was transferred to a new 40 ml-volume centrifugal tube and the extraction operation using chloroform:isoamyl alcohol (24:1) was repeated. To the obtained upper layer, 1/10 volume of 10% CTAB was added and thoroughly mixed and centrifuged (2,800 rpm, 15 minutes, room temperature). The upper layer was transferred to a new centrifugal tube and 1 volume of cold isopropanol was added thereto, well mixed and centrifuged (4,500 rpm, 20 minutes, room temperature). After removing the supernatant by an aspirator, a TE buffer solution containing 5 ml of 1M sodium chloride was added and completely dissolved at 55 to 60 °C. Thereto, 5 ml of cold isopropanol was added and when DNA was observed, the DNA was taken up using the end of a stick, transferred to an Eppendorf tube (containing 80% cold ethanol) and rinsed. The DNA was further rinsed with 70% ethanol and the dry precipitate was dissolved in an appropriate amount of TE buffer. Thereto, 5 μl of RNAaseA (10 mg/ml) was added and reacted at 37 °C for 1 hour. The 2×CTAB solution had a composition of 2% CTAB, 0.1M Tris-HCl (pH: 8.0), 1.4M sodium chloride and 1% polyvinyl pyrrolidone (PVP), and the 10% CTAB solution had a composition of 10% CTAB and 0.7M sodium chloride.

The Southern analysis was performed as follows.

(i) Electrophoresis and Alkali Modification of DNA:

After completely degrading 40 μg of the obtained chromosome DNA using a restriction enzyme, 1.5% agarose gel electrophoresis (50 V) was performed by a standard method. The gel was stained with ethidium bromide, photographed and shaken for 20 minutes in 400 ml of 0.25M HCl. Thereafter, the solution was discarded and the gel was immersed in 400 ml of modified solution (1.5M, NaCl, 0.5M, NaOH) and gradually shaken for 45 minutes. Subsequently, the solution was discarded and 400 ml of a neutralization solution (1.5M NaCl, 0.5M Tris-Cl (pH: 7.4)) was added and gradually shaken for 15 minutes. After discarding the solution, 400 ml of the neutralization solution was again added and gradually shaken for 15 minutes.

(ii) Transfer:

The DNA after the electrophoresis was transferred to a nylon membrane (Hybond-N Amersham) using 20×SSC. The transfer was performed for 12 hours or more. The blotted membrane was dried at room temperature for 1 hour and subjected to UV fixing for 5 minutes. The 20×SSC had a composition of 3M NaCl and 0.3M sodium citrate.

(iii) Preparation of DNA Probe

The DNA probe was prepared using Random prime Labeling Kit (produced by Takara Shuzo). In an Eppendorf tube, a reaction solution shown below was prepared and after heating at 95 ° C for 3 minutes, rapidly cooled in ice: template DNA 25 ng, Random Primer 2 μl, water added to make 5 μl. 10×Buffer and dNTP each in 1.5 μl and [α-^{32}P]dCTP (1.85 MBq, 50 mCi) in 5 μl were added, followed by filling up to 24 μl with H_2O. Thereto, 1 μl of Klenow fragment was added

and after keeping at 37 ° C for 10 minutes, eluted through NAP10 column (produced by Pharmacia) to purify the DNA. This purified DNA was heated at 95 ° C for 3 minutes and then rapidly cooled in ice to obtain a hybridization probe.

(iv) Hybridization:

To the following prehybridization solution, 0.05 mg/ml of 0.5% (w/v) SDS was added. In the resulting solution, the membrane of (ii) above was immersed and the prehybridization was performed at 42 ° C for 2 hours or more. Thereafter, the DNA probe prepared in (iii) was added and the hybridization was performed at 42 °C for 12 hours or more. The prehybridization solution had a composition of 5×SSC. 50 mM sodium phosphate, 50% (w/v) formamide, 5×Denhardt's solution (obtained by diluting 100×Denhardt's solution), 0.1% (w/v) SDS. The 100×Denhardt's solution had a composition of 2% (w/v) BSA, 2% (w/v) Ficol 400, 2% (w/v) polyvinyl pyrrolidone (PVP).

(v) Autoradiography:

After the cleaning in the following sequence, the autoradiography was performed by a standard method. Twice in 2×SSC and 0.1% SDS at 65 °C for 15 minutes and then, once in 0.1×SSC and 0.1% SDS at 65 °C for 15 minutes.

Fig. 3 shows the results of the Southern analysis of genomic DNA prepared each resistant strain obtained above. As seen from Fig. 3, it was confirmed that the hGT gene was integrated in four strains of GT1, 6, 8 and 9.

3. Analysis of Glycoprotein Secreted by Galactosyl Transferase Transformant

(Preparation of Glycoprotein by Extracellular Secretion of

Tobacco Cultured Cell GT6 Strain)

A culture medium of tobacco cultured cell GT6 strain resulting from culturing in modified Murashige-Skoog medium prepared using mixed salts for Murashige-Skoog medium (produced by Wako Junyaku) for 7 days was centrifuged at 2, 000 rpm for 10 minutes at room temperature and the obtained supernatant was recovered as the GT6 strain culture medium and used in this Example. The obtained supernatant was dialyzed against dH_2O (deionized water) (1×10^5 times dilution) and then freeze-dried.

(Preparation of N-Linked Type Sugar Chain)

The sample obtained by the freeze-drying was hydrazinolyzed at 100 °C for 10 hours to excise the sugar chain. To the hydrazinolysis product, excess acetone was added and by centrifugation at 4 °C and 10,000 rpm for 20 minutes, sugar chains were precipitated. The sugar chains were N-acetylated in the presence of an aqueous saturated sodium hydrogencarbonate solution and acetic anhydride, then desalted using Dowex 50×2 (produced by Muromachi Kagaku Kogyo), and passed through Sephadex G-25 super fine gel filter column (1.8×180 cm) equilibrated with 0.1N aqueous ammonia, thereby recovering the N-linked sugar chains.

(Preparation of Pyridylaminated (PA) Sugar Chain)

The recovered N-linked sugar chains were PA-formed. The PA-sample was passed through Sephadex G-25 super fine gel filter column (1.8×180 cm) equilibrated with an aqueous 3% acetic acid solution to purify the PA-sugar chains.

(Fractionation and Analysis of PA-Sugar Chains by HPLC)

The PA-sugar chain structure was analyzed by

reversed-phase (RP) and size-fractionation (SF) HPLC, two-dimensional sugar chain mapping using the exoglycosidase digestion, and IS-MS/MS analysis. In the HPLC (high-performance liquid chromatography) analysis. Jasco 880-PU HPLC having Jasco 821-FP Intelligent Spectrofluorometer was used and the fluorescence intensity was measured at excitation wavelength of 310 nm and fluorescence wavelength of 380 nm.

In the RP-HPLC analysis using Cosmosil 5C18-P column (6×250 mm, produced by Nakaraitesc), the concentration of acetonitrile in an aqueous 0.02% TFA solution was increased from 0% to 6% over 40 minutes at a flow rate of 1.2 ml/min and thereby PA-sugar chains were eluted. In the SF-HPLC analysis using Asahipak NH2P-50 column (4.6×250 mm, produced by Showa Denko K.K.), the concentration of acetonitrile in a dH_2O-acetonitrile mixed solution was increased from 26% to 50% over 25 minutes at a flow rate of 0.7 ml/min and thereby PA-sugar chains were eluted.

(Analysis of PA-Sugar Chains by Exoglycosidase Digestion)

In the enzymatic digestion reaction using β-galactosidase (*Diplococcus pneumoniae*, Roche), each PA-sugar chain was reacted at 37 °C for 2 days in a 0.1M sodium acetate buffer (pH: 5.5) containing 5 mU of β-galactosidase. Similarly, in the enzymatic digestion reaction using N-acetylglucosamidase (*Diplococcus pneumoniae*, Roche), each PA-sugar chain was reacted at 37 °C for 2 days in a 0.1M sodium acetate buffer (pH: 5.5) containing 5 mU of N-acetylglucosamidase. Furthermore, in the enzymatic digestion reaction using α-mannosidase (Jack bean, Sigma), each PA-sugar chain was reacted at 37 °C for 2 days in a 0.1M

sodium acetate buffer (pH: 3.88) containing 10 mM zinc acetate and 10 μU of α-mannosidase. Each enzymatic digestion reaction was stopped by boiling the solution at 100 °C for 3 minutes. Then, the reaction solution was centrifuged at 12,000 rpm for 10 minutes and the supernatant was subjected to HPLC. The elution time of each sample sugar chain was compared with the elution time of a known sugar chain.

(IS-MS/MS Analysis)

The IS-MS/MS Analysis was performed using Perkin-Elmer Sciex API-III triple-quadrupole mass spectrometer. The scan interval was 0.5 Da.

(Sialic Acid Transferase Reaction In Vitro)

The GT6 strain cell culture medium-derived glycoprotein prepared above after the dialysis and freeze-drying was used as the substrate. A sialic acid transferase reaction was performed at 37 °C for 5 hours in 62.5 mM sodium cacodylate buffer solution (pH: 6.0) containing 1 mg/ml of BSA, 0.5% of Triton CF-54, 2 μM of CMP-sialic acid, 6 mU of α2,6-sialyl transferase (derived from rat liver) (produced by Wako Junyaku) and 400 μg of GT6 strain cell culture medium-derived glycoprotein. In the control test, 400 μg of BY2 strain cell culture medium-derived glycoprotein and 40 μg of asialo fetuin fetal (fetal bovine serum, Sigma) were used as the substrate.

(Lectin Staining)

The sialic acid transferase reaction product was subjected to SDS-PAGE at 130 V for 2 hours using 12.5% polyacrylamide gel and then transferred to nitrocellulose

membrane at a constant current of 1 mA/cm^2 for 50 minutes. In the lectin blotting, horseradish peroxidase-linked SNA lectin (produced by EY Laboratories, Inc.) 200-fold diluted with a PBS solution containing 0.05% of Tween-20 was used. After the blotting, the staining was performed using POD Immunostain Kit (produced by Wako Junyaku).

(Purification of Tobacco Cultured Cell GT6 Strain Culture Medium-Derived PA-Sugar Chain)

The PA-sugar chain prepared from the GT6 strain culture medium was purified using RP-HPLC and SF-HPLC (shown in Fig. **4A** and Fig. **4B**, respectively). Fig. **4A** shows the peak of PA-product by RP-HPLC. After the recovery of peaks (1 to 6), each was subjected to SF-HPLC (see, Fig. **4B**).

Exclusive of 6 peaks (shown by A to F in Fig. **4B**) obtained by the SF-HPLC, the peaks were not an N-linked sugar chain. This was verified because, in IS-MS/MS analysis, signals agreeing with m/z 299.33 (GlcNAc-PA) and m/z 502.52 ($GlcNAc_2$-PA) were not obtained. Fig. **5** shows N-linked sugar chain structures analyzed on the peaks (A-F). In Fig. **5**, the numerals in parentheses indicate the molar ratio of sugar chain having each structure shown in the Figure.

As shown in Fig. **5**, A to F are all a human-type sugar chain having a galactose residue bound to an N-acetylglucosamine residue, contain no fucose residue and except for B and D, have no xylose residue.

(Structural Analysis of Tobacco Cultured Cell GT6 Culture Medium-Derived PA-Sugar Chain)

The molecular weight (m/z 1354.8) obtained by the IS-MS analysis of the peak A (I in Fig. 6) agreed with $GalGlcNAcMan_3GlcNAc_2$-PA (1354.27). The signals obtained by the IS-MS/MS analysis, namely, m/z 1192.5, m/z 990.5, m/z 827.5, m/z 665.5, m/z 503.0, m/z 300.0, are presumed to be $GlcNAcMan_3GlcNAc_2$-PA (1192.13), $Man_3GlcNAc_2$-PA (988. 94), $Man_2GlcNAc_2$-PA (826.80), $ManGlcNAc_2$-PA (664.66), $GlcNAc_2$-PA (502.52) and GlcNAc-PA (299.33), respectively, and this suggests that the peak A contains these structures (the data are not shown).

The β-galactosidase digestion product from the peak A was $GlcNAcMan_3GlcNAc_2$-PA (II in Fig. 6) and the N-acetylglucosaminidase digestion product thereof was $Man_3GlcNAc_2$-PA (III in Fig. 6).

The molecular weight (m/z 1486.8) obtained by the IS-MS analysis of the peak B (I in Fig. 7) agreed with $GalGlcNAcMan_3XylGlcNAc_2$-PA (1486.38). The signals obtained by the IS-MS/MS analysis, namely, m/z 1354.5, m/z 1324.0, m/z 1324.0, m/z 1122.0, m/z 991.5. m/z 960.0, m/z 666.0, m/z 503.0 and m/z 300.0, are presumed to be $GalGlcNAcMan_3GlcNAc_2$-PA (1354.27), $GlcNAcMan_3XylGlcNAc_2$-PA (1324.24), $Man_3XylGlcNAc_2$-PA (1121.05), $Man_3GlcNAc_2$-PA (988.94), $Man_2XylGlcNAc_2$-PA (958.91), $ManGlcNAc_2$-PA (664.66), $GlcNAC_2$-PA (502.52) and $GlcNAc_2$-PA (299.33), respectively, and this suggests that the peak B contains these structures (the data are not shown).

The β-galactosidase digestion product from the peak B was $GlcNAcMan_3XylGlcNAc_2$-PA (II in Fig. 7) and the N-acetylglucosaminidase digestion product thereof was $Man_3XylGlcNAc_2$-PA (III in Fig. 7).

The molecular weight (m/z 1355.0) obtained by the IS-MS analysis of the peak C (I in Fig. 8) agreed with $GalGlcNAcMan_3GlcNAc_2$-PA (m/z 1354.27). The signals obtained by the IS-MS/MS analysis, namely, m/z 1193.5, m/z 989.0. m/z 827.0, m/z 665.5, m/z 503.0, m/z 300.0, are presumed to be $GlcNAcMan_3GlcNAc_2$-PA (m/z 1192.13), $Man_3GlcNAc_2$-PA (m/z 988.94), $Man_2GlcNAc_2$-PA (m/z 826.80). $ManGlcNAc_2$-PA (m/z 664.66), $GlcNAc_2$-PA (m/z 502.52) and GlcNAc-PA (m/z 299.33), respectively, and this suggests that the peak C contains these structures (the data are not shown).

The β-galactosidase digestion product from the peak C was $GlcNAcMan_3GlcNAc_2$-PA (II in Fig. 8) and the N-acetylglucosaminidase digestion product thereof was $Man_3GlcNAc_2$-PA (III in Fig. 8).

The molecular weight (m/z 1487.0, A in Fig, 10) obtained by the IS-MS analysis of the peak D (I in Fig. 9) agreed with $GalGlcNAcMan_3XylGlcNAc_2$-PA (m/z 1486. 38). The signals obtained by the IS-MS/MS analysis, namely, m/z 1354.0, m/z 1325.0, m/z 1191.0, m/z 1121.5, m/z 989.5, m/z 828.5, m/z 503.0 and m/z 300.5, are presumed to be $GalGlcNAcMan_3GlcNAc_2$-PA (m/z 1354.27), $GlcNAcMan_3XylGlcNAc_2$-PA (m/z 1324.24), $GlcNAcMan_3GlcNAc_2$-PA (m/z 1192.13), $Man_3XylGlcNAc_2$-PA (m/z 1121.05), $Man_3GlcNAc_2$-PA (m/z 988.94), $Man_2GlcNAc_2$-PA (m/z 826.80), $GlcNAc_2$-PA (m/z 502.52) and GlcNAc-PA (m/z 299.33), respectively, and this suggests that the peak D contains these structures (B in Fig. 10).

The β-galactosidase digestion product from the peak

D was $GlcNAcMan_3XylGlcNAc_2$-PA (II in Fig. 9) and the N-acetylglucosaminidase digestion product thereof was $Man_3XylGlcNAc_2$-PA (III in Fig. 9).

The molecular weight (m/z 1516.6) obtained by the IS-MS analysis of the peak E (I in Fig. 11) agreed with $GalGlcNAcMan_4GlcNAc_2$-PA (1516.41). The signals obtained by the IS-MS/MS analysis, namely, m/z 1355.0, m/z 1193.0, m/z 990.0, m/z826.5, m/z 665.0, m/z 503.5 and m/z 300.0, are presumed to be $GalGlcNAcMan_3GlcNAc_2$-PA (m/z 1354.27), $GalNAcMan_3GlcNAc_2$-PA (m/z 1192.13), $Man_3GlcNAc_2$-PA (m/z 988.94), $Man_2GlcNAc_2$-PA (m/z 826.80), $ManGlcNAc_2$-PA (m/z 664.66) $GlcNAc_2$-PA (m/z 502.52) and GlcNAc-PA (m/z 299.33), respectively, and this suggests that the peak E contains these structures (the data are not shown).

The β-galactosidase digestion product from the peak E was $GlcNAcMan_4GlcNAc_2$-PA (II in Fig. 11) and the N-acetylglucosaminidase digestion product thereof was $Man_4GlcNAc_2$-PA (III in Fig. 11).

The molecular weight (m/z 1679.8) obtained by the IS-MS analysis of the peak F (I in Fig. 12) agreed with $GalGlcNAcMan_5GlcNAc_2$-PA (1678.55). The signals obtained by the IS-MS/MS analysis, namely, m/z 1517.5, m/z 1313.5, m/z 1152.0, m/z 827.5, m/z 665.5, m/z 503.0 and m/z 300.0, are presumed to be $GlcNAcMan_5GlcNAc_2$-PA (m/z 1516.41). $Man_5GlcNAc_2$-PA (1313.22), $Man_4GlcNAc_2$-PA (1151.08), $Man_2GlcNAc_2$-PA (826.80), $ManGlcNAc_2$-PA (664.66), $GlcNAc_2$-PA (502.52) and GlcNAc-PA (m/z 299.33), respectively, and this suggests that the peak F contains these structures (the data are not shown).

The β-galactosidase digestion product from the peak F was $GlcNAcMan_5GlcNAc_2$-PA (II in Fig. 12) and the N-acetylglucosaminidase digestion product thereof was $Man_5GlcNAc_2$-PA (III in Fig. 12).

From these results, the peak A or C is considered to be either α-D-Man-(1→6) [β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA(GalGN$_1$M3-PA) or [β-D-Gal-(1→4)-β-D-GlcNAc(1→2)-α-D-Man-(1→6)] α-D-Man-(1→3) β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GalGN^{1}M3-PA).

The peak B or D is considered to be either α-D-Man-(1→6) [β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GalGN$_1$M3X-PA) or [β-D-Gal-(1→4)-β-D-GlcNAc(1→2)-α-D-Man-(1→6)] [α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAC-PA (GalGN^{1}M3X-PA).

The peak E is considered to be either α-D-Man-(1→6)-α-D-Man-(1→6) [β-D-Gal-(1→4)-β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4) β-D-GlcNAc-(1→4)-GlcNAc-PA (GalGNM4-PA) or α-D-Man-(1→3)-α-D-Man-(1→6) [β-D-Gal-(1→4)-β-D-GlcNAc(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GalGNM4-PA).

The peak F is considered to be α-D-Man-(1→6) [α-D-Man-(1→3)] α-D-Man-(1→6) [β-D-Gal-(1→4)-β-D-GlcNAc(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GalGNM5-PA).

(Sialic Acid Transferase Reaction In Vitro)

A sialic acid transferase reaction *in vitro* was performed using GT6 strain culture medium-derived glycoprotein, BY2 strain culture medium-derived glycoprotein or asialo fetuin as the substrate. Each reactant was transferred to nitrocellulose membrane and subjected to lectin staining. As a result, the lectin staining was positive in the case where the substrate was asialo fetuin (lane 1 in Fig. 13) or GT6 strain culture medium-derived glycoprotein (lane 2 in Fig, 13), and the lectin staining was negative in the case where the substrate was BY2 strain culture medium-derived glycoprotein (lane 3, Fig. 13). From this, it is revealed that tobacco cultured cell GT6 strain culture medium-derived glycoprotein acts as the substrate in the sialic acid transferase reaction.

(Comparative Example)

(Analysis of Glycoprotein Secreted by Non-Transformant BY2 Strain Cultured Cell)

(Preparation of Glycoprotein by Extracellular Secretion)

A freeze-dried culture supernatant sample was obtained by the same method as in GT6 strain except for using BY2 strain in place of GT6 strain.

(Preparation of N-Linked Sugar Chain)

N-Linked sugar chains were recovered by the same method as in GT6 strain except for using TSK gel TOYO PERAL HW-40 (produced by TOSO) gel filter column (2.5×30 cm) in place of the Sephadex G-25 super fine gel filer column (1.8×180 cm).

(Preparation of Pyridylaminated (PA) Sugar Chain)

The recovered N-linked sugar chains were PA-formed using 2-aminopyridine. The PA-sample was filtered through

TSK gel TOYO PERAL HW-40 (produced by TOSO) gel filter column (2.5×30 cm) to purify the PA-sugar chains.

(Fractionation and Analysis of PA-Sugar Chain by HPLC)

The fractionation and analysis of PA-sugar chains were performed in the same manner as in GT6 strain except for using HITACHI HPLC system having a HITACHI FL Detector L-7480 in place of Jasco 880-PU HPLC having a Jasco 821-FP Intelligent Spectrofluorometer.

(Analysis of PA-Sugar Chain by Exoglycosidase Digestion)

In the enzymatic digestion reaction using N-acetylglucosaminidase (*Diplococcus pneumoniae*, Roche), each PA-sugar chain was reacted at 37 °C for 2 days in a 0.1M sodium acetate buffer (pH: 5.45) containing 3 mU of N-acetylglucosaminidase. In the enzymatic digestion reaction using α-mannosidase (Jack bean, Sigma), each PA-sugar chain was reacted at 37 °C for 2 days in a 0.1M sodium acetate buffer (pH: 4.0) containing 10 mM zinc acetate and 10 μU of α-mannosidase. Each enzymatic digestion reaction was stopped by boiling the solution at 100 °C for 3 minutes. Then, the reaction solution was centrifuged at 12,000 rpm for 10 minutes and the supernatant was subjected to HPLC. The elution time of each sample sugar chain was compared with the elution time of a known sugar chain.

(IS-MS/MS Analysis)

This analysis was performed in the same manner as in GT6 strain.

(Preparation of BY2 Strain Culture Medium-Derived PA-Sugar Chain)

The PA-sugar chains prepared from BY2 culture medium were also purified using RP-HPLC and SF-HPLC (Figs. **14A** and **14B**). Fig. **14A** shows the peaks of PA-product by RP-HPLC. After the recovery, each fraction (I to X) was subjected to SF-HPLC (Fig. **14B**).

In the fractions (I, II and III) shown in Fig. **14A**, N-linked sugar chain was not detected by SF-HPLC analysis. This was verified because in IS-MS/MS analysis, signals agreeing with m/z 299.33 (GlcNAc-PA) and m/z 502.52 ($GlcNAc_2$-PA) were not obtained. The peaks of fractions IV to X were analyzed by SF-HPLC, as a result, 21 peaks were obtained (see, Fig. **14B**). Figs. **15A** and **15B** show analyzed N-linked sugar chain structures.

(Structural Analysis of BY2 Strain Culture Medium-Derived PA-Sugar Chain)

As a result of IS-MS/MS analysis of the peaks V-4, VII-3 and VIII-4 shown in Fig. **14B**, the molecular weights (m/z) were 1639.0, 1476.5 and 1314.5, respectively and, on considering the elution sites in SF-HPLC together, the sugar chain structures present in the peaks agreed with PA-high mannose sugar chain $Man_7GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA, respectively (the data are not shown).

Also, as a result of SF-HPLC analysis, the α-mannosidase digestion product of sugar chain of each peak agreed with Man $GlcNAc_2$-PA (M1) (the data are not shown). When the isomers M7A, M7B and M7D of $Man_7GlcNAc_2$-PA were compared, the elution site of the peak V-4 agreed with the elution site of M7A. Among the isomers M6B and M6C of $Man_6GlcNAc_2$-PA, the peak VII-3 agreed with M6B. Also, the isomer M5A of $Man_5GlcNAc_2$-PA and the peak VIII-4 agreed.

From these data, the peaks V-4, VII-3 and VIII-4 shown in Fig. **14B** were, as shown in Fig. **15A**, α-D-Man-(1→2)-α-D-Man-(1→6) [α-D-Man-(1→3)] α-D-Man-(1→6) [α-D-Man-(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (M7A), α-D-Man-(1→6) [α-D-Man-(1→3)] α-D-Man-(1→6) [α-D-Man(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (M6A), and α-D-Man-(1→6) [α-D-Man-(1→3)] α-D-Man-(1→6) [α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (M5A). In the figure, the numerals in parentheses indicate the molar ratio of sugar chain having each structure shown in the Figure.

The molecular weight (m/z 1267.5) determined by the IS-MS analysis of the peaks IV-1 and V-1 shown in Fig. **14B** agreed with the calculated value of $Man_3XylFucGlcNAc_2$-PA (M3FX: 1267.19). Also, the elution site in HPLC completely agreed with M3FX standard product on the two-dimensional sugar chain map. Furthermore, as a result of SF-HPLC analysis and IS-MS/MS analysis, the α-mannosidase digestion product of sugar chain of each peak agreed with the calculated value of $ManXylFucGlcNAc_2$-PA (MFX: 942.91) (see, Figs. **17A** and **17B**).

From these data, the peaks IV-1 and V-1 shown in Fig. **14B** were, as shown in Fig. **15B**, α-D-Man-(1→6) [α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→3)]GlcNAc-PA (M3FX).

The molecular weight (m/z 1417.0) determined by the IS-MS analysis of the peak VII-2 shown in Fig. **14B** agreed with the calculated value of $GlcNAcMan_3XylFucGlcNAc_2$-PA

(GNM3FX: 1470.38). Also, as a result of SF-HPLC analysis and IS-MS/MS analysis, the N-acetylglucosaminidase digestion product of sugar chain of the peak agreed with $Man_3XylFucGlcNAc_2$-PA (M3FX: 1267.19). Furthermore, after the digestion by α-mannosidase, the SF-HPLC analysis and IC-MS/MS analysis revealed that the product agreed with $ManXylFucGlcNAc_2$-PA (MFX: 942.91) (C to E in Fig. **17**). On the two-dimensional sugar chain map, the elution site in RP-HPLC of the peak VII-2 (D-2 in Fig. **16**) completely agreed with the standard product GN^1M3FX, β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-[α-L-Fuc-(1-3)]GlcNAc-PA (D-1 in Fig. **16**).

From these data, the peak VII-2 was, as shown in Fig. **15B**, β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→3)]GlcNAc-PA (GN^1M3FX).

The molecular weight (m/z 1324.0) of the peaks VII-1 and VIII-3 shown in Fig. **14B** agreed with the calculated value of $GlcNAcMan_3XylGlcNAc_2$-PA (GNM3FX: 1324.24). Also, as a result of SF-HPLC analysis and IS-MS/MS analysis, the N-acetylglucosaminidase digestion product of the peak VIII-1 agreed with $Man_3XylGlcNAc_2$-PA (M3X: 1121.05) (B in Fig. **18A**). Furthermore, after the digestion by α-mannosidase, the SF-HPLC analysis and IC-MS/MS analysis revealed that the product agreed with $ManXylGlcNAc_2$-PA (MX: 796.77) (C in Fig. **18A**). As the structure of GNM3X, two isomer types are considered, that is, α-D-Man-(1→6) [β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN_1M3X) and β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [α-D-Man-(1→3)] [β-D-Xyl-

(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN^1M3X). On the ODS column, it has been reported that GN_1M3X elutes in advance of GN^1M3X. When the elution sites of the peaks VII-1 and VIII-3 are considered, as shown in Fig. **15B**, the peak VII-1 was α-D-Man-(1→6) [β-D-GlcNAc-(1→2)-α-D-Man-(1→3)][β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN_1M3X) and the peak VIII-3 was β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN^1M3X).

The elution site of the peak X-1 shown in Fig. **14B** in HPLC completely agreed with the standard GN2M3 (1395.32) on the two-dimensional sugar chain map. Also, as a result of SF-HPLC analysis and IS-MS/MS analysis, the N-acetylglucosaminidase digestion product of sugar chain of the peak agreed with $Man_3GlcNAc_2$-PA (M3: 988.94). Furthermore, after the digestion by α-mannosidase, the SF-HPLC analysis and IC-MS/MS analysis revealed that the product agreed with $ManGlcNAc_2$-PA (M1: 664.66) (the data are not shown). From these results, the peak X-1 was, as shown in Figs. **15A** and **15B**, β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN2M3).

The molecular weight (m/z 1529.5) of the peak X-2 shown in Fig. **14B** agreed with the calculated value of $GlcNAc_2Man_3XylGlcNAc_2$-PA (GN2M3FX: 1527.43). Also, the elution site in HPLC completely agreed with the M3FX standard product on the two-dimensional sugar chain map. Furthermore, the elution site of the α-mannosidase digestion product of sugar chain of this peak was not changed by the HPLC analysis. However, as a result of the

SF-HPLC analysis and IC-MS/MS analysis, the N-acetylglucosaminidase digestion product agreed with $Man_3XylGlcNAc_2$-PA (M3X: 1121.05) (B in Fig. **18B**). After further digestion by α-mannosidase, the SF-HPLC analysis and IC-MS/MS analysis revealed that the product agreed with $ManXylGlcNAc_2$-PA (MX: 796.77) (C in Fig. **18A**). From these data, the peak X-2 was, as shown in Fig. **15B**, β-D-GlcNAc-(1→2)-α-D-Man-(1→6) [β-D-GlcNAc-(1→2)-α-D-Man-(1→3)] [β-D-Xyl-(1→2)] β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-PA (GN2M3X).

Other peaks IV-2, IV-3, V-2, V-3, V-5, VI-1, VI-2, VI-3, VI-4, VIII-2 and IX shown in Fig. **14B** were not an N-linked sugar chain, because as a result of the IS-MS/MS analysis, signals agreeing with m/z 299.33 (GlcNAc-PA) and m/z 502.52 ($GlcNAc_2$-PA) were not obtained.

4. Secretion of Horse radish peroxidase (HRP)

A foreign gene, horseradish peroxidase gene, which was obtained from 35S-C1a (Kawaoka et al.,J.Ferment.Bioeng.,78, 49-53 (1994)), was inserted at Hind III and SacI site of vector pBI101 HmB (Akama et al., Plant Cell Rep.12,7-11(1992)) and introduced into GT6 Strain. After cultivating the obtained clones of GT6 Strain (GT-HRP-5, GT-HRP-19), the supernatant was collected, and subjected to a standard isoelectric focusing electrophoresis. As a result, as indicated in Figure **19**, electrophoresis band of pI 7.8 of HRP was detected in the supernatant of clones GT-HRP-5, GT-HRP-19, and thus confirmed that an foreign protein was also secreted from the plant cell transformed with the GalT gene. Further, figure **20** indicates the results of lectin staining of secreted proteins by clone GT-HRP-5, separated by standard SDS-PAGE electrophoresis

as described above. RCA120 staining indicates that GT-HRP-5 has a positive signal (Fig. 20(c)), and thus it was indicated that secreted HRP had a galactose added sugar chain structure.

INDUSTRIAL APPLICABILITY

According to the present invention, a method for the secretory production of a heterologous glycoprotein having a human-type sugar chain using a plant cell, a plant cell which can secrete this glycoprotein, and a glycoprotein having a human-type sugar chain which is secreted by a plant cell. The glycoprotein of the present invention has a human-type sugar chain and therefore, is free of antigenicity and is useful for the administration to animals including human.

CLAIMS

1. A method for the secretory production of a glycoprotein having a human-type sugar chain, comprising:

a step of introducing a gene of an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and a gene of a heterologous glycoprotein, to obtain a transformed plant cell, and

a step of culturing said plant cell.

2. The method according to claim 1, wherein said glycoprotein having a human-type sugar chain comprises a core sugar chain and an outer sugar chain, said core sugar chain substantially comprises a plurality of mannoses and acetylglucosamines, and said outer sugar chain has a terminal sugar chain moiety containing a non-reducing terminal galactose.

3. The method according to claim 2, wherein said outer sugar chain has a linear structure.

4. The method according to claim 2, wherein said outer sugar chain has a branched structure.

5. The method according to claim 4, wherein said branched sugar chain moiety is a mono-, bi-, tri- or tetra-structure.

6. The method according to any one of claims 1 to 5, wherein said glycoprotein is free of fucose or xylose.

7. The method according to claim 1, which further comprises a step of recovering the culture solution of said plant

cells.

8. The method according to any one of claims 1 to 7, which further comprises a step of adding sugar or a sugar chain *in vitro*.

9. A plant cell which comprises a sugar chain adding mechanism capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and can secrete a protein with an added sugar chain added by said sugar chain-adding mechanism, wherein said sugar chain-adding mechanism adds a sugar chain comprising a core sugar chain and an outer sugar chain, said core sugar chain substantially comprises a plurality of mannoses and acetylglucosamines, and said outer sugar chain has a terminal sugar chain moiety containing a non-reducing terminal galactose.

10. A glycoprotein having a human-type sugar chain obtained by the method according to any one of claims 1 to 8.

11. A method for the secretory production of a glycoprotein having a human-type sugar chain, comprising:

a step of introducing a gene for an enzyme capable of performing a transfer reaction of a galactose residue to a non-reducing terminal acetylglucosamine residue and a gene for a heterologous glycoprotein, to obtain a transformed plant cell, and

a step of expressing said enzyme within an intracellular organelle.

12. A plant cell transformed with a gene for an enzyme capable of performing a transfer reaction of a galactose

residue to a non-reducing terminal acetylglucosamine residue,

wherein said enzyme is localized in the plant cell such that the plant cell is capable of synthesizing a glycoprotein having a human-type sugar chain structure.

13. A plant cell according to claim 12, wherein said sugar chain structure includes an added galactose residue.

14. A plant cell according to claim 13, wherein said sugar chain structure is free of β1,2-xylose or α1,3-fucose.

15. A plant cell according to claim 12, wherein said sugar chain structure includes an galactose residue added to a N-linked type sugar chain of $(N\text{-acetylglucosamine})_{1-2}(Mannose)_{2-5}(N\text{-acetylglucosamine})_2$ selected from the group consisting of $GlcNAc_1Man_3GlcNAc_2$, $GlcNAc_1Man_5GlcNAc_2$, $GlcNAc_2Man_3GlcNAc_2$ and $GlcNAc_1Man_4GlcNAc_2$.

16. A plant cell according to claim 12, wherein said enzyme is localized within an intracellular organelle in the plant cell.

17. A plant regenerated from the plant cell of claim 12.

18. A seed produced from the plant of claim 16.