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MODIFICATION OF PLANT CELL *N*-GLYCOSYLATION TO PRODUCE GLYCOPROTEINS WITH HUMAN COMPATIBLE TYPE STRUCTURE

ヒト適応型糖鎖構造を持つ糖タンパク質生産のための
植物細胞*N*-結合型糖鎖機構の改変

1999

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PREFACE

It is no longer appropriate to view plants simply as a source of biological compounds. Transformation systems for the introduction and stable expression of foreign genes into plants have revolutionized plant biology. Plants are now earning its place among eukaryotic systems as a host for recombinant glycoprotein production. Indeed, with the "green revolution", several mammalian proteins have been produced in plants, including erythropoietin, blood proteins (e.g. albumin, enkephalins, interferons), monoclonal antibodies, and antibody fragments (e.g. scFv, IgG). Attempts to produce subunit vaccines, therapeutic proteins and peptides are, likewise, being done using various recombinant gene expression systems and plant viruses. As plant transformation becomes more routine, the post-translational modifications, including glycosylation, demands increasing attention. Glycosylation has been shown to be involved in various physiological activities of mammalian glycoproteins. Consequently, it became imperative to examine the presence and the extent of glycosylation on recombinant mammalian glycoproteins produced from transgenic plants.

N-glycosylation, or the addition of oligosaccharides to proteins through the asparagine: *N*-acetylglucosamine linkages, is a metabolic process that has been conserved during evolution. In all eukaryotes, biosynthesis of *N*-glycoproteins is initiated with the dolichol pathway in the endoplasmic reticulum. However, the fine details and further modification of glycans in the Golgi differ in mammalian and plant cells. Differences in the complex type glycans lie not only in their sugar residues but also in the manner of linkages. Plants can produce glycosylated complex recombinant mammalian proteins that are biologically active *in vitro*, but the altered glycosylation

status affected the proteins' *in vivo* biological activity, subcellular targeting, and/or immunogenicity. Furthermore, when plant glycoproteins are injected into animals to produce antibodies, the resulting sera are often non-specific due to the presence of xylose and fucose residues on the plant complex type glycans. Both sugar residues are implicated as key IgE-binding epitopes and may play critical roles in allergenicity. Thus, when human glycoproteins are produced in plants, these undesirable sugar residues will be present. To produce glycoproteins of pharmaceutical importance in plants, the modification of plant glycosylation pathway towards those in human cells is, therefore, essential.

The small diversity in the complex glycan structures of plant glycoproteins compared to that of the mammalian counterparts reflects the minimum machinery of plants in Golgi glycosyltransferases. In this respect, the feasibility to enlarge the spectrum of glycosyltransferases in plant cells can augment the *N*-glycosylation machinery in plants. Indeed, the mammalian *N*-acetylglucosaminyl transferase I (GnT-I), in mutant *Arabidopsis* plants that lack the plant GnT-I, was functional. The size of the resulting complex type glycans having only terminal glucosamine residues attached to the α-mannose branches was, nonetheless, still limited. Further modification of the plant *N*-glycosylation machinery is deemed necessary to yield the ideal extensively processed *N*-linked oligosaccharides akin to mammalian glycans.

In the strict order of *N*-glycosylation events, complementation of plant cells with human β1,4-galactosyltransferase may render an exquisite solution to the effective modification of the plant *N*-glycosylation machinery. The enzyme is the first glycosyltransferase that initiates the further branching of complex *N*-linked glycans in mammalian cells after the action of GnT-I and -II. Complementing with the human enzyme, instead of the establishment of a mutant system, may bestow

renewed impetus to the generation of human recombinant proteins in plants with restricted, defined glycosylation status and predictable pharmacokinetic properties. As a possible offshoot, the strategy may also aid in the resolution of critical issues (e.g. antigenicity and authenticity) on the production of pharmaceutically important glycoproteins in plants. Moreover, the developed system may show the plasticity of the plant glycosylation pathway, the regulatory features of *N*-glycosylation, and the functional consequence of glycosylation on plant glycoproteins.

The first chapter provides a brief review of the current understanding on the *N*-linked glycoprotein pathway including differences in the pathway and in the final glycan structures of mammalian and plant cells. A brief introduction on β1,4-galactosyltransferase is included. The chapter also addresses the importance of modifying the *N*-linked glycosylation, as well as its contribution to the production in plants of therapeutically important glycoproteins.

Chapter 2 evaluates tobacco BY2 suspension cultured cells as a possible model host system to assess the consequence of plant *N*-glycosylation modification. Structural analysis on the *N*-linked oligosaccharides from glycoproteins infers that BY2 cells have the appropriate *N*-glycan structures suitable for further modification.

Chapter 3 deals with the expression of the human β1,4-galactosyltransferase gene in BY2. The *N*-linked sugar chains from transformed cells confirmed the alterations in *N*-glycosylation and denoted that the introduced human galactosyltransferase could function in the plant cell *N*-linked oligosaccharide processing pathway.

The successful production of a specific foreign protein with the expected *N*-glycan modification in the transformed cell line described in Chapter 4, validates

transformed cells as an efficient host for the production of a foreign glycoprotein with defined, extended and human compatible glycan.

In the final chapter, the implications of the results obtained are discussed. Some future research aspects are also presented.

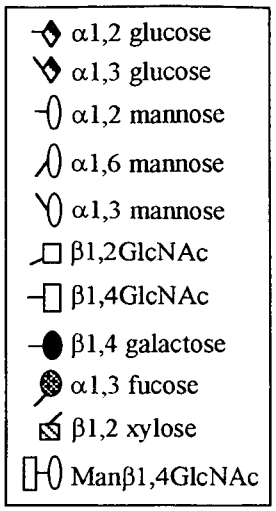
Abbreviations for commonly referred enzymes:

N-acetylglucosaminyl transferase-I or -II	GlcNAc transferase I or II; GnT-I or -II
β1,4-galactosyltransferase	Gal-T
Mannosidase-I or -II	Man-I or –II
Xylosyltransferase	Xyl-T
Fucosyltransferase	Fuc-T
Sialyltransferase	Sia-T
Human β1,4-galactosyltransferase	hGT
Horseradish peroxidase isozyme C	HRP

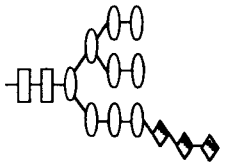
Abbreviations for commonly referred sugars:

Glucose	Glc
Mannose	Man
N-acetylglucosamine	GlcNAc
Galactose	Gal
N-acetylneuraminic (sialic) acid	NeuNAc
Xylose	Xyl
Fucose	Fuc

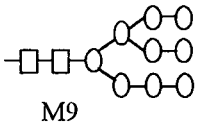
Guide for commonly referred glycan structures:

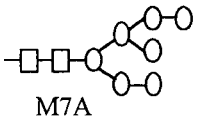


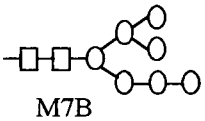
$Glc_3Man_9GlcNAc_2$
(tetradecasaccharide *N*-linked precursor sugar)

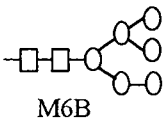


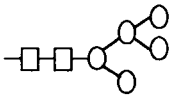
$Man_nGlcNAc_2$
(oligomannose or high mannose *N*-glycans)


M9

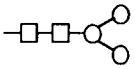

M7A


M7B

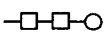

M6B


M5A

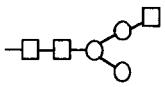
$Man_3GlcNAc_2$; M3
(trimannosyl core)

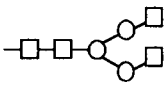


$ManGlcNAc_2$; M1
(N,*N*'-diacetylchitobiose unit with a β1,4-mannose residue)

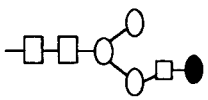


$GlcNAc_nMan_3GlcNAc_2$; GNM3
(non-reducing end GlcNAc residues linked to M3)

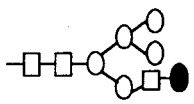

$GlcNAc_1Man_3GlcNAc_2$


$GlcNAc_2Man_3GlcNAc_2$

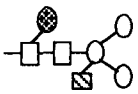
$Gal_1GlcNAc_1Man_3GlcNAc_2$; GalGNM3
(non-reducing end galactose residue linked to GNM3)



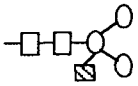
$Gal_1GlcNAc_1Man_5GlcNAc_2$; GalGNM5
(non-reducing end galactose residue linked to GNM5)



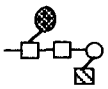
$Man_3Fuc_1Xyl_1GlcNAc_2$; M3FX
(trimannosyl core with α1,3-fucose and β1,2-xylose residues)



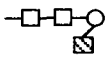
$Man_3Xyl_1GlcNAc_2$; M3X
(trimannosyl core with a β1,2-xylose residue)



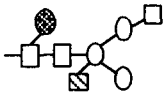
$Man_1Fuc_1Xyl_1GlcNAc_2$; MFX
(N,*N'*-diacetylchitobiose unit with β1,4-mannose, α1,3-fucose and β1,2-xylose residues)



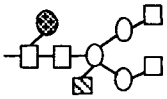
$ManXylGlcNAc_2$; MX
(N,*N'*-diacetylchitobiose unit with β1,4-mannose and β1,2-xylose residues)



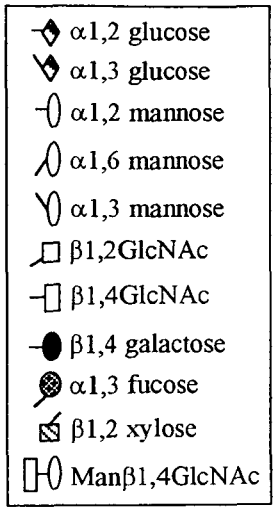
$GlcNAc_nMan_3Fuc_1Xyl_1GlcNAc_2$; GN_nM3FX
(one or two GlcNAc residues linked to M3FX)



GN1M3FX



GN2M3FX



CHAPTER 1

INTRODUCTION

Production systems for various recombinant proteins ranged from bacteria, filamentous fungi, yeast, mammalian cells, insect cells, transgenic animals and transgenic plants (Pen, 1996; Kusnadi et al., 1997). A suitable host is selected based on its ability to fulfil all the technical and economic requirements for commercialization (Table 1, as modified from Pen, 1996). Focusing on the technical requirements, the first issue is on the expression of the protein of interest in the host, which is gene dependent and varies in accord with the order of organisms, from prokaryote to eukaryote. Therefore, some production systems may well be suited for industrial enzyme production but not for the production of eukaryotic proteins, especially therapeutic proteins, in which the glycosylation status is to be considered (Chrispeels and Faye, 1996; Kusnadi et al., 1997). The major drawback of high yield bacterial production systems is their inability to effect post-translational modifications that are critical for the function of many non-bacterial proteins (Chrispeels and Faye, 1996; Kusnadi et al., 1997). Yeast, fungi, insect cells and plants are all capable of modifying eukaryotic recombinant proteins, but the glycan moieties are highly antigenic against mammals (Pen, 1996; Maras et al., 1997; Chiba et al., 1998). Though mammalian cells and transgenic animals are able to glycosylate and process the recombinant protein, the production system is threatened by contamination with animal pathogens/viruses and production costs (Ganz et al., 1996; Pen, 1996). In

Table 1. Comparison of host systems for the production of recombinant proteins.

	Transgenic plants	Transgenic animals	Mammalian cells	Insect cells	Fungi	Yeast	Bacteria
Research and Development aspects							
Development time	Intermediate	Long	Intermediate	Intermediate	Short	Short	Short
Process Characteristics							
Crude non-formulated product	Seed	Milk	Medium	Medium	Medium	Medium	Medium
Stability	+++	±	±	±	±	±	±
Storage period	Indefinite	Limited	Limited	Limited	Limited	Limited	Limited
Upscaling							
Time	Fast	Fast	Intermediate	Intermediate	Intermediate	Intermediate	Intermediate
Cost	Low	Intermediate	High	High	High	High	High
Production volume	Unlimited	Unlimited	Limited	Limited	Limited	Limited	Limited
Product Characteristics							
Authenticity							
Glycosylation	yes	yes	yes	yes	yes	yes	no
High-mannose	yes	yes	yes	yes	yes	yes	no
Complex	yes	yes	yes	yes/no	no	no	no
'Human'	no	yes	yes	no	no	no	no
Capability other modifications	?	+++	+++	+++	+	+	-
Folding capabilities	+++	+++	+++	+++	+++	++	-
Safety/public acceptance							
Food use	+++	+++	-	-	+++	+++	-
Mammalian pathogens	-	++	++	-	-	-	-
Public acceptance	+	±	++	++	++	++	++

-: absent; ±: very low or doubtful; +: low; ++: intermediate; +++: high (as modified from Pen, 1996)

this point of view, plants had earned its place among one of several novel hosts systems for the production of therapeutic proteins.

The biofarming concept, in which plants are used as 'natural' bioreactors, has a number of unique advantages: (1) ease of genetic manipulation as compared to mammalian or transgenic animals; (2) ease of scale-up, *e.g.* increase in planted acreage; (3) the availability of natural storage organs, thereby, the possibility of expressing proteins in different organs and cellular compartments where expression in a compartment may optimize protein accumulation; (4) lack of potential contamination with human pathogens; (5) the feasibility to use plant products directly with little or no further processing, *i.e.*, the concept of oral vaccines; and (6) the capacity to carry out most post-translational modifications akin to mammalian cells (Holzmann, 1994; Sturm, 1995; Aspergren et al., 1996; Cramer et al., 1996; Ganz et al., 1996; Ma and Hiatt, 1996; Owen et al., 1996; Verwoerd and Pen, 1996; Arntzen, 1997; Kusnadi et al., 1997; Takase and Hagiwara, 1998). Eukaryote-specific post-translational modifications, including glycosylation, are necessary for the biological property or efficacy of most recombinant glycoproteins (Kusnadi et al., 1997; Kukuruzinska and Lennon, 1998).

1.1 Protein glycosylation

Glycosylation, the addition of sugar residues to a peptide backbone, introduces an incredible degree of diversity into glycoproteins by the wide array of available monosaccharides as well as the potential for different chemical linkages between each pair of carbohydrates (Furukawa and Kobata, 1992; Jenkins and Curling, 1994; Sturm, 1995; Wyss and Wagner, 1996). A single site can be occupied by a variety of glycan

structures giving rise to extremely heterogenous glycoprotein populations known as glycoforms (Lerouge and Faye, 1996). The glycosylation pattern of a glycoprotein is a function not only of the primary sequence but also of the host expression system (Lu and Wold, 1991; Furukawa and Kobata, 1992; Faye, et al., 1993a; Sturm, 1995; Chrispeels and Faye, 1996; Pen, 1996; Verwoerd and Pen, 1996; Lerouge et al., 1998). The final oligosaccharide structure assembled on a glycoprotein is dictated to a large extent by the order in which the glycoprotein encounters the processing glycosidases and glycosyltransferases (Kornfeld and Kornfeld, 1985). The presence or absence of the enzymes is in part dependent on the organism, tissue, cell type, state of development, differentiation of the tissue and cell types, and environmental conditions (Yet, et al., 1988; Lu and Wold, 1991; Kukuruzinska and Lennon, 1998). In many cases, the structural diversity defines the biological role of the modified proteins (Furukawa and Kobata, 1992; Imperiali and Hendrickson, 1995).

Carbohydrate modifications of proteins fall into three general categories: *N*-linked modification of asparagine, *O*-linked modification of serine or threonine and glycosylphosphatidylinositol derivatization of the C-terminus carboxyl group (Varki, 1991; Furukawa and Kobata, 1992; Imperiali and Hendrickson, 1995). *N*-linked glycosylation represents a major post-translational protein modification (Sonnewald, et al., 1990; Lerouge, et al., 1998). The knowledge on protein *N*-glycosylation machinery and the role of glycoproteins in diverse biological processes, including the availability of several cloned glycosyltransferases, has provided the impetus to develop transgenic plants for the production of foreign glycoproteins with modified glycosylation status.

1.2 Asparagine (*N*)-linked glycosylation

N-linked glycosylation is catalyzed by oligosaccharyltransferase (OT), a single multi-subunit enzyme. The pathway begins in the rough endoplasmic reticulum (ER) with the co-translational transfer of a lipid-linked tetradecasaccharide made up of three glucose, nine mannose and two *N*-acetylglucosamine residues ($Glc_3Man_9GlcNAc_2$) from dolichol pyrophosphate oligosaccharide to an asparagine (Asn) residue that must be in an Asn-X-Ser/Thr triplet, where X is any amino acid except proline (Fig. 1; Kornfeld and Kornfeld, 1985; Jenkins and Curling, 1994; Roth, 1995; Imperiali and Hendrickson, 1995; Sturm, 1995; Wyss and Wagner, 1996). This first step in the *N*-linked glycosylation process appears to be conserved throughout eukaryotic evolution (Narimatsu, 1994; Imperiali and Hendrickson, 1995; Kukuruzinska and Lennon, 1998).

N-glycan biosynthetic pathway may be conveniently divided into 3 stages. The first is the initial synthesis of the dolichol-linked precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, at the cytoplasmic side and in the lumen of the endoplasmic reticulum (ER) (Narimatsu, 1994; Roth, 1995; Sturm, 1995; Kukuruzinska and Lennon, 1998). The lipid dolichol anchors the oligosaccharide precursor in the ER membrane and during protein synthesis on membrane bound ribosomes $Glc_3Man_9GlcNAc_2$ is transferred *en bloc* to the growing polypeptide chain (Moremen et al., 1994; Sturm, 1995). The second stage, catalyzed by ER glucosidases I and II, begins immediately with the removal of the three terminal glucose units (Fig. 1; Kaushal and Elbein, 1989; Sturm, 1995). The resulting high mannose structure ($Man_9GlcNAc_2$, Fig. 2) can be further trimmed by α-mannosidases in the ER and Golgi complex (Yet et al., 1988; Sturm, 1995). The number of mannose residues removed varies according to the differences in

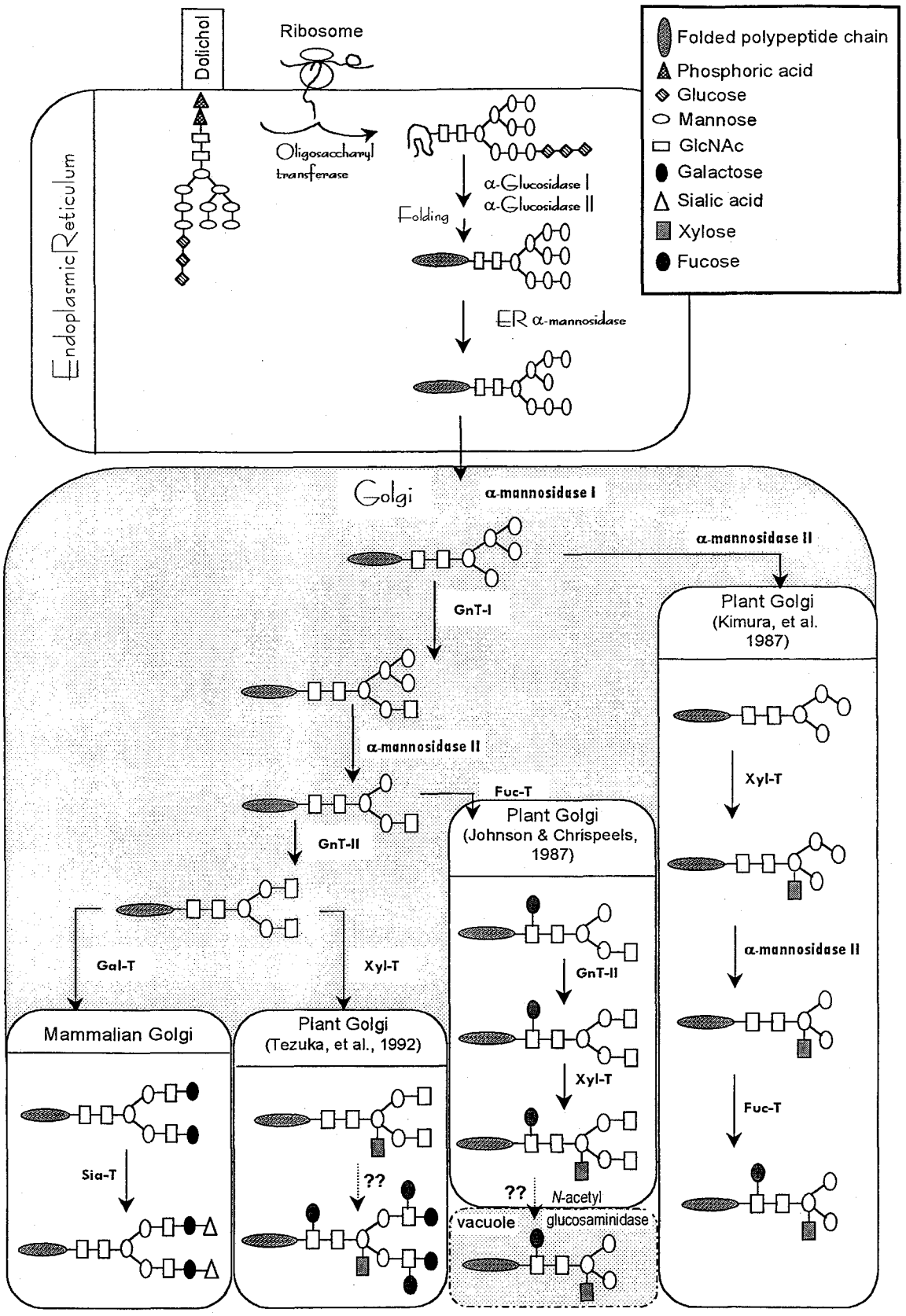


Fig. 1. Comparison of *N*-glycosylation pathway in mammalian cells and plants.

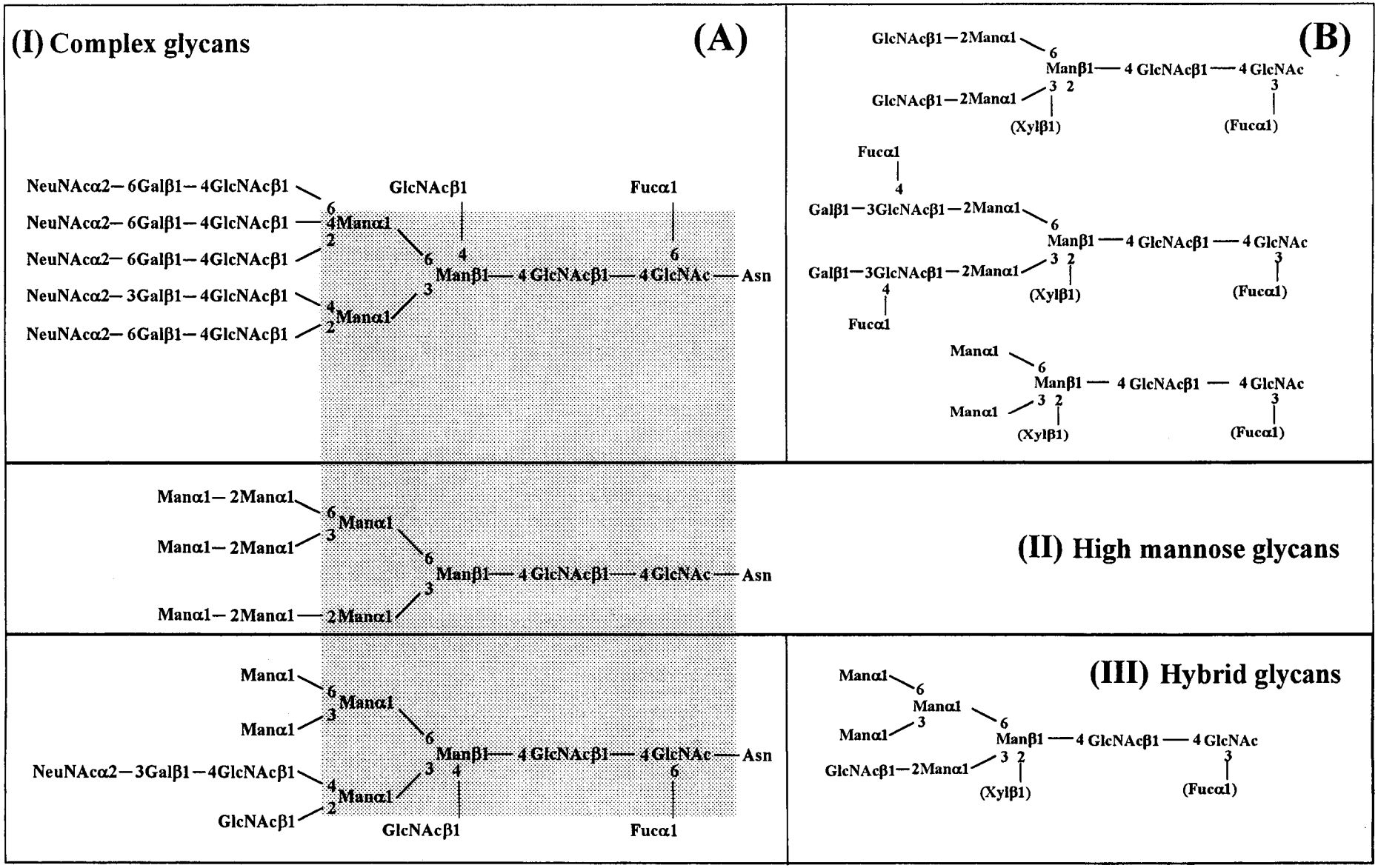


Fig. 2. Sub-groups of *N*-linked sugar chains. The structure within the shaded box is the trimannosyl core common to all *N*-linked glycans. (A) Representatives from mammalian glycoproteins; (B) representatives from plant glycoproteins.

the accessibility of the different glycans to the processing enzymes. The particular isomers Man_8-, Man_7-, Man_6-, and $Man_5GlcNAc_2$ is a reflection of the pattern of mannose removal during processing (Kornfeld and Kornfeld, 1985; Moremen et al., 1994). Glycans completely or partly buried in the folded polypeptide, remain in their high mannose form (Sturm et al., 1987; Yet et al., 1988; Faye et al., 1993a; Moremen et al., 1994; Rayon et al., 1996). The third stage is the conversion of high mannose type glycans to elaborate branched oligosaccharide chains by Golgi glycosyltransferases and glycosidases (Kornfeld and Kornfeld, 1985; Narimatsu, 1994; Field and Wainwright, 1995).

The removal of four α1,2-linked mannose residues from $Man_9GlcNAc_2$, catalyzed by mannosidase I (Man I) in the Golgi, yield the smallest high mannose oligosaccharide structure, $Man_5GlcNAc_2$, which serves as a precursor to hybrid and complex *N*-glycans (Schachter et al., 1983; Kornfeld and Kornfeld, 1985). In the medial Golgi compartment, *N*-acetylglucosaminyl transferase I (GnT-I) transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to $Man_5GlcNAc_2$, resulting to $GlcNAcMan_5GlcNAc_2$. GlcNAc transferase I is a key enzyme in the biosynthesis of hybrid and complex-type glycans, and must act before the removal of two mannose residues. The removal of two mannose residues by mannosidase II (Man II) is a prerequisite for the addition of a second GlcNAc residue by GlcNAc transferase II (GnT-II), yielding $GlcNAc_2Man_3GlcNAc_2$ (Fig. 1; Kornfeld and Kornfeld, 1985; Kaushal and Elbein, 1989; Moremen et al., 1994). The $GlcNAc_2Man_3GlcNAc_2$ oligosaccharide can be bisected or extended by *N*-acetylglucosaminyl transferases III to VI (GnT-III to -VI), terminal glycosyltransferases α1,3-, β1,3-, or β1,4-galactosyltransferases (Gal-T), and α2,3- or α2,6- sialyltransferases

(Sia-T), which are located in the trans-Golgi cisternae and trans-Golgi network (Shaper and Shaper, 1992; Field and Wainwright, 1995).

1.2.1 A comparison of the N*-linked glycan biosynthesis in plants and animals*

The early steps of *N*-linked glycan biosynthesis, particularly the reactions of the dolichol pathway, are highly conserved in eukaryotes (Narimatsu, 1994; Imperiali and Hendrickson, 1995). Although, an ER mannosidase in mammalian cells, which specifically cleaves a single mannose residue to yield $Man_8GlcNAc_2$ (Kornfeld and Kornfeld, 1985; Moremen et al., 1994), has not been isolated in plants so far (Lerouge et al., 1998), structural analysis of ER resident spinach and maize chaperone (calreticulin) revealed major *N*-glycans identified as $Man_9GlcNAc_2$ or $Man_8GlcNAc_2$ indicating the presence of a specific mannosidase that can be analogous to the mammalian ER mannosidase (Lerouge et al., 1998). Similarities in the plant and mammalian *N*-glycan biosynthesis has also been proposed based on: (1) the presence of key enzymes in the Golgi (*N*-acetylglucosaminyl transferases I and II, and α-mannosidases I and II) and (2) the glycosyltransferase substrate specificities in bean cotyledons (Johnson and Crispeels, 1987; Kaushal and Elbein, 1989) and in sycamore cells (Tezuka et al., 1992; Fig. 1). The trimming sequence may infer that the Golgi resident enzymes in plants are similarly situated as in mammalian cells. For example, enzymes involved in the trimming (Man I) and elongation (GnT-I) are located in the cis- and medial- Golgi network, respectively (Kornfeld and Kornfeld, 1985; Shaper and Shaper, 1992).

N-acetylglucosaminyl transferases III to VI (GnT-III to -VI), terminal glycosyltransferases α1,3-, β1,4-galactosyltransferases (Gal-T) and α2,3- or α2,6-

sialyltransferases (Sia-T) are apparently absent in plants (Sturm, 1995). Instead, β1,2-xylosyltransferase (Xyl-T) and α1,3- or α1,4-fucosyltransferase (Fuc-T) catalyze further modifications of the sugar chain after GlcNAc transferase I, and are responsible for plant-specific complex-type glycans (Faye et al., 1993b; Staudacher et al., 1995; Zeng et al., 1997; Lerouge et al., 1998).

The study on substrate specificity of α1,3 Fuc-T and β1,2 Xyl-T has shown that the presence of at least one terminal GlcNac residue is necessary for the transfer of α1,3-fucose and β1,2-xylose. However, based on the structural diversity of mature sugar chains *N*-linked to *Ricinus communis* lectin and in developing castor bean cotyledons (Kimura et al., 1987, 1990), it is proposed that a xylose residue could immediately be transferred to $Man_4GlcNAc_2$ and thus would not have a strict requirement for the prior action of GnT-I. Plant *N*-glycans having only a β1,2-xylose or only a α1,3-fucose residue has been identified in plant glycoproteins (Ohsuga et al., 1996). Consequently, fucosylation and xylosylation appear to be independent events (Staudacher et al., 1995; Zeng et al., 1997). Based on immunocytochemistry of xylosyl- and fucosyl-containing sugar chains in plant dictyosomes, the two steps occur mostly in the *medial* and *trans* Golgi cisternae, respectively (Lerouge et al., 1998). Such findings may indicate that during the transport of the glycoprotein through the Golgi, β1,2-xylose transfer starts before the addition of the α1,3-fucose residue.

After the transfer of xylose and/or fucose, complex *N*-glycans can be further processed by the addition of terminal galactose and fucose residues to yield one or two antennae of Galβ1,3(Fucα1,4)GlcNAc sequences, known as Lewis a (Le^a) antigen, a

common cell surface glycoconjugate in mammals (Fitchette-Laine et al., 1997; Lerouge et al., 1998). Substrate specificity of α1,4-fucosyltransferase has shown that the enzyme specifically transfers fucose to Galβ1,3GlcNAc (Fitchette-Laine et al., 1997; Melo et al., 1997). The presence of this sugar chain in a wide array of plant glycoproteins suggests a putative function for plant *N*-glycans similar to mammalian *N*-glycans, *i.e*, cell/cell communication (Fitchette-Laine et al., 1997).

Glycosidases and glycosyltransferases in plants that have been purified to homogeneity and characterized are: glucosidases I and II (Kaushal and Elbein, 1989), mannosidases I and II (Kaushal and Elbein, 1989; Kaushal et al., 1990); GlcNAc transferase I (Szumilo et al., 1986; Johnson and Chrispeels, 1987) and II (Szumilo et al., 1987); α1,3-fucosyltransferase (Staudacher et al., 1995); and β1,2-xylosyltransferase (Zeng et al., 1997). The studies (except for the later two enzymes that are apparently absent in most animal cells) showed that the enzymes have similar substrate specificities as those found in mammalian cells.

1.2.2 Structures of N*-linked glycans in plants and animals*

All asparagine (*N*)-linked glycans contain the pentasaccharide $Man_3GlcNAc_2$ (Fig. 2) as a common (trimannosyl) core constitutive of two *N*-acetylglucosamine residues (*i.e.*, N, *N'*-diacetylchitobiose unit), a β1,4-mannose residue linked to the chitobiose and two α-mannose residues linked to the hydroxyl 3 and 6 of the β1,4-mannose (Kornfeld and Kornfeld, 1985; Wyss and Wagner, 1996). The heterogeneity of *N*-linked glycans originates primarily from the presence or absence, and type or length of the sugar chains attached to the common core (Schachter et al., 1983). There are at least

three sub-groups: (i) high mannose *N*-glycans which contain only mannose (Man) residues attached to the core; (ii) complex type *N*-glycans with outer chains which contain other sugars such as xylose (Xyl), fucose (Fuc), *N*-acetylglucosamine (GlcNAc), galactose (Gal), and sialic acid (NeuNAc); and (iii) hybrid glycans which have the features of both complex type and high mannose type glycans (Fig. 2).

High mannose (oligomannose) type glycans have identical structures in plants, mammals and other organisms, and results from limited trimming of mannose residues from the precursor oligosaccharide ($Glc_3Man_9GlcNAc_2$) (Fig. 2-II; Kornfeld and Kornfeld, 1985; Kaushal and Elbein, 1989; Faye et al., 1993a; Jenkins and Curling, 1994; Ma and Hiatt, 1996; Lerouge et al., 1998). The preservation of a high mannose structure may result from its location within the folded protein that is inaccessible to mannosidases (Yet et al., 1988; Moremen et al., 1994; Kukuruzinska and Lennon, 1998).

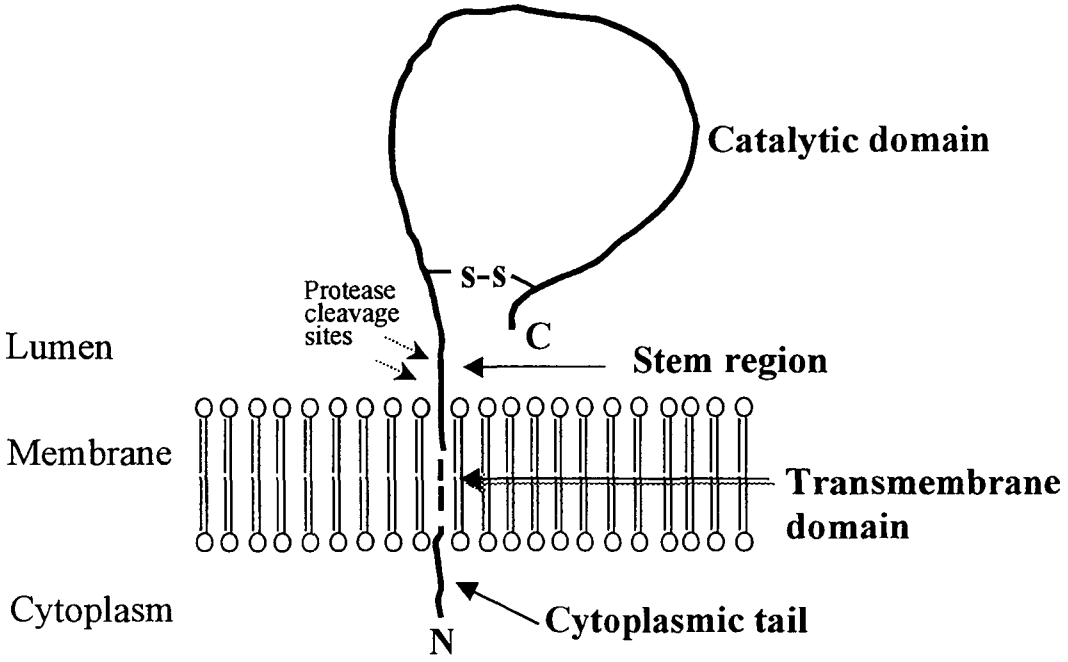
Complex *N*-glycans in plants, as in other eukaryotes, result from the specific action of glycosidases and glycosyltransferases (Kornfeld and Kornfeld, 1985; Lu and Wold, 1991; Sturm, 1995; Lerouge and Faye, 1996; Kimura, 1997; Lerouge et al., 1998). An ever-growing diversity is observed from recently reported oligosaccharide structures (Sturm, 1991; Kimura, 1997), although plant complex type glycans are smaller and differ in the terminal sugars compared to mammalian complex type glycans (Fig. 2-I-A *vs* 2-I-B). Plant *N*-glycans are characterized by the presence of α1,3-fucose and/or β1,2-xylose residues respectively linked to the proximal *N*-acetylglucosamine (GlcNAc) and to the β1,4-mannose residues of the core (termed paucimannosidic type glycans by Lerouge et al., 1998 and Rayon et al., 1998). Further branching may occur with the presence of β1,2-*N*-acetylglucosamine (GlcNAc) residues linked to the α-mannose units. Larger

complex types *N*-glycans contain additional α1,4-fucose and β1,3-galactose residues (Fitchette-Laine et al., 1997; Melo et al., 1997; Lerouge et al., 1998). Fucose residues α1,3-linked to the chitobiose core and α1,2-linked arabinose are not found in mammalian glycoproteins (Sharon and Lis, 1979; Priem et al., 1993; Staudacher et al., 1995; Sturm, 1995; Chrispeels and Faye, 1996). Mammalian glycoproteins with xylose residues were only found in *Helix pomatia*, an invertebrate (Kimura et al., 1990; Faye et al., 1993b; Sturm, 1995). *N*-acetylneuraminic (sialic) acid, a predominant terminal sugar residue in mammalian glycoproteins, has never been reported in plants (Sturm, 1995; Ma and Hiatt, 1996).

Hybrid type *N*-glycans result from the processing of only the α1,3-mannose branch. In mammalian glycoproteins, hybrid structures reflect in part the absence or low activity of α-mannosidase II (Schachter et al., 1983). In plants, hybrid glycans have been reported in *Nicotiana alata N*-glycans (Fig. 2-III-B; Oxley, et al., 1996).

1.3 β1,4-galactosyltransferase

β1,4-galactosyltransferase (Gal-T) is a ubiquitous, constitutively expressed, trans-Golgi resident, type II integral membrane glycoprotein that catalyzes the transfer of UDP-galactose to terminal *N*-acetylglucosamine residues forming Galβ1,4GlcNAc moiety in glycoproteins and glycolipids (Fig. 3; Paulson and Colley, 1989; Chatterjee, 1991; Shaper and Shaper, 1992; Narimatsu, 1994; Field and Wainwright, 1995; Lo et al., 1998). The enzyme is the only glycosyltransferase known to alter its substrate specificity in the presence of another protein (Uejima et al., 1992; Narimatsu, 1994). With α-



Narimatsu, 1994; Field and Wainwright, 1995

UDP-Gal + GlcNAc-R ⟶ Galβ1,4GlcNAc-R+ UDP

Fig. 3. Schematic diagram of the domain structure of galactosyltransferase, showing the orientation of the catalytic, stem and transmembrane domains. The catalytic domain and the amino termini is designated by C and N, respectively. The enzyme is known to catalyze the following reaction.

lactalbumin, the affinity for GlcNAc residues is diminished and the affinity for glucose is dramatically increased resulting to the synthesis of lactose in the mammary gland (Chatterjee, 1991; Nakazawa et al., 1991).

β1,4-galactosyltransferase is the first group of glycosyltransferases to be studied at the molecular level and represents the largest single group in the database (Yamaguchi and Fukuda, 1995; Lo et al., 1998). The cDNAs encoding Gal-T have been isolated from human, bovine, murine, nematode, chicken, and swine sources (Paulson and Colley, 1989; Aoki et al., 1990; Nakazawa et al., 1991; Uejima et al., 1992). Nucleotide sequences reveal six family members: β1,4-galactosyltransferase -I, -II, -III, -IV, -V, and –VI, each mapped to human chromosome 9p13, Ip33-34, Iq21-23, 3q13, 11 and 18q11, respectively (Lo et al., 1998). The enzyme has a single conserved potential *N*-glycosylation site (Narimatsu, 1994). Amino acid sequence similarity is over 90% in the transmembrane anchor and C-terminal catalytic domains, but less than 50% in the stem region (Uejima et al., 1992; Schwientek and Ernst, 1994).

Similar to all type II integral membrane glycoproteins, the protein domain structure consists of: (i) a short NH_2-terminal cytoplasmic domain of 4 to 24 amino acids (a.a.) depending on the protein isomer; (ii) a large COOH-terminal lumenal domain (268-277 a.a.); and (iii) a single transmembrane domain (19-20 a.a.) (Narimatsu, 1994; Schwientek and Ernst, 1994). A glycosylated peptide segment of 42-86 a.a., called the stem region, links the COOH-terminal to the transmembrane domain and acts as a flexible tether to hold the catalytic domain within the Golgi lumen (Narimatsu, 1994; Schwientek and Ernst, 1994). The stem region (spacer) is accessible to proteases (Yeh and Cummings, 1997).

The amino-terminal region of Gal-T and the transmembrane domain contain information for trans-Golgi retention (Nilsson et al., 1991; Shaper and Shaper, 1992; Schwientek et al., 1996). Specifically, Cys^{29} and His^{32} in the transmembrane domain are critical amino acids for a functional Golgi retention signal (Aoki et al., 1992; Schwientek and Ernst, 1994; Yamaguchi and Fukuda, 1995). The amino-terminal and transmembrane domains are, however, not required for catalytic activity. In contrast, photoaffinity labeling and site-directed mutagenesis demonstrate that the C-terminal domain contains all the necessary information required for catalysis (Aoki et al., 1990; Field and Wainwright, 1995). A disulfide linkage between 2 cysteine residues, Cys^{134} and Cys^{247}, is necessary for Gal-T activity (Fig. 3; Aoki et al., 1992). Chemical labeling experiments on purified Gal-T suggested two lysine residues, Lys^{341} and Lys^{351}, to be involved in UDP-galactose (UDP-Gal) binding within the catalytic domain (Uejima et al., 1992; Narimatsu, 1994). The expression of truncated recombinant enzymes proves that Gal-T can be cleaved at the stem region to yield an active soluble enzyme (Schwientek and Ernst, 1994).

A full-length Gal-T is estimated to have a molecular weight of about 43-53 kDa. Isoelectric focusing of purified Gal-T shows multiple bands between pH 4.5 and 6.0, representing several isozymes (Uemura et al., 1992). Substrate specificity studies show: (i) the preferential galactosylation of the GlcNAcβ1,2Manα1,3 arm both in the absence and in the presence of a bisecting GlcNAc residue; and (ii) the sequential addition of a galactose residue onto the GlcNAcβ1,2Manα1,6 arm (Paquet et al., 1984; Narasimhan et al., 1985).

After a mean half-retention time of 19 hours in the Golgi, the enzyme is released from the cells in soluble form by proteolytic cleavage on the luminal side (Arg^{77}) close to the transmembrane domain (Aoki et al., 1990; Uejima et al., 1992; Krezdorn et al., 1993; Narimatsu, 1994).

1.4 Effect of oligosaccharides on glycoprotein properties

Glycans on mammalian glycoproteins are involved in many cellular activities, in embryogenesis and development, and are often altered structurally and functionally in disease (Priem et al., 1993; Chrispeels and Faye, 1996; Wyss and Wagner, 1996; Kukuruzinska and Lennon, 1998; Brockhausen et al., 1998). In particular, *N*-linked carbohydrates participate in diverse biological processes such as protein folding and conformation, stability, and targeting to subcellular and extracellular sites as well as cell-matrix and cell-cell interactions (Table 2; Furukawa and Kobata, 1992; Faye et al., 1993a; Hammond et al., 1994; Sturm, 1995; Chrispeels and Faye, 1996; Ganz et al., 1996; Pen, 1996; Lerouge et al., 1998; Brockhausen et al., 1998). Transgenic and gene disruption techniques revealed that *N*-glycosylation influence the conformational dynamics of nascent polypeptides and, in some cases, confer the biological activity of proteins. There still remains an apparent paradox that although *N*-glycosylation is indispensable to cell viability, many *N*-glycoproteins remain functional regardless of their glycosylation status. To wit, the consequences of altering the spectrum of carbohydrates expressed by a cell or an organism range from none to death.

In plants, *N*-linked glycans strongly influence the glycoprotein conformation, stability and biological activity. For example, *N*-glycosylation of Con A is important for

Table 2. Functional importance of the *N*-linked oligosaccharides of some glycoproteins.

Protein	Effect of lack/alteration of oligosaccharides	Selected references
AIDS virus glycoprotein (GP120)	Lowered fusion activity/decreased infectivity	Yet et al., 1988 Varki, 1991 Wyss and Wagner, 1996
Gonadrophic hormones	Agonist converted into antagonist	"
Granulocyte/macrophage Colony-stimulating factor	Graded increase in activity with decrease in glycosylation	"
Viral coat proteins	Increased reactivity with polyclonal antisera	"
Lysosomal enzymes	Loss of targeting signal for delivery of enzymes to lysosomes	"
Erythropoietin	Failure of secretion/decrease in biological activity	"
Immunoglobulins	Reduced solubility/ alteration of some secondary effector functions of the Fc region	"
Many glycoprotein receptors	Failure of disulfide bond formation/activation	"
Concanavalin A	Reduce protection solubility and transport; loss of lectin activity	Chrispeels et al., 1986

Con A transport to the vacuole and the regulation of its lectin activity (Lerouge et al., 1998). Free *N*-glycans were shown to stimulate ripening of tomato fruits (Priem et al., 1993) and to act as growth factors during the development of flax seedlings (Lerouge et al., 1998). Complex *N*-glycans in *Arabidopsis* has been implicated to be involved in some defense reaction against *Phytophthora parasitica* infection (Lerouge et al., 1998). New putative biological functions for plant glycans are also emerging in the light of the recent identification of Lewis a (Le^a) epitopes on plant *N*-linked glycans. Le^a-containing *N*-glycans were found attached to secreted proteins and to cell-surface proteins suggesting putative role in cell signaling, similar to its role in cell-cell recognition or adhesion processes in mammalian glycoproteins (Lerouge et al., 1998; Rayon et al., 1998).

1.5 Constraints and concerns in foreign gene expression in plants

Since oligosaccharide moieties on glycoproteins modulate or mediate to a large extent important biological activities, the choice of an expression system for producing recombinant proteins include considerations on the glycosylation capabilities. In any eukaryotic system and particularly in plants, *N*-glycosylation of the recombinant protein is one of the major concerns for the production of functional glycoproteins. Studies suggest that the level and/or the pattern of glycosylation of recombinant proteins in plants vary (Kusnadi et al., 1997).

Recombinant rabies virus glycoprotein (G-protein) when produced from tomatoes appeared in two major forms with molecular masses of about 4-6 kDa less than that obtained from virus-infected BHK cells. The obtained mass, however, was still larger

than the predicted 58.6 kDa for the unglycosylated polypeptide chain (McGarvey et al., 1995). The size differences were largely attributed to the extent of glycosylation in plants and in animal cells. Vesicular stomatitis virus (VSV) G-protein produced in transiently transfected tobacco protoplast was, similarly, 5 kDa smaller than the native VSV G-protein (Galbraith et al., 1992). The apparent molecular mass of erythropoietin (EPO) produced in tobacco cells was 30 kDa while that produced in mammalian cells was 37 kDa (Matsumoto et al., 1995). The half-life of the protein was, in general, increased by the presence of terminal sialic acid when EPO was produced in BHK cells, while, on the contrary, the presence of terminal mannose, galactose or *N*-acetylglucosamine when EPO was produced in insects, yeast, fungal or plant cells initiates clearance from the body (Furukawa and Kobata, 1992; Pen, 1996). Differences in the level and pattern of glycosylation also accounted for EPO's lack of *in vivo* activity (Matsumoto et al., 1995) and was presumed to be comparable to that of desialylated recombinant human EPO. The lack of terminal sialic acid results to rapid clearance of the glycoprotein from circulation on hepatocytes. EPO, though correctly processed, remained attached to the tobacco cell wall and was not released into the culture medium, as opposed to the excretion of EPO when produced in BHK cells (Matsumoto et al., 1995).

Plant glycoproteins have been found to be uncommonly antigenic in mammals, and the likely cause are two residues/linkages not found in mammalian glycoproteins (Hase et al., 1986; Fournet et al., 1987; Kurosaka et al., 1991; Faye et al., 1993b; Staudacher et al., 1995; Garcia-Casado et al., 1996; Ohsuga et al., 1996; Zeng et al., 1997; Altmann, 1998; Wilson et al., 1998). Plant complex-type *N*-glycans have a α1,3-

fucose residue instead of the α1,6-fucose residue in mammals (Staudacher et al., 1995; Roitinger et al., 1988); and a β1,2-xylose residue which has only been observed in invertebrates and insects (Zeng et al., 1997; Wilson et al., 1998). These type of glycans present on recombinant human glycoproteins produced in plants, are frequently observed on pollen grain and food allergens (Owen and Pen, 1996). When plant glycoproteins are used as immunogens in mammals, they are non-specific, often recognizing numerous proteins (Pen, 1996; Garcia-Casado et al., 1996; Wilson et al., 1998). Hypersensitivity reactions related to food allergens of plant origin occur in 6-8% of children (Lerouge et al., 1998). These findings then pose certain limitations to the use of plants or plant cell cultures for the production of pharmacologically important proteins.

The effectivity of food plant-based antigens/subunit toxins (HIV, heat labile enterotoxin, cholera, hepatitis B antigen, diabetes autoantigen) suggests that plant-based toxins elicit lower immune response compared to bacterial purified antigens (Mason et al., 1992; Thanavala et al., 1995; Ma et al., 1997; Yusibov et al., 1997; Arakawa et al., 1998; Tacket et al., 1998) due to differential delivery (gavage *vs.* oral/eaten), and/or alterations in glycosylation (Haq et al., 1995; Mehta et al., 1997; Arakawa et al., 1997; Yusibov et al., 1997).

Due to the constraints presented, further development of plants as 'bioproduction' systems should focus on either the production of glycoproteins analogous to authentic human glycoproteins, or the production of modified glycoproteins with restricted/defined glycosylation status for more predictable pharmacokinetic properties (Chrispeels and Faye, 1996; Pen, 1996).

1.6 Modifying *N*-link glycosylation in plants

In the biotechnology field, transgenic plants are aggressively investigated for the production of recombinant glycoproteins intended for therapeutic use (Dezoeten et al., 1989; Hiatt et al., 1989; Vandekerckhove et al., 1989; Sijmons et al., 1990; Edelbaum et al., 1992; Mason et al., 1992; Hamamoto et al., 1993; Higo et al., 1993; Kumagai et al., 1993; Matsumoto et al., 1993; Fiedler and Conrad, 1995; Haq et al., 1995; Ma et al., 1995; Featherstone, 1996; Salmanian et al., 1996; Dalsgaard et al., 1997; Ehsani et al., 1997; Arakawa et al., 1998; Takase and Hagiwara, 1998). The impetus stemmed from the Children's Vaccine Initiative (CVI) (1990 World Summit for Children) call for alternatives to cell culture systems in the production of therapeutically important recombinant proteins. The expression of candidate vaccine antigens with the goal of using edible plant organs for economical delivery of oral vaccines could render the development of heat-stable vaccines that would not need the refrigeration currently limiting success and coverage of immunizations in the developing countries. From the industrial viewpoint, with the realization that the recombinant proteins and subunit vaccines produced in plants were biologically active, the potential benefits are straightforward: bulk production at minimal cost. The use of plants as production system, however, is not without difficulties. Focus is now on the authenticity of glycosylation, especially with regards to undesirable pharmacokinetics, and too long or too short plasma half-lives (Owen and Pen, 1996). The production of pharmaceutically important glycoproteins in plants entail the modification in the glycosylation pathway towards that of higher eukaryotes.

Two approaches to modify the glycosylation of recombinant proteins in plants are presented by Chrispeels and Faye (1996) and Lerouge et al (1998). One is to retain the recombinant human glycoprotein in the endoplasmic reticulum (ER) by fusing C-terminal retention signals, inferred from ER resident proteins, to the recombinant glycoproteins (Chrispeels and Faye, 1996; Lerouge et al., 1998). By preventing any further modification in the glycan structure, the recombinant proteins that remain in the ER lumen possess oligomannose type glycans. The mere presence of the retention signals, however, does not guarantee that the proteins will be retained in the ER and remain unmodified (Chrispeel and Faye, 1996).

The second strategy, which allows storage of glycoproteins downstream from the Golgi (e.g. vacuole or apoplast), is to modify the enzyme compliment of the Golgi by knocking out genes that encode Golgi enzymes or by expressing heterologous glycosyltransferases (Chrispeels and Faye, 1996; Pen, 1996; Lerouge et al., 1998).

In yeast and mammals, mutants provided valuable insight into the structure and function of enzymes involved in *N*-glycosylation. With at least 60 glycosyltransferase and glycosidase genes cloned, the details and functions of protein *N*-glycosylation is unraveling (Kukuruzinska and Lennon, 1998). In plants, particularly in *Arabidopsis thaliana*, only two mutants present a clearly defined mutation affecting the biosynthesis of *N*-linked glycans (Lerouge et al., 1998). *A. thaliana cgl* mutant, that lack GlcNAc transferase I (GnT-I), is unable to synthesize complex type *N*-glycans and accumulate $Man_5GlcNAc_2$ oligosaccharides (von Schaewen et al., 1993; Gomez and Chrispeels, 1994). The study has confirmed that GnT-I, as in other eukaryotes, is the key enzyme in the biosynthesis of complex *N*-glycans. The other *Arabidopsis* mutant, *mur1*, do not

synthesize L-fucose (Lerouge et al., 1998; Rayon et al., 1999). Major non-fucosylated glycans have been identified in a ratio similar to that found in wild-type plants. Thus, the addition of the α1,3-fucose residue was confirmed to be a late event in the processing of plant *N*-linked glycans. The minor population of sugar chains (5%), consisting of a novel plant *N*-glycan $Man_3Xyl(L\text{-}Gal)GlcNAc_2$, indicated that in the absence of L-fucose, α1,3-fucosyltransferase transfer L-galactose from GDP-L-galactose instead of fucose (Lerouge et al., 1998; Rayon et al., 1999). The plants have a normal phenotype despite the absence of fucose and the alteration in the *N*-glycans of xyloglucan, an important plant cell wall polysaccharide. Complex glycans in plants must play fundamentally different roles from that in mammals. Hence, knocking out plant *N*-glycosylation enzymes can produce glycans without either fucose or xylose.

The expression of mammalian glycosyltransferases in yeast and insect cells has altered the glycosylation pathway of such cells, thereby suggesting that heterologous glycosyltransferases can be functionally integrated into their biosynthetic machinery (Jarvis and Finn, 1996; Hollister et al., 1998; Chiba et al., 1998). The feasibility to enlarge the spectrum of glycosyltransferases in plant cells has also been demonstrated. Complementation with the human GnT-I in mutant *Arabidopsis* plants that lack the plant GnT-I proved that the mammalian enzyme could contribute to the plant *N*-glycosylation pathway (Gomez and Chrispeels, 1994). The transgenic plant cells have glycoproteins with complex glycans, albeit of limited size, having only terminal glucosamine residues attached to the α-mannose branches. The characterization of the alterations in *N*-glycan structure and *N*-glycosylation pathway has been hampered by the very limited amount of

material for structural analysis due to the small size of *Arabidopsis* plants (Lerouge et al., 1998; Rayon et al., 1999).

1.7 Development of a modified *N*-glycosylation system in tobacco

To date, only a few studies have completely characterized proteins produced in transgenic plants. The presence or absence of glycosylation was inferred from immunodetection studies. In a non-mutant background, the pattern of glycosylation varied according to the host plant glycosylation capability (Kusnadi et al., 1997). Lack of structural analysis makes it difficult to predict with certainty the resulting carbohydrate heterogeneity of the recombinant protein.

Moreover, attempts to modify the plant *N*-glycosylation pathway have, so far, concentrated on the use of mutant plants that are unable to synthesize fucose and xylose (Chrispeels and Faye, 1996), sugar residues implicated to be highly immunogenic in humans. The resulting complex type glycans were expectedly smaller in terms of size, reflecting the plants' limited capacity to yield larger biantennary complex glycans similar to mammalian cells. It was not demonstrated if the mutant plants could yield heterologous proteins with predictable glycan structures.

In this thesis, modification of *N*-glycosylation in non-mutant tobacco cells was done by complementation of an enzyme found in the mammalian *N*-glycosylation pathway. Hereafter, the following were considered in the development of tobacco cells with altered *N*-glycosylation: (i) to yield more extensively processed sugar chains resembling mammalian *N*-linked glycans, (ii) to evaluate the structural diversity of the *N*-glycans found in the glycoproteins of transgenic cells, and (iii) to characterize the glycan

structure of a foreign protein produced from transgenic cells. Modification of glycosylation activities is deemed important not only for the production of recombinant proteins for medical use but also as a tool to investigate the role of plant glycan structures and elucidate the mechanisms of the *N*-link glycosylation pathway in plants.

CHAPTER 2

STRUCTURES OF *N*-LINKED OLIGOSACCHARIDES FROM GLYCOPROTEINS OF TOBACCO BY2 SUSPENSION CULTURED CELLS

2.1 Introduction

Among the first isolated glycoproteins in higher plants were an *N*-linked glycan from soya-bean agglutinin and the proteolytic bromelain from pineapple (Sharon and Lis, 1979). Since then, several studies showed that glycoproteins are of wide distribution in plants (Table 3). Percentage molar ratios of the *N*-glycans suggest that sugar chains of plant glycoproteins are highly processed and have common structural features.

Structural analysis of glycoproteins revealed the large heterogeneity of *N*-glycans in plants, shedding light on the *N*-glycan processing pathway and the subsequent individuality of the processing steps in different cell types (Kimura, et al. 1987, 1990, 1992; Sturm et al., 1987; Lu and Wold, 1991; Takahashi et al., 1990; Tezuka et al., 1993; Rayon et al., 1996; Kimura, 1997; Melo et al., 1997; Rayon et al., 1998). The biological roles of sugar moieties have been discussed on the basis of increasing knowledge on the structures of *N*-glycans (Kimura et al., 1996).

As shown in Table 3, predominant plant complex type glycans described, so far, follows the Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc [M3FX] motif. Glycans with larger antennae have fucose α1,3-linked to the proximal GlcNAc residue of the chitobiose core and/or α1,4-linked to the terminal GlcNAc residue(s), suggesting the existence of two different fucosyltransferases (Sturm, 1995; Lerouge et al., 1998). These large complex *N*-glycans have the

Table 3. Some plant glycoproteins and their major oligosaccharide structures.

	Source	Major Structures of Oligosaccharides [a]	Reference
Protease Inhibitor	*Caesalpinia pulcherrima* (barbados pride)	M3FX (38%)[b]	Hase et al., 1986
Bromelain	*Ananas comosus* (pineapple)	M2FX	Hase et al., 1986; Wilson et al., 1998
Laccase	*Acer pseudoplatanus* (sycamore)	GN2M3FX (+++); GFGN2M3FX (++)	Takahashi et al., 1986
Ascorbate oxidase	*Cucurbita pepo* (zucchini)	M3FX (43%); M3X (38%)	Altmann, 1998
Miraculin	*Richadella dulcifica* (miracle berries)	M3FX (58%); M2FX (18.8%)	Takahashi et al., 1990
Peroxidase	*Amoracia rusticana* (horseradish)	M3FX	Kurosaka et al., 1991; Yang et al., 1996
	Glycine max (soybean hull)	M3FX (60-65%)	Gray et al., 1996
Golgi enzymes	*Phaseolus vulgaris* (red kidney bean)	GNM3	Lu and Wold, 1991
Pollen allergen- BG60	*Cyanodon dactylon* (bermuda grass)	M3F (68.3%); M2F (11.9%)	Ohsuga et al., 1996
Storage glycoproteins in seeds	*Pisum sativum* (mature pea)	M6B (24.7%); M8A (19.7%); M3FX (18.8%)	Kimura et al., 1996
Phaseolin	*Phaseolus vulgaris* (common bean)	Oligomannose; M3X	Sturm et al., 1987
Microsomal (Endosperms)	*Ricinus communis* (castor bean)	M3FX (33.8%); M6B (43.8%)	Kimura et al., 1992
Self-incompatibility RNAses			
S_8-allele	*Brassica campestris* (brassica)	GNM3FX; M3FX	Takayama et al., 1987
S_1 and S_2 allele	*Nicotiana alata* (tobacco)	GNM3X (76-88%)	Oxley and Bacic, 1995
S_3 and S_6 allele	*Nicotiana alata*	GNM4X (71-89%)	Oxley et al., 1996
S_3 allele	*Lycopersicum peruvianum* (tomato)	GNM3X; GNM3	Parry et al., 1998

Table 3. (con't)

	Source	Major Structures of Oligosaccharides	Reference
Lectins	*Clerodendron trichotomum*	M3FX	Kitagaki-Ogawa et al., 1986
	Erythrina cristagalli	M3FX	Sturm, 1991
	Sophora japonica	M3FX	Fournet et al., 1987
	Ricinus communis	M3FX	Kimura et al., 1987; 1988a
Toxins: Ricin	*Ricinus communis*	M3FX	Kimura et al., 1988b
Secretory and Vacuolar			
Laccase	*Acer pseudoplatanus*	M2FX	Tezuka et al., 1993
Medium and cell wall			
β-fructosidase	*Daucus carota* (carrots)	M3FX	Sturm, 1991
Peroxidase	*Vaccinium myrtillus* (bilberry)	G2F2GN2M3FX (49%); GFGN2M3FX (23%)	Melo et al., 1997
Free N-glycans			
in tomato pericarp	*Lycopersicon esculentum* (tomato)	Oligomannose	Priem et al., 1993
in pea seedlings	*Pisum sativum* (pea)	Oligomannose	Kimura et al., 1997

[a]**M2FX**, Manα1,6(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; **GFGN2M3FX**, Galβ1,3(Fucα1,4)GlcNAcβ1,2Manα1,6(GlcNAcβ1,2 Mana1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; **G2F2GN2M3FX**, Galβ1,3(Fucα1,4)GlcNAcβ1,2Manα1,6[Galβ1,3(Fucα1,4) GlcNAcβ1,2Mana1,3](Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; **GNM4X**, Manα1,3Manα1,6(GlcNAcβ1,2Manα1,3) (Xylβ1,2) Manβ1,4GlcNAcβ1,4GlcNAc; **M3F**, Manα1,6(Manα1,3) Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc; **M2F**, (Manα1,3)Manβ1,4GlcNAcβ1,4 (Fucα1,3)GlcNAc; **M8A**, Manα1,2Manα1,6(Manα1,3)Manα1,6(Manα1,2Manα1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc

[b]percentage molar ratios

Galβ1,3(Fucα1,4)GlcNAc carbohydrate motif, giving rise to mammalian Lewis a (Le^a) structures (Lerouge et al., 1998; Rayon et al., 1998).

Glycans bearing *N*-acetylglucosamine residues are believed to result from both ER and Golgi processing (Lerouge et al., 1998); whereas glycoproteins which bear only M3FX, are suggested to have undergone further post-Golgi trimming by *N*-acetylglucosaminidase and/or by successive action of exoglycosidases in the compartment where the glycoproteins accumulated (Tezuka et al., 1992, 1993; Lerouge et al., 1998).

Structural analysis of glycans from soybean and horseradish peroxidase yielded minor glycans of M6F (soybean peroxidase; Gray et al., 1996) and M6X (horseradish peroxidase; Yang et al., 1996) whose presence cannot be explained in terms of the current knowledge of the processing pathway, since, neither $Man_6GlcNAc_2$ nor $Man_7GlcNAc_2$ was found to be substrates for xylosyltranferase (Tezuka et al., 1992; Gray et al., 1996; Yang et al., 1996).

Obviously, the analysis of more plant glycoproteins is essential to further streamline knowledge on plant *N*-glycosylation pathway and determine the different functional glycosidases and glycosyltransferases in plants. Among suspension-cultured plant cells, the tobacco cell line BY2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) is often preferred as a model system for the production of heterologous proteins because of its fast doubling time, capacity to grow well at high densities (50-60% of wet weight), and amenability to genetic manipulation (Hogue et al., 1990; Wahl et al., 1995; Magnuson et al., 1996; Gomord et al., 1997; LaCount et al., 1997). However, to date, the structures of *N*-linked sugar chains of glycoproteins in tobacco BY2 cells is still unreported. To fully utilize tobacco BY2 cells for the production of recombinant proteins of biological interest, structural studies of *N*-linked

oligosaccharides of glycoproteins is necessary. Analysis of *N*-linked glycans is relevant to evaluate, modify and improve the plant *N*-glycosylation pathway.

2.2 Materials and Methods

2.2.1 Materials

Tobacco BY2 cell line was established from *Nicotinia tabacum* L. cv. Bright Yellow 2 (kindly donated by Dr. K. Yoshida, Nara Institute of Science and Technology). BY2 cells were subcultured weekly in modified Linsmaier and Skoog medium (LS, as reported by Nagata et al., 1981) containing B5-MS vitamins (10 mg thiamine-HCl, 1mg nicotinic acid, 1mg pyridoxin-HCl and 100 mg myo-inositol per liter), at pH 5.8. The cell line was maintained by regularly transferring 2-ml onto 95-ml fresh medium in a 300-ml Erlenmeyer flask, incubated in the dark at 26°C on a gyratory shaker (New Brunswick Scientific) at 120-125 rpm. A Cosmosil 5C18-AR column (6 x 250 mm) was purchased from Nacalai Tesque, Inc., and an Asahipak NH2P-50 column (4.6 x 250 mm) from Showa Denko Co. Authentic PA-sugar chains (GN2M3FX, M3FX, M3X, and M5A) were prepared as described (Kimura et al., 1992).

2.2.2 Preparation of the cell free extract from tobacco BY2 suspension cultured cells

Seven-day-old cultures of BY2 cells (50 g fresh weight) were washed once with 50 mM sodium phosphate buffer, pH 7.0. Cells were disrupted by homogenization and centrifuged at 6,000 rpm for 20 min at 4°C. The resulting

supernatant was extensively dialyzed against deionized water and the dialysate was lyophilized (5 mg).

2.2.3 Pyridylamination of N-glycans

N-Glycans were released by hydrazinolysis (100°C, for 12 hr) from crude glycoproteins in the cell free extract. After *N*-acetylation of the hydrazinolysate with saturated sodium bicarbonate and acetic anhydride, the acetylated hydrazinolysate was desalted by Dowex 50 X 2 followed by Sephadex G-10 column (18 x 40 cm) in 50 mM NH_4OH. Pyridylamination (PA-) of the desalted *N*-glycan fraction was carried out by the method of Kondo *et al.* (1990). Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column or an Asahipak NH2P-50 column. The eluate was monitored by measuring fluorescence intensity using excitation and emission wavelengths of 310 nm and 380 nm, respectively. For the reversed-phase (RP-) HPLC on the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% trifluoroacetic acid (TFA) linearly from 0 to 15% for 40 min at a flow rate 1.2 ml/min. In the case of size-fractionation (SF-) HPLC, using the Asahipak NH2P-50 column, the PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 30% to 50% linearly for 25 min at a flow rate of 0.8 ml/min.

2.2.4 Glycosidase digestions

All digestions with α-mannosidase (jackbean, Sigma Co.) or β-*N*-acetylglucosaminidase (*Diplococcus pneumoniae*, Boehringer Mannheim) was performed using about 1 nmole of the PA-sugar chains under the same conditions as

described (Kimura et al., 1992). Digestion with α-mannosidase was done in 50 mM citrate buffer, pH 4.0 containing 1 mM $ZnCl_2$ and 30 mU of enzyme. For β-*N*-acetylglucosaminidase, digestion was carried out in 50 mM of sodium acetate buffer, pH 5.5 using 200 mU of the enzyme. Reaction mixtures were incubated overnight at 37°C under an atmosphere of toluene. The reactions were stopped by boiling the mixtures for 3 minutes and a part of the digest was analyzed by SF-HPLC. In the analysis of the glycosidase digests, the PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 18% to 40% linearly at a flow rate of 0.8 ml/min. The molecular masses of the resulting digests were analyzed by ion-spray mass spectrometry.

2.2.5 Ion-spray mass spectrometry

The mass spectrometer used was a Perkin Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source. The mass spectrometer was operated in the positive mode with an ion spray voltage of 4200 V. Samples were typically dissolved in 50% acetonitrile/water (containing 0.05% formic acid) at a concentration of approximately 10 pmol/μl and introduced into the electrospray needle by mechanical infusion through a micro syringe at a flow rate of 5 μl/min. The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas. The collision energy was 60-100 eV. Scanning was done with a step size of 0.5 Da. and the CAD daughter ion spectrum was recorded from a mass-to-charge (*m/z*) value of 200.

2.3 Results

2.3.1 Purification of pyridylaminated (PA-) sugar chains from the cell extract of tobacco BY2 suspension cultured cells

The sugar chains of glycoproteins in the cell extract from tobacco BY2 cells were prepared by hydrazinolysis and labeled with 2-aminopyridine. The general strategy for the purification and analysis of the sugar chains is presented in Fig. 4. PA-sugar chains that gave fragment ions at *m/z* 300, 503 and 665 were considered *N*-linked glycans and, thus, further characterized.

Figure 5 shows the RP-HPLC pattern of the PA-derivatives. A run-through fraction (10 min-20 min), albeit a fragment ion at *m/z* 300 by MS/MS analyses, have PA-sugar chains detected as $Man_1GlcNAc_1$-PA (*m/z*: 463) fragment instead of the $GlcNAc_2$-PA. The results suggest these *N*-glycans to be by-products during hydrazinolysis as previously described by Hase *et al.* (1986). Several peaks found between 24 min to 28 min could not be *N*-glycans, since MS/MS analyses of the PA-derivatives showed no daughter ion at *m/z* 300. On the contrary, several PA-sugar chains found in F-I (*m/z* 1268.0), F-II (*m/z* 1675.0), F-III (*m/z* 1471.5), and F-IV (*m/z* 1121.5 and *m/z* 1314.5) gave $GlcNAc_2$-PA (*m/z*: 503) and GlcNAc-PA (*m/z*: 300) fragments by MS/MS, implying that the fractions should contain *N*-glycans. Since IS-MS analysis of F-IV contained two *m/z* values, the fraction was further subjected to SF-HPLC for purification. As shown in Fig. 5-B, F-IV was purified to two PA-sugar chains: F-IV-a (*m/z* 1121.5) and F-IV-b (*m/z* 1314.5).

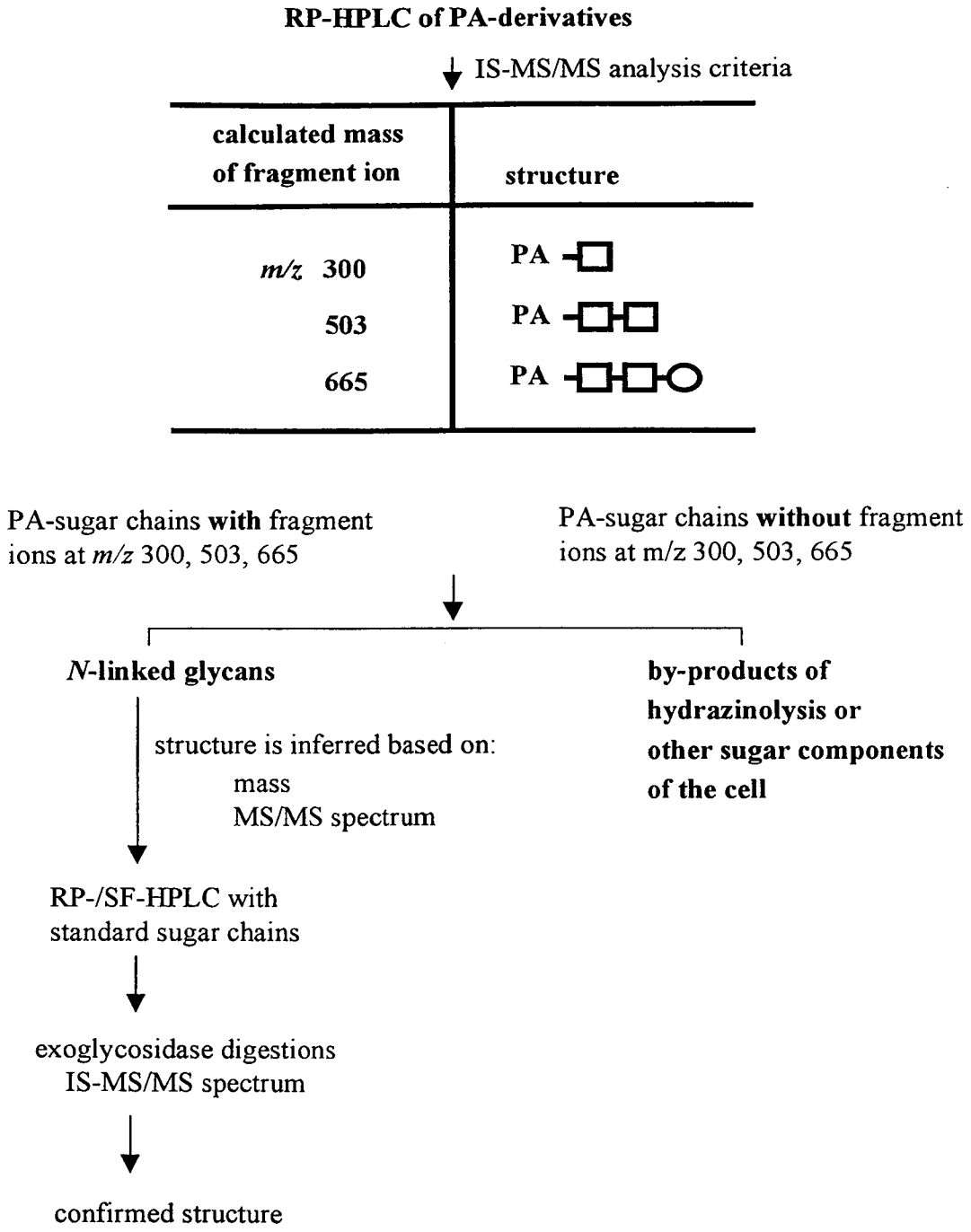


Fig. 4. The general strategy for the purification and identification of PA-labeled sugars from BY2.

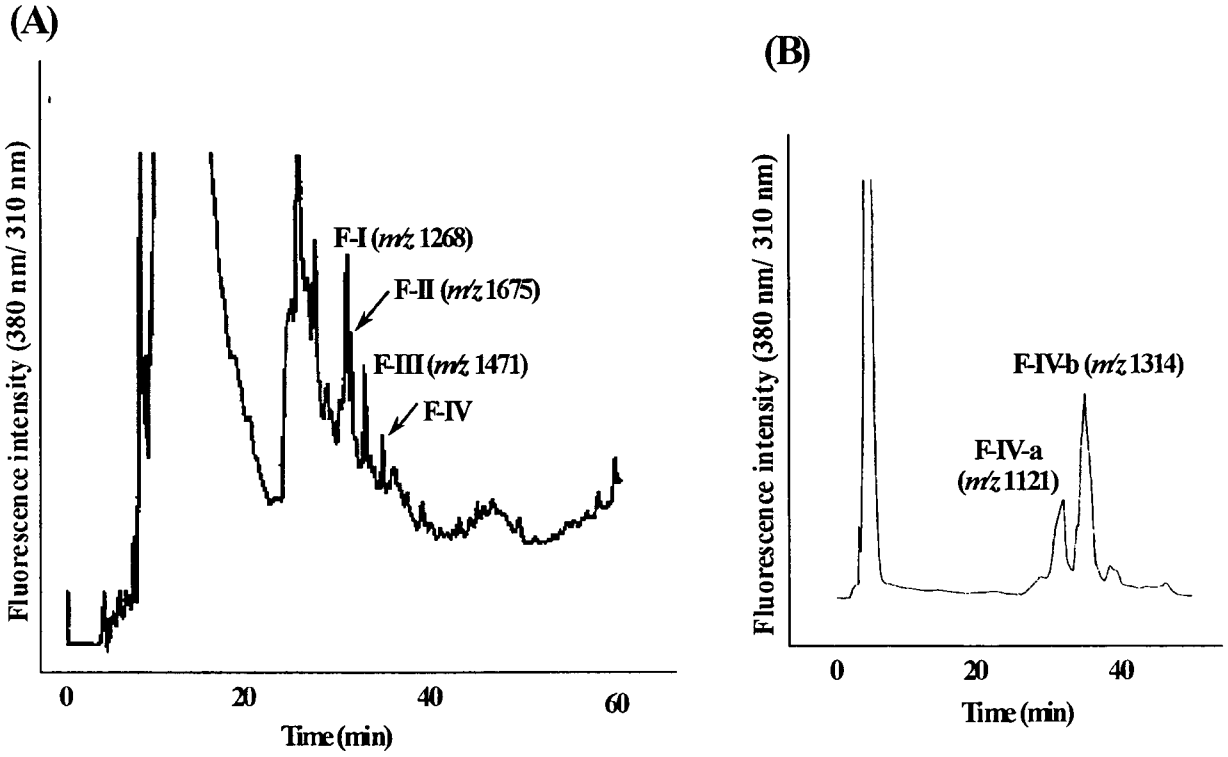


Fig. 5. HPLC profiles of PA-derivatives from glycoproteins expressed in tobacco BY2 cultured cells. **(A).** Reversed-phase HPLC of glycans prepared from glycoproteins in the cell free extract. The PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 15% for 40 min at a flow rate of 1.2 ml/min. **(B).** Size-fractionation HPLC of fraction F-IV in (A). The PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 30% to 50% linearly for 25 min at a flow rate of 0.8 ml/min.

2.3.2 Structures of N-glycans from BY2 cells

The elution position of F-I (*m/z* 1268.0) corresponded exactly to authentic M3FX on the 2D sugar chain map. The relevant signals observed by IS-MS/MS analysis of F-I could be assigned as fragment ions derived from the M3FX: m/z 1122.0 ($Man_3Xyl_1GlcNAc_2$-PA), *m/z* 990.0 ($Man_3GlcNAc_2$-PA), *m/z* 960.0 ($Man_2Xyl_1GlcNAc_2$-PA), *m/z* 827.5 ($Man_2GlcNAc_2$-PA), *m/z* 665.0 ($Man_1GlcNAc_2$-PA), *m/z* 503.0 (GlcNAc-GlcNAc-PA), *m/z* 446.0 (FucGlcNAc-PA), *m/z* 300 (GlcNAc-PA) (Fig. 6). These results suggested the structure of F-I as Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc-PA (M3FX, Fig. 7).

The elution position of F-II (*m/z* 837.5 for $[M+2H]^{2+}$, *m/z* 1675.0 for $[M+H]^{+}$) corresponded exactly to authentic GN2M3FX on the 2D sugar chain map. The relevant signals observed by IS-MS/MS analysis of F-II could be designated as fragment ions derived from the GN2M3FX: *m/z* 1528.5 ($GlcNAc_2Man_3Xyl_1GlcNAc_2$-PA), *m/z* 1471.0 ($GlcNAc_1Man_3Fuc_1Xyl_1GlcNAc_2$-PA), *m/z* 1396.5 ($GlcNAc_2Man_3GlcNAc_2$-PA), *m/z* 1326.0 ($GlcNAc_1Man_3Xyl_1GlcNAc_2$-PA), *m/z* 1193.0 ($GlcNAc_1Man_3GlcNAc_2$-PA), *m/z* 1164.0 ($GlcNAc_1Man_2Xyl_1GlcNAc_2$-PA), *m/z* 1032.0 ($GlcNAc_1Man_2GlcNAc_2$-PA), *m/z* 990 ($Man_3GlcNAc_2$-PA), *m/z* 960.5 ($Man_2Xyl_1GlcNAc_2$-PA), *m/z* 828.0 ($Man_2GlcNAc_2$-PA), *m/z* 665.0 ($Man_1GlcNAc_2$-PA), *m/z* 503.0 (GlcNAc-GlcNAc-PA), *m/z* 446.0 (FucGlcNAc-PA), *m/z* 300.0 (GlcNAc-PA) (Fig. 8). Furthermore, a product derived from F-II by diplococcal β-*N*-acetylglucosaminidase, which is specific for GlcNAcβ1,2-linkage (Yamashita et al., 1983), gave a signal at *m/z* 1268.0 on IS-MS analysis, suggesting the removal of two GlcNAc residues that were previously bound to the non-reducing end mannose residues by β1,2-linkages. Thus, the deduced structure of F-II is

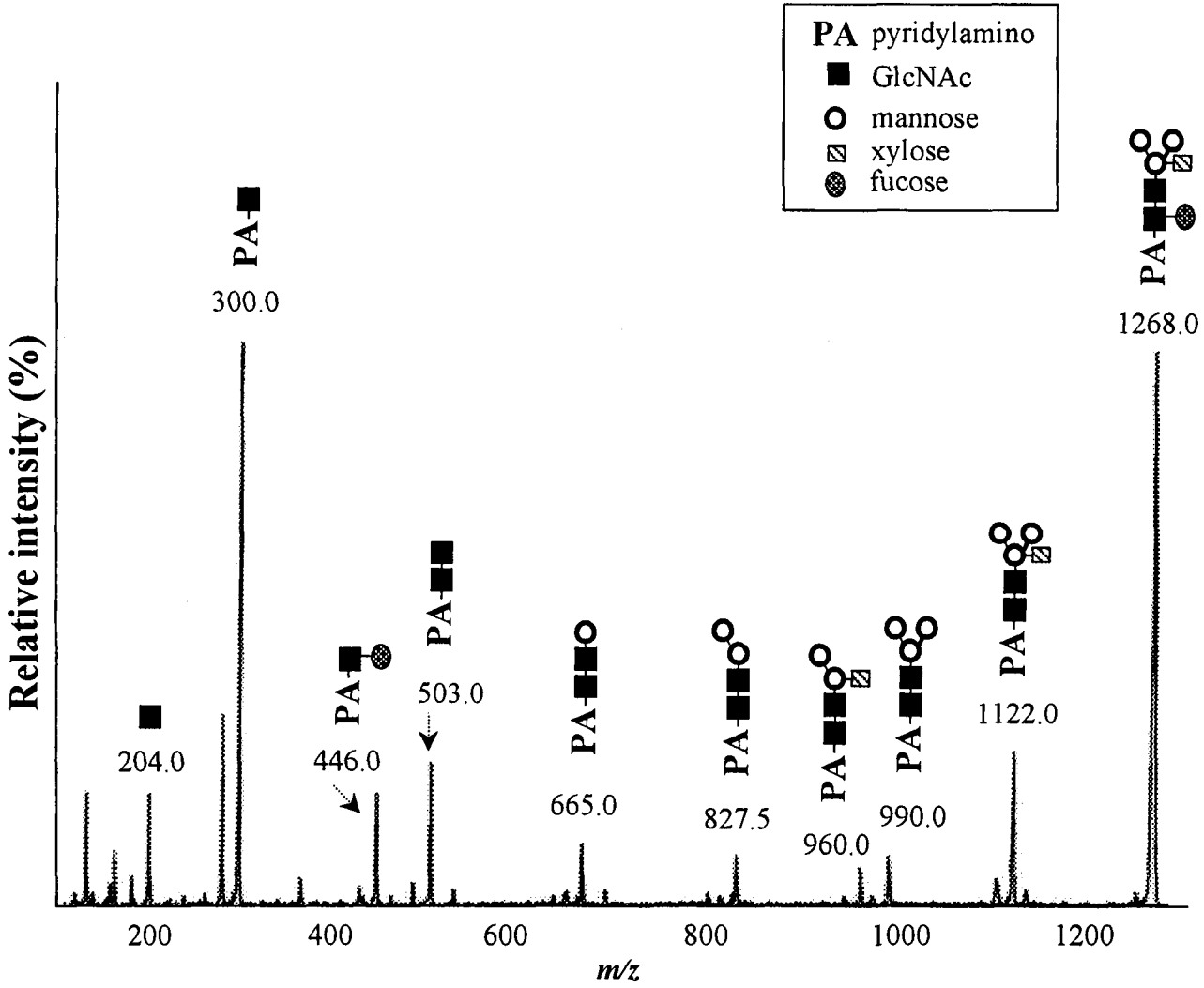
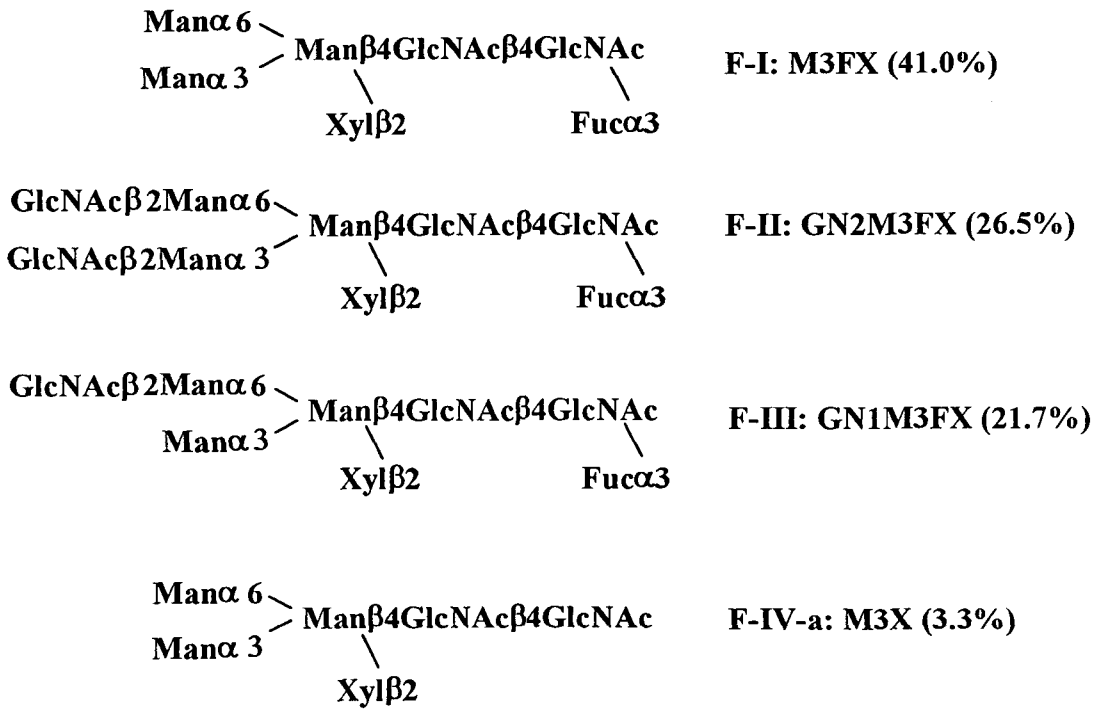


Fig. 6. MS/MS spectrum of PA-sugar chain F-I (*m/z* 1268.0).

Complex type glycans:



High mannose type glycans:

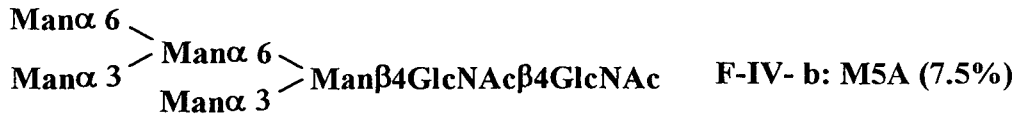


Fig. 7. Proposed structures for sugar chains of BY2 cells and their relative amounts.

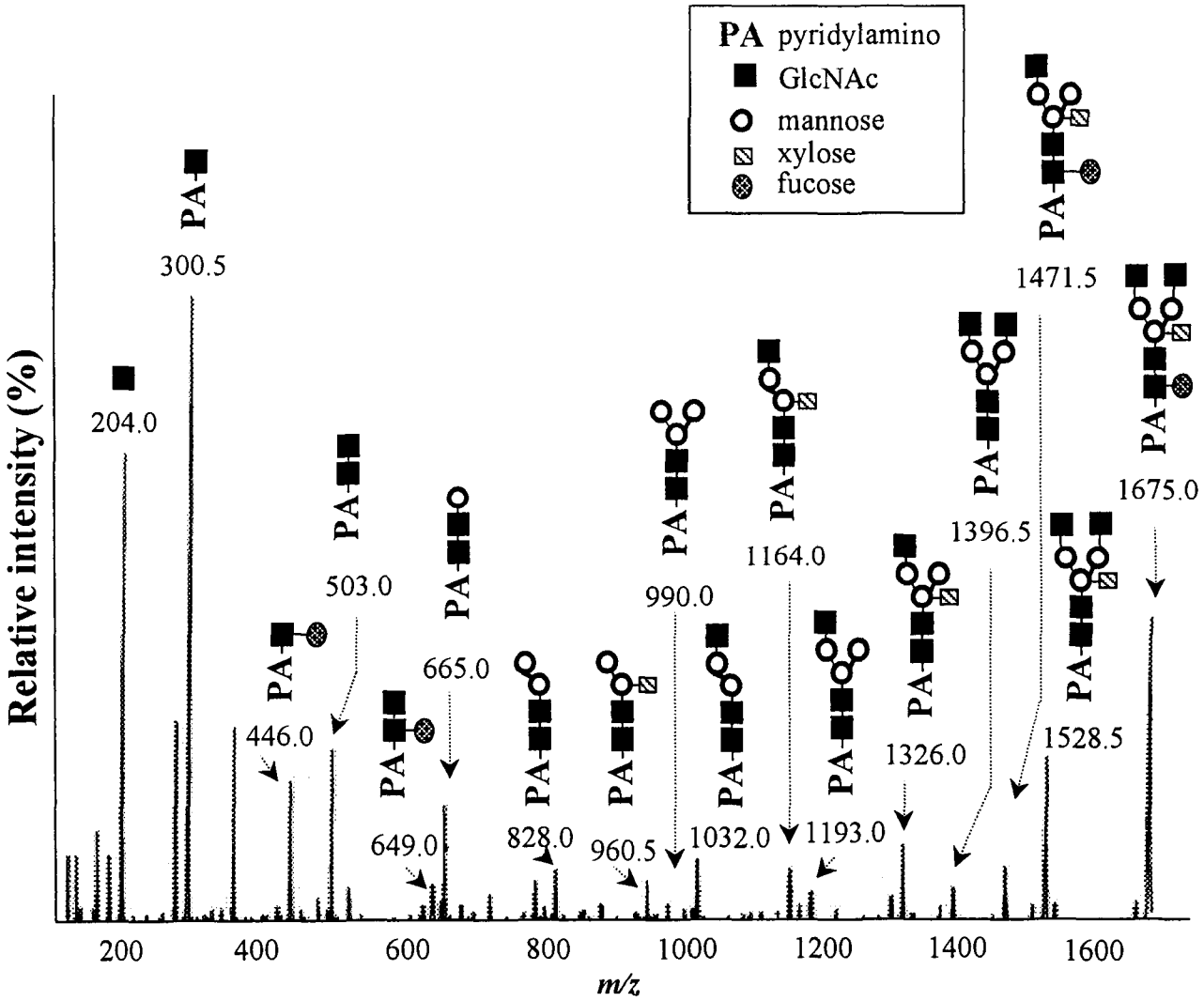


Fig. 8. MS/MS spectrum of PA-sugar chain F-II (*m/z* 1675.0).

GlcNAcβ1,2Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1, 3)GlcNAc-PA (GN2M3FX, Fig. 7).

The molecular mass of F-III (*m/z* 1471.5) corresponded to that of $GlcNAc_1Man_3Fuc_1Xyl_1GlcNAc_2$-PA (GN1M3FX). Relevant signals observed by IS-MS/MS analysis of F-III were ascribed as fragment ions derived from the GN1M3FX: *m/z* 1325.5 ($GlcNAc_1Man_3Xyl_1GlcNAc_2$-PA), *m/z* 1268.0 ($Man_3Fuc_1Xyl_1GlcNAc_2$-PA), *m/z* 1193.0 ($GlcNAc_1Man_3GlcNAc_2$-PA), *m/z* 1122.0 ($Man_3Xyl_1GlcNAc_2$-PA), *m/z* 990.0 ($Man_3GlcNAc_2$-PA), *m/z* 959.5 ($Man_2Xyl_1GlcNAc_2$-PA), *m/z* 827.5 ($Man_2GlcNAc_2$-PA), *m/z* 665.0 ($Man_1GlcNAc_2$-PA), *m/z* 649.0 ($Fuc_1GlcNAc_2$-PA) *m/z* 503.0 (GlcNAc-GlcNAc-PA), *m/z* 446.0 (FucGlcNAc-PA), *m/z* 300.5 (GlcNAc-PA) (Fig. 9). Furthermore, the product derived from F-III by the *diplococcal* β-*N*-acetylglucosaminidase digestion, with a signal at *m/z* 1268.0 on IS-MS analysis, suggested one β1,2-linked GlcNAc residue bonded to one non-reducing end mannose residue. For the GN1M3FX structure, two isomeric forms could occur: one is Manα1,6(GlcNAcβ1,2Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc-PA and the other is GlcNAcβ1,2Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4 (Fucα1,3)GlcNAc-PA. It has been reported that on RP-HPLC the former structure is eluted before GN2M3FX and the latter structure is eluted after GN2M3FX (Takahashi et al., 1986). Considering the elution position of F-III which was eluted after GN2M3FX (F-II) on the Cosmosil 5C18-AR column (Fig. 5), the structure of F-III is GlcNAcβ1,2Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4GlcNAcβ1,4(Fucα1,3)GlcNAc-PA (Fig. 7).

The elution position of F-IV-a (*m/z* 1121.5) corresponded exactly to authentic M3X on the 2D sugar chain map. By IS-MS/MS analysis of F-IV-a, fragment ions

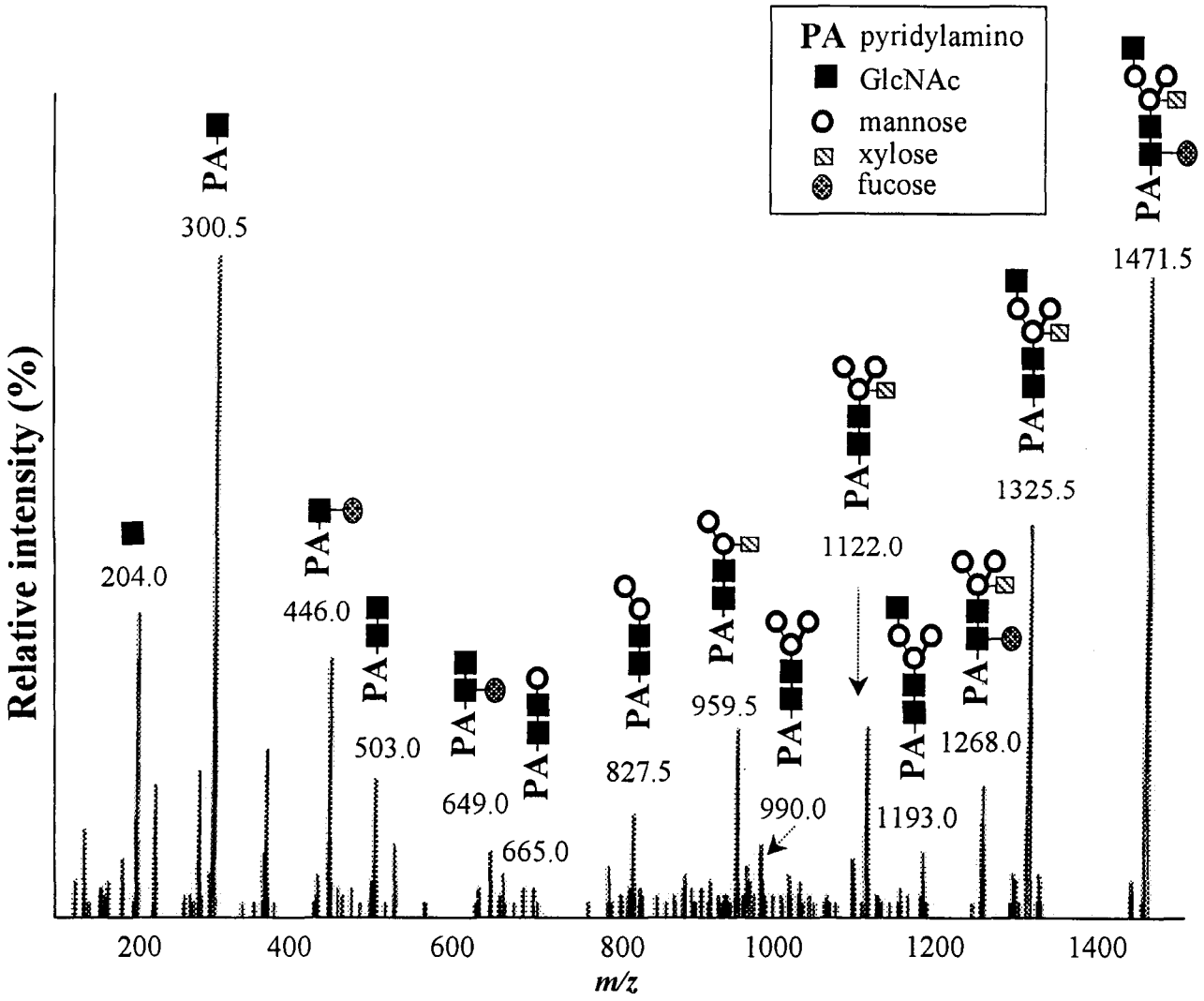


Fig. 9. MS/MS spectrum of PA-sugar chain F-III (*m/z* 1471.5).

could be assigned as derived from M3X: *m/z* 989.5 ($Man_3GlcNAc_2$-PA), *m/z* 959.5 ($Man_2Xyl_1GlcNAc_2$-PA), *m/z* 827.0 ($Man_2GlcNAc_2$-PA), *m/z* 665.5 ($Man_1GlcNAc_2$-PA), *m/z* 503.0 (GlcNAc-GlcNAc-PA), *m/z* 300.5 (GlcNAc-PA) (Fig. 10). The deduced structure of F-IV-a is Manα1,6(Manα1,3)(Xylβ1,2)Manβ1,4 GlcNAcβ1,4GlcNAc-PA (M3X, Fig. 7).

The elution position of F-IV-b (*m/z* 1314.5) corresponded exactly to authentic M5A on the 2D sugar chain map. IS-MS/MS analysis of F-IV-a showed fragment ions which could be derived from M5A: *m/z* 1152.0 ($Man_4GlcNAc_2$-PA), *m/z* 990.0 ($Man_3GlcNAc_2$-PA), *m/z* 828.0 ($Man_2GlcNAc_2$-PA), *m/z* 665.5 ($Man_1GlcNAc_2$-PA), *m/z* 503.0 (GlcNAc-GlcNAc-PA), *and m/z* 300.0 (GlcNAc-PA) (Fig. 11). Furthermore, a product derived from F-IV-b by α-mannosidase (jackbean) digestion, which exhibits activity toward α1-2, α1-3 and α1-6 mannose residues, gave a signal at *m/z* 665.5 on IS-MS analysis, suggesting F-IV-b is a typical high-mannose type structure. These results suggested the structure of F-IV-b as Manα1,6(Manα1,3)Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-PA (M5A, Fig. 7).

2.4 Discussion

The characterization of *N*-linked glycans in tobacco BY2 suspension cultured cells is summarized in Table 4. Five *N*-glycans found in the cell extract from tobacco BY2 cells were $Man_3Fuc_1Xyl_1GlcNAc_2$ (41.0 % as molar ratio), $GlcNAc_2Man_3Fuc_1Xyl_1GlcNAc_2$ (26.5 %), $GlcNAc_1Man_3Fuc_1Xyl_1GlcNAc_2$ (21.7 %), $Man_3Xyl_1GlcNAc_2$ (3.3 %), and $Man_5GlcNAc_2$ (7.5%) (Fig. 12). Nearly 90% (as

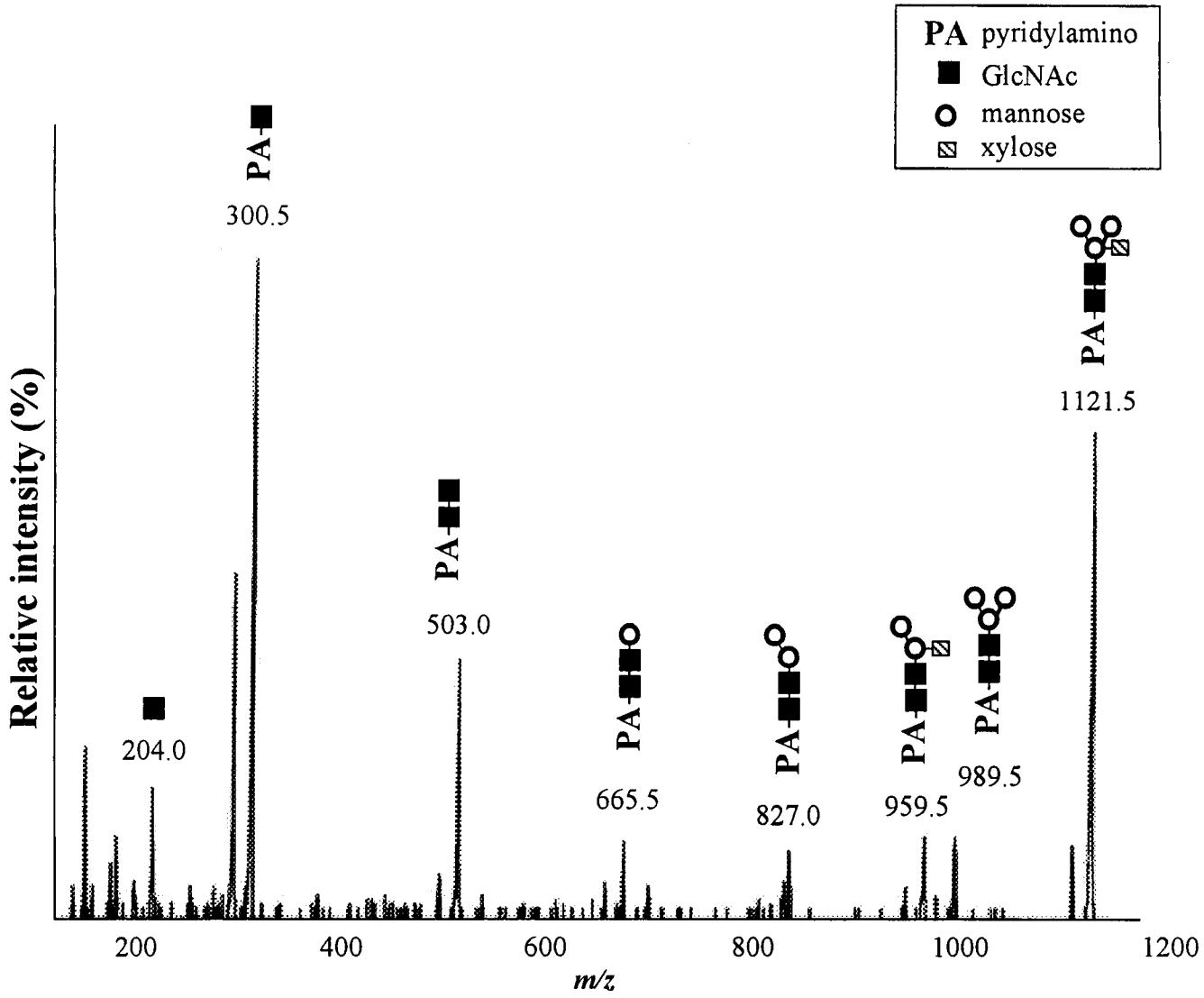


Fig. 10. MS/MS spectrum of PA-sugar chain F-IV-a (*m/z* 1121.5).

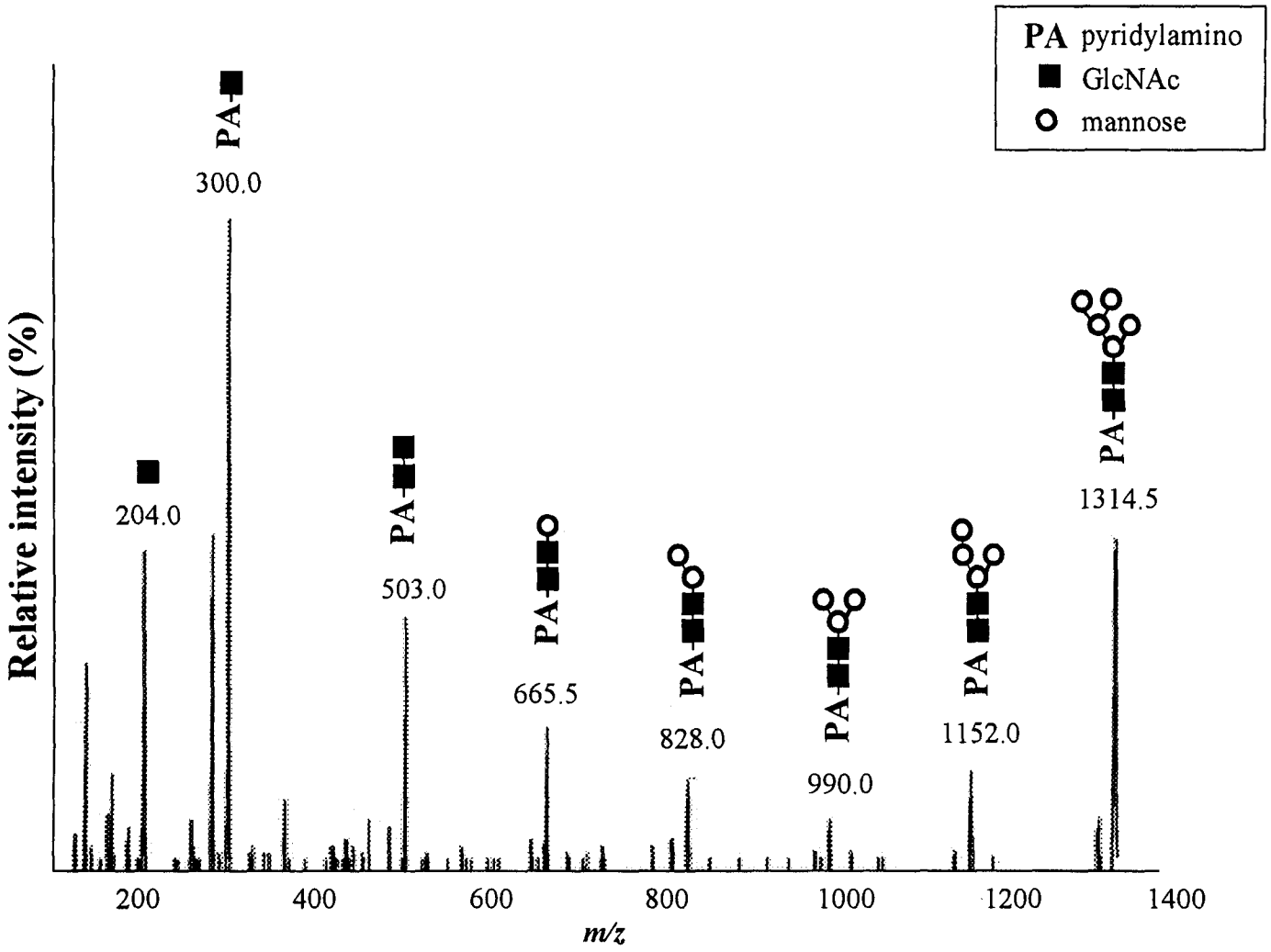
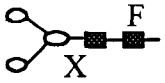
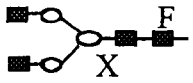
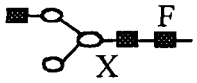
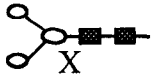
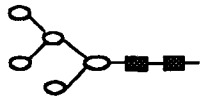
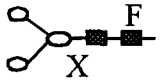
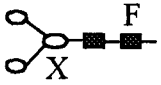
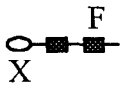
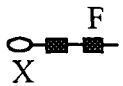
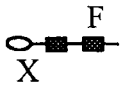
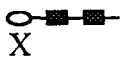


Fig.11. MS/MS spectrum of PA-sugar chain F-IV-b (*m/z* 1314.0).

Table 4. Characterization of PA-sugar chains from tobacco BY2 cultured cells.

Peak name	**F-I**	**F-II**	**F-III**	**F-VIa**	**F-VIb**
Expected structure based on elution position with standards: **RP-/SF-HPLC**	**M3FX**	**GN2M3FX**	**GNM3FX**	**M3X**	**M5A**
IS-MS [M+H]$^+$ (Obtained *vs.* Calculated Mass)	**1268.0** (1267.2)	**1675.0** (1673.6)	**1471.5** (1470.4)	**1121.5** (1121.0)	**1314.5** (1313.2)
Deduced structures ■, GlcNAc; ○, mannose; **X**, xylose; **F**, fucose					
Glycosidase digestions β-*N*-acetylglucosaminidase					
SF-HPLC		**M3FX**	**M3FX**		
IS-MS [M+H]$^+$		**1268.0**	**1268.0**		
Jackbean mannosidase					
SF-HPLC	**MFX**	**MFX**	**MFX**	**MX**	**M1**
IS-MS[M+H]$^+$	**943.0**	**943.0**	**943.0**	**797.0**	**665.5**
					

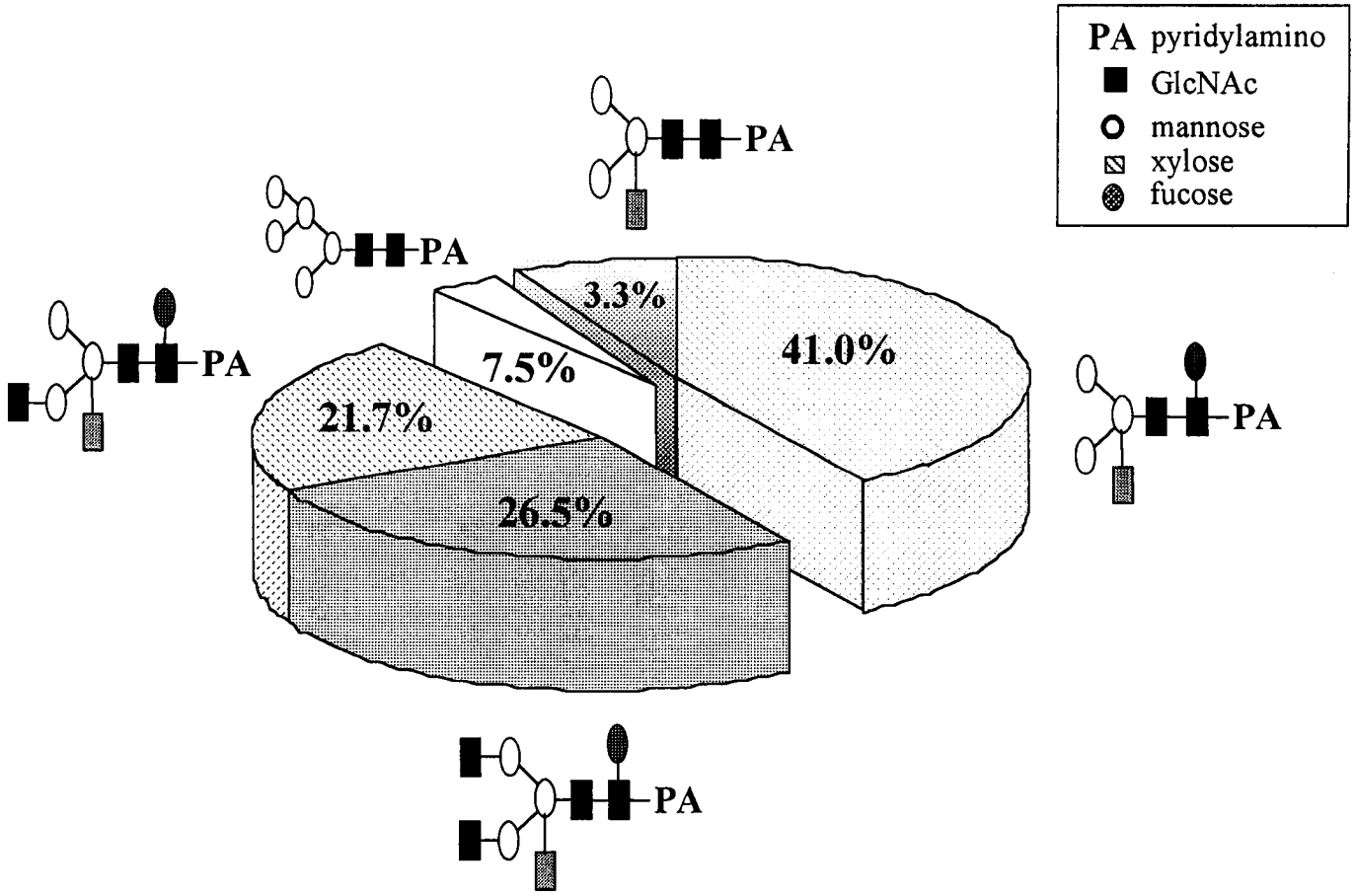


Fig. 12. Relative molar ratios of *N*-glycans obtained from tobacco BY2 suspension cultured cells.

molar ratio) of *N*-glycans occurring in the BY2 tobacco cultured cells are the fucosylated plant complex-type structure. On the contrary, no fucosylated *N*-glycan has been found on *N. alata S*-glycoproteins (Oxley and Bacic, 1995; Oxley et al., 1996). The predominance of sugar chains bearing both xylose and fucose residues indicates that the sugar chains are highly processed. Furthermore, the amount of a high-mannose type structure, $Man_5GlcNAc_2$, which bears no α1,2 mannosyl residue, occupies only 7.5% of total *N*-glycans from BY2 cell glycoproteins, suggesting that *N*-glycan modification proceed rapidly in the cultured cells. The presence of xylose and fucose residues as sugar modifications implies that BY2 cells contain highly immunogenic *N*-glycans.

Galactosylated *N*-glycans found in the *N*-glycans of sycamore cultured cells (Takahashi et al., 1986) or miracluin (Takahashi et al., 1990), could not be found in BY2. The most highly processed complex type sugar chains obtained have only terminal GlcNAc residues. Nevertheless, the presence of the sugar chains bearing GlcNAc residues (48.2% of total *N*-glycans) implies that theoretically, glycoproteins in BY2 cells could be modified to yield larger, biantennary *N*-glycans containing penultimate galactose and terminal sialic acid residues similar to complex type sugar chains in mammalian cells. Tobacco BY2 cells, thus, is a good model system for the evaluation and modification of plant *N*-glycosylation gearing towards the production of human compatible glycoproteins.

2.5 Summary

The five *N*-linked sugar chains of glycoproteins obtained from tobacco BY2 cells can be categorized as follows: a major class (92.5% as molar ratio) consisting of

xylose-containing complex type sugar chains; and a minor class of high-mannose type (7.5% as molar ratio). The predominance of complex type sugar chains and the presence of M5 as the only high mannose type sugar chain suggest that the glycans are highly processed in BY2. Results also indicate the heterogeneity of *N*-linked glycans, and the presence of complex sugar chains bearing the M3FX motif characteristic of plant *N*-glycans. Sugar chains bearing GlcNAc residues (48.2% of total *N*-glycans) imply that glycoproteins in BY2 cells could be further extended to yield larger, bi-antennary *N*-glycans similar to complex type sugar chains in mammalian cells.

CHAPTER 3

EXPRESSION OF THE HUMAN β1,4-GALACTOSYLTRANSFERASE (hGT) GENE IN TOBACCO CELLS AND ITS CHARACTERIZATION

3.1 Introduction

To make transgenic plants suitable hosts for the production of therapeutic proteins it is necessary to modify the glycosylation pattern of the cell (Chrispeels and Faye, 1996). Previous works have shown that the *N*-glycosylation capabilities of eukaryotic expression systems can be modified by transfecting cells with genes encoding new processing enzymes (Krezdorn et al., 1993; Kleene et al., 1994; Jarvis and Finn, 1996; Schwientek et al., 1996; Chiba et al., 1998; Hollister et al., 1998). Genes encoding α1,2-sialyltransferase or α1,3-galactosyltransferase has been expressed in Chinese hamster ovary or BHK-21 cells, with the resulting clones having altered terminal glycosylation (Jarvis and Finn, 1996). Mouse cells transfected with the human gene encoding α1,2-fucosyltransferase synthesized blood group H determinants (Schwientek, et al., 1996). Bovine β1,4-galactosyltransferase expressed under the viral *ie*1 promoter, can contribute to the lepidopteran insect cell *N*-glycosylation pathway and add galactose to at least one *N*-linked oligosaccharide side chain on gp64, a major baculovirus virion glycoprotein (Jarvis and Finn, 1996). Stable transformation of insect cells, instead of the viral vector, also showed *de novo* mammalian β1,4-galactosyltransferase and was able to add β1,4-linked galactose to two different foreign glycoproteins (Hollister et al., 1998). In plants, transformation of mutant *Arabidopsis cgl* cells (plants lacking in GnT-I activity) with the cDNA encoding human *N*-acetylglucosaminyl transferase I (GnT-I) resulted to the

conversion of high-mannose glycans into complex glycans that maybe fucose-rich and xylose-poor, implying that the human enzyme can be functionally integrated in the normal pathway for biosynthesis of complex glycans in *Arabidopsis* (Gomez and Chrispeels, 1994; Chrispeels and Faye, 1996; Lerouge et al., 1998). These studies suggest a possible conservation of the glycosyltransferase mechanisms among eukaryotes. Complementing the plant *N*-glycan machinery with heterologous glycosyltransferases may, therefore, help achieve the production of glycoproteins with human-compatible-type oligosaccharide structures (Chrispeels and Faye, 1996; Lerouge et al., 1998).

Tobacco BY2 cells, proved to be a suitable model system for studying modifications in plant *N*-glycosylation as shown in Chapter 2, were transformed with the human β1,4-galactosyltransferase gene (UDP-galactose: β-*N*-acetylglucosaminide β-1,4-galactosyltransferase; E.C. 2.4.1.22) as an initial step to evaluate the possibility of enlarging the spectrum of glycosyltransferases in plant suspension cultured cells. The mammalian glycosyltransferase was chosen for two reasons: β1,4-galactosyltransferase is the first glycosyltransferase in mammalian cells that initiates further branching of complex *N*-linked glycans after the action of GnT-I and -II (Kornfeld and Kornfeld, 1985; Narimatsu, 1994); and, although the glycosyltransferase is ubiquitous in the vertebrate kingdom (Narimatsu, 1994), its presence has not yet been conclusively proven in plants (Lerouge et al., 1998). Moreover, as shown in Chapter 2, the tobacco BY2 suspension cultured cells do not contain any galactosylated *N*-glycan suggesting the absence or low activity of the glycosyltransferase in these cells. Structural analyses of glycoproteins from transformed cells confirm the alterations in the *N*-link processing pathway upon introduction of β1,4-galactosyltransferase, demonstrating the plasticity of the glycosylation pathway in plants.

3.2 Materials and Methods

3.2.1 Organisms, plasmids and DNA

The organisms, strains and plasmids characteristics are listed below:

Strains/Plasmids	Genotype and characteristics	References
Strains		
Escherichia coli		
DH5α	*sup*E44,Δ*lac*U169, (ϕ 80*lac*ZΔM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1	Hanahan, 1983
JM109	*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, Δ(*lac*-proAB)/F`[*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15]	Yanisch-Perron et al., 1985
Agrobacterium tumefaciens		
EHA101	Km^r carrying the trans-acting virulence functions necessary to facilitate the transfer of the T-DNA region of binary vectors to plants	Hood et al., 1986
Plasmids		
pUC19	Amp^r, *lacZ*	Yanisch-Perron et al., 1985
M13mp19	*lacZ*	"
pBI221	pBI121 GUS casette in pUC19	Clonetech
pBluescriptIISK$^+$	Amp^r, lacZ	Stratagene
pGA482	Km^r, Tc^r	An, 1987
pRK2013	Km^r [conjugative plasmid used to mobilize plasmids from *E. coli* to *Agrobacterium tumefaciens* via triparental mating]	Ditta et al., 1980

3.2.2 Construction of the plant expression vector

The gene for human β1,4-galactosyltransferase (hGT) was amplified by PCR using two sets of primers based on the cDNA sequence reported by Masri *et al.* (1988). With Taq polymerase (Takara Shuzo), an 813 bp fragment from human placenta cDNA (Clonetech) was amplified using primers 1 (5' AAGACTAGTGGGCCCCATGCTGATTGA 3', *Spe*I site underlined) and 2 (5'

GTAGTCGACGTGTACCAAAACGCTAGCT 3', *Sal*I site underlined). For the N-terminal region of the enzyme, primers 3 (5' AAAGAATTCGCGATGCCAGGCGCGTCCCT 3', *Eco*RI site underlined) and 4 (5' AATACTAGTAGCGGGGACTCCTCAGGGCA 3', *Spe*I site underlined) were used to obtain a 376 bp fragment from human genomic DNA (Clonetech). The PCR products were cloned in M13mp18, sequenced and analyzed using an AutoRead Sequencing Kit (Pharmacia), and an ALF DNA sequencer (Pharmacia). The truncated coding sequences were juxtaposed to obtain the complete β1,4-galactosyltransferase gene (1.2 kbp; Fig. 13). Sequence alignment of the entire gene shows 99% homology to that reported by Watzele and Berger (1990; GenBank accession number X55415) and Uejima et al. (1992; accession number X13223).

The 1.2 kbp DNA fragment was subcloned into pBluescript –KS (Stratagene), designated as pSKhGT (Moran et al., 1997). Plasmid pSKhGT was digested with *Eco*RI and *Sal* I, and the resulting fragment was inserted between the cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase terminator (nos-t) in pBI221 (Clonetech). The resulting 2.2 kbp fragment, CaMV35S/hGT/nos-t chimeric gene, was excised and ligated to the binary vector, pGA482 (An, 1987, 1995), to construct the hGT expression vector pGAhGT (Fig. 13).

3.2.3 Plant transformation

The pGAhGT plasmid was mobilized into *Agrobacterium tumefaciens* strain EHA101 by triparental mating (Ditta et al., 1980). Tobacco BY2 suspension cultured cells (*Nicotiana tabacum* L. *cv.* Bright Yellow 2) were transformed as described by Rempel and Nelson (1995). Transformants were selected and maintained on modified

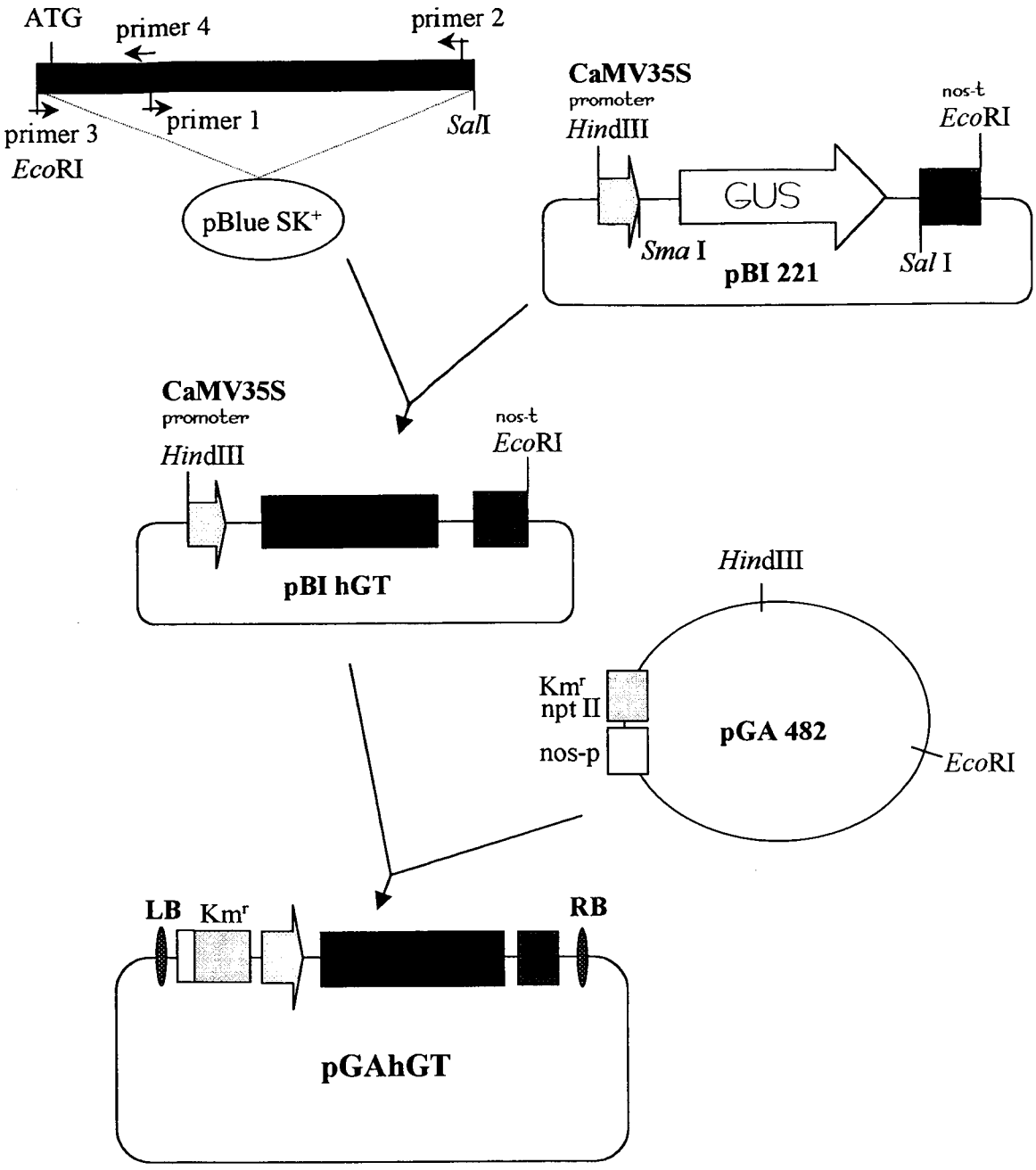


Fig. 13. Construction of the expression plasmid, pGAhGT. The hGT coding region lies downstream of the cauliflower mosaic virus 35S (CaMV35S) promoter, followed by the nopaline synthase terminator (nos-t) from pBI221. The construct also has the neomycin phosphotransferase II (nptII) gene under the control of the nopaline synthase promoter (nos-p) from pGA482. RB/LB, indicate right and left borders, respectively.

Linsmaier and Skoog medium (LS, see Chapter 2) supplemented with 150 μg/ml kanamycin and 250 μg/ml carbenicillin. Kanamycin resistant transformants were screened for hGT expression by Southern hybridization (McCabe et al., 1997; DIG Nucleic Acid Detection and DNA Labeling protocol, Boehringer Mannheim) and Western blotting (Uejima, et al., 1992; Uemura, et al., 1992).

3.2.4 DNA isolation and Southern blot analyses

Chromosomal DNA (40 μg) was isolated from tobacco cell transformants (Doyle and Doyle, 1990), and digested with *Eco*RI and *Hin*dIII. DNA fragments were electrophoresed, blotted onto nylon membranes, and hybridized with a [^{32}P] random-primed 2.2 kbp *Eco*RI and *Hin*dIII fragment from pGAhGT (Takara random priming labeling kit).

3.2.5 Preparation of cell extracts for Western blotting, enzyme assay and affinity chromatography

Five- to seven-day old tobacco suspension cultures were harvested, suspended in extraction buffer (25 mM Tris-HCl, pH 7.4; 0.25 M sucrose, 1 mM $MgCl_2$, 50 mM KCl), and disrupted by brief sonication (200W; Kaijo Denki Co.) or homogenized. Cell extracts/lysates were obtained from homogenates after centrifugation at 10,000 x*g* for 20 min at 4°C. To obtain microsome fractions, the supernatant was further centrifuged at 100,000 x*g* for 60 min at 4°C. Membrane bound proteins were further extracted from the 100,000 x*g* pellet using 0.02% to 1% Triton X-100 in 25 mM sodium cacodylate (pH 7.4) containing 10 mM $MgCl_2$.

Protein concentration was measured by the Coomassie blue dye-binding assay (BioRad) with bovine serum albumin (Sigma) as standard.

3.2.6 Immunoblotting and β1,4-galactosyltransferase activity

Cell homogenates were solubilized in Laemmli sample buffer (Laemmli, 1970) and subjected to 10% SDS-PAGE. The hGT protein was detected by Western blotting using a monoclonal anti-human galactosyltransferase (Gal-T) antibody (MAb 8628; 1:5000 in phosphate buffered saline [PBS: 140 mM NaCl, 2.6 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4] + 5% skim milk) kindly provided by Dr. H. Narimatsu, Soka University (Uejima et al., 1992; Uemura et al., 1992; Kawano et al., 1994). After one hour at room temperature, blots were then incubated with a horseradish peroxidase conjugated goat anti-mouse IgG (1:1000 in PBS + 5% skim milk; EY Laboratories, Inc.) and washed three times in PBS with 0.05% Tween-20 prior to the peroxidase color reaction carried out using POD immunostain kit (Wako). Immunoblot analysis for plant specific complex glycans was carried out using a polyclonal antiserum raised against carrot cell wall β-fructosidase (Lauriere et al., 1989). The apparent molecular masses were calculated based on the following molecular weight standards (NEB Prestained protein molecular weight markers): maltose binding protein (MBP)-β-galactosidase (175,000), MBP-paramyosin (83,000), glutamic dehydrogenase (62,000), aldolase (47,500), triosephosphate isomerase (32,500), β-lactoglobulin A (25,000), lysozme (16,500), and aprotinin (6,500).

β1,4-galactosyltransferase activity was assayed using UDP-galactose and pyridylamino-labeled (PA-) $GlcNAc_2Man_3GlcNAc_2$ ($GlcNAc_2Man_3GlcNAc_2$-PA) as substrate (Morita et al., 1988). The enzyme reaction contained 1-120 μg protein, 25 mM sodium cacodylate (pH 7.4), 10 mM $MnCl_2$, 200 μM UDP-galactose, and 100 nM $GlcNAc_2Man_3GlcNAc_2$-PA. Reaction products were analyzed by HPLC using a

PALPAK Type R and Type N column (Takara) according to the manufacturer's recommendation. PA-labeled standards $GlcNAc_2Man_3GlcNAc_2$-PA, $Gal_2GlcNAc_2Man_3GlcNAc_2$-PA and two isomers of $Gal_1GlcNAc_2Man_3GlcNAc_2$-PA were from Takara and Honen Co. (Tokyo), respectively.

3.2.7 Affinity chromatography on Ricinus communis120 *agglutinin* (RCA_{120})

Crude cell extracts and microsome fractions of transformed cells with highest enzymatic activity were loaded onto an RCA_{120}-agarose column (Wako). The column was washed with 15 volumes of 10 mM ammonium acetate, pH 6.0. Bound proteins were subsequently eluted with 0.2 M lactose, and fractionated on SDS-PAGE prior to silver (Wako Silver Staining Kit) or lectin staining. For lectin staining, membrane blots were washed in TTBS buffer (10 mM Tris-HCl, pH 7.4; 0.15 M NaCl, 0.05% Tween 20), incubated with horseradish-peroxidase-labeled RCA_{120} (Honen Co.), and galactosylated glycans visualized using POD immunostain kit (Wako).

3.2.8 Preparation, derivatization, and characterization of N-linked glycans

Structures of *N*-linked glycans in transformed cells were analyzed by a combination of reversed-phase and size-fractionation HPLC, two dimensional (2D) sugar chain mapping, exoglycosidase digestions and ion-spray tandem mass spectrometry (IS-MS/MS) as described in Chapter 2, except for the following modifications. Cell extracts were defatted with acetone and sugar chains were released by hydrazinolysis (100°C, 12 hr). The hydrazinolysate was *N*-acetylated, desalted by Dowex 50X2 and Dowex 1X2, and fractionated on a Sephadex G-25 gel filtration column (1.8 x 180 cm) in 0.1 N ammonia. After pyridylamination (PA-), sugar chains were separated on a Jasco 880-PU HPLC apparatus equipped with a

Jasco 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-P column or an Asahipak NH2P-50 column. Elution positions of sugar chains were compared with authentic sugar chains (Kimura et al., 1992) or purchased (Wako).

Glycosidase digestions using β-*N*-acetylglucosaminidase (*Diplococcus pneumoniae*, Boehringer Mannheim) or α-mannosidase (jackbean, Sigma) were done using about 1 nmol of PA-sugar chains under the same conditions as in Chapter 2. For β-galactosidase (*D. pneumoniae*, Boehringer Mannheim) and α1,2-mannosidase (*Aspergillus saitoi*, a kind gift from Dr. T. Yoshida, Tohoku University) digestions, 1 nmol of PA-sugar chain in 50 mM sodium acetate buffer (pH 5.5) were incubated with either 200 mU β-galactosidase or 60 μg α1,2-mannosidase at 37°C. The reactions were stopped by boiling and an aliquot of the digests was analyzed by size-fractionation HPLC. The molecular masses of the resulting digests were analyzed by ion-spray mass spectrometry (IS-MS/MS) and/or compared with authentic sugar chains (Kimura et al., 1992). IS-MS/MS experiments were performed as described previously (Chapter 2).

3.3 Results

3.3.1 Transformation of tobacco BY2 cells with the hGT gene

The constructed hGT gene encoding human β1,4-galactosyltransferase cDNA for expression in tobacco cells is depicted in Fig. 13. The gene was placed under the control of the CaMV35S promoter. pGAhGT also carries a neomycin phosphotransferase expression cassette conferring kanamycin resistance in transformed cells. Southern hybridization of genomic DNA obtained from

transformed cells confirmed the presence of a 2.2 kbp fragment derived from the T-DNA of pGAhGT (Fig. 14A). No hybridization signal was observed in nontransformed control cells, establishing that BY2 cells were transformed with the human β1,4-galactosyltransferase gene.

Western immunoblotting of the proteins from transformed cells gave a positive signal at a molecular mass of about 50 kDa (Fig. 14B). The obtained mass is higher than that estimated from the amino acid sequence (which is about 40 kDa), but is similar to the galactosyltransferase purified from ascitic fluids or obtained from yeast-expressed bovine galactosyltransferase (Uemura et al., 1992; Schwientek et al., 1996). Strong immunoreactive bands observed in microsome fractions compared to cell lysates may suggest that hGT was preferentially localized intercellularly. No immunoreactive bands were detected from wild type cells.

3.3.2 Selection of transformed cells exhibiting highest β1,4-galactosyltransferase activity

β1,4-galactosyltransferase activity was assayed using Triton-X 100 solubilized proteins from the microsomal fraction. Based on HPLC analysis, the β1,4-galactosyltransferase in transformed cells was able to transfer galactose from UDP-galactose to $GlcNAc_2Man_3GlcNAc_2$-PA (data not shown). No reaction product was obtained from that of wild type BY2 cells. Thus, the enzyme is present and active in transformed tobacco suspension cells alone. β1,4-galactosyltransferase activity varied among the different cell lines (GT1: 1.2×10^2, GT6: 9.0×10^2, GT8: 1.0×10^1, WT: $<2.1 \times 10^{-1}$ pmol/hr/mg protein). The transformant with the highest activity (9.0×10^2 pmol/h/mg protein), designated GT6, was used for further study.

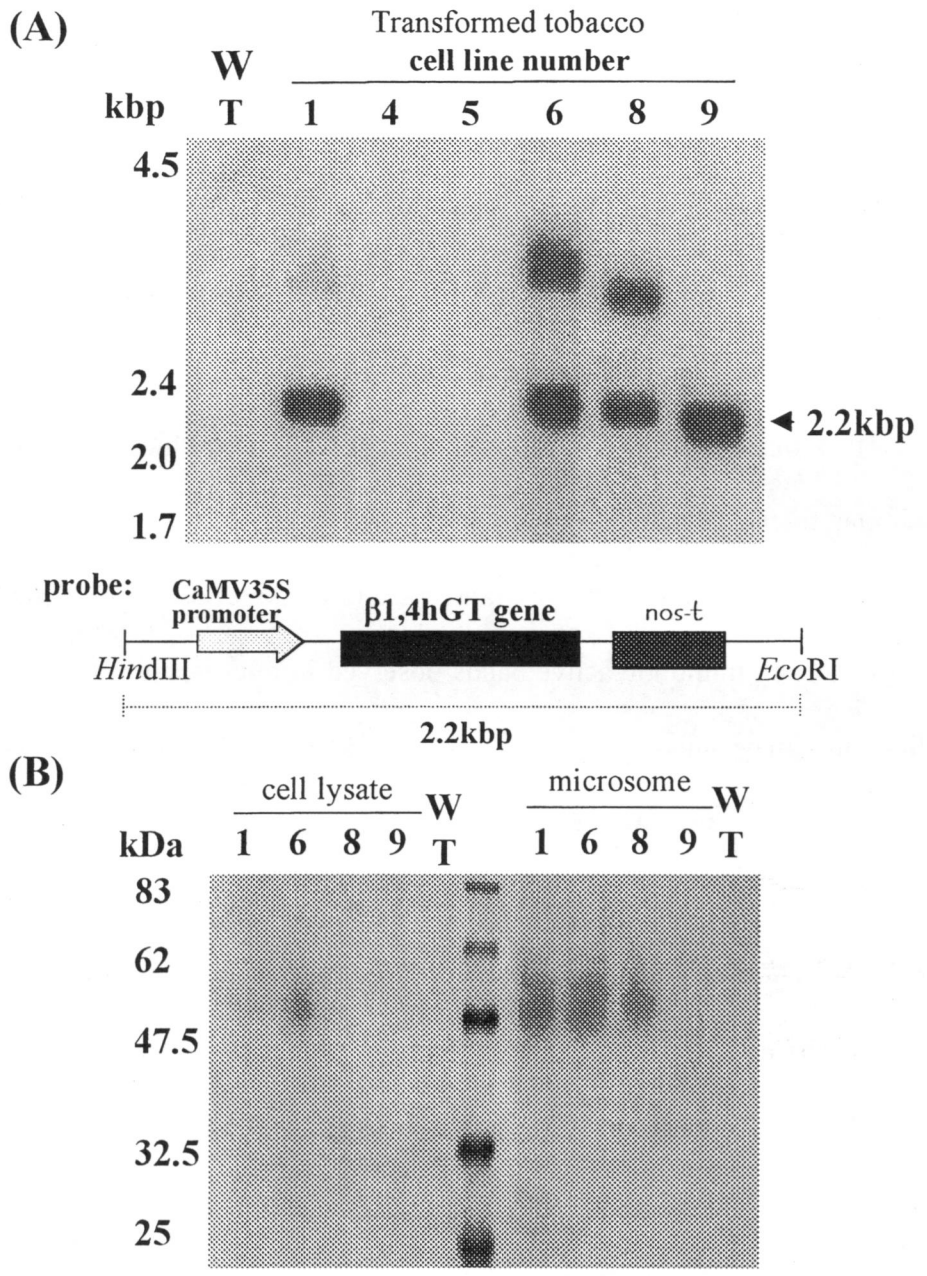


Fig. 14. **(A).** Southern blot analyses of genomic DNAs from transformed and wild type BY2 tobacco cells. DNAs were digested with *Hin*dIII and *Eco*RI, and probed with [^{32}P] labeled hGT gene fragment. WT: wild type; 1, 4, 5, 6, 8, and 9: denotes the cell lines for transformed tobacco cells. The numbers to the left indicate the sizes (kbp) and positions of lambda DNA fragments digested with *Hin*dIII. Size of the hybridizing fragment (2.2 kbp) is indicated. **(B).** Western blots of immunoreactive proteins from transgenic and non-transgenic tobacco cells. Proteins were denatured, electrophoresed through a 10% SDS-PAGE gel, and electroblotted onto a nitrocellulose membrane. The blots were probed with anti-hGT antibody as described in Materials and Methods. Lanes contain proteins from: cell lysates of cell line 1, 6, 8, and 9; microsome fractions of cell line 1, 6, 8, and 9. Molecular mass of marker proteins are indicated on the left.

3.3.3 Effect of the hGT activity on the glycan structure in plant cells

RCA_{120} affinity chromatography, which is specific for β1,4-linked galactose, was used to examine whether or not galactosyltransferase does transfer galactose residue to glycoproteins in transformed cells. Proteins, from both cell lysates and microsome fractions of the GT6 cell line, were bound to the RCA_{120} column and subsequently eluted using a hapten sugar (Fig. 15A). Eluted proteins blotted onto nitrocellulose membranes were positive in a subsequent RCA_{120} lectin staining analysis (Fig. 15B). RCA_{120} binding was not observed in wild type BY2 cells which further substantiates that GT6, unlike BY2, has glycoproteins with galactose at the non-reducing end of their glycan moieties.

Total protein extracts from BY2 and GT6 cells and eluted proteins of GT6 from RCA120 affinity chromatography were, likewise, probed with a polyclonal antiserum specific for plant complex glycans (Fig. 15C). The antiserum used primarily binds to the β1,2-xylose residue on plant glycoproteins (Lauriere et al., 1989). BY2 cells contained several glycoproteins that reacted strongly with the antibody (Fig. 15C, lane 1). GT6 cells contained glycoproteins that crossreacted, although the intensity was much less (lane 2). In contrast, the glycoproteins eluted from RCA_{120} showed no reactive band (lane 3), suggesting that galactosylated glycans may not contain any β1,2-xylose residues.

3.3.4 Analysis of glycans in GT6 cells

Structural elucidation of *N*-linked glycans in transformed cells further confirmed the presence of galactose residues, and allowed analysis and comparison of glycan processing between wild type and transformed cells. The PA-sugar chains obtained from GT6 were purified and characterized by a combination of reversed-

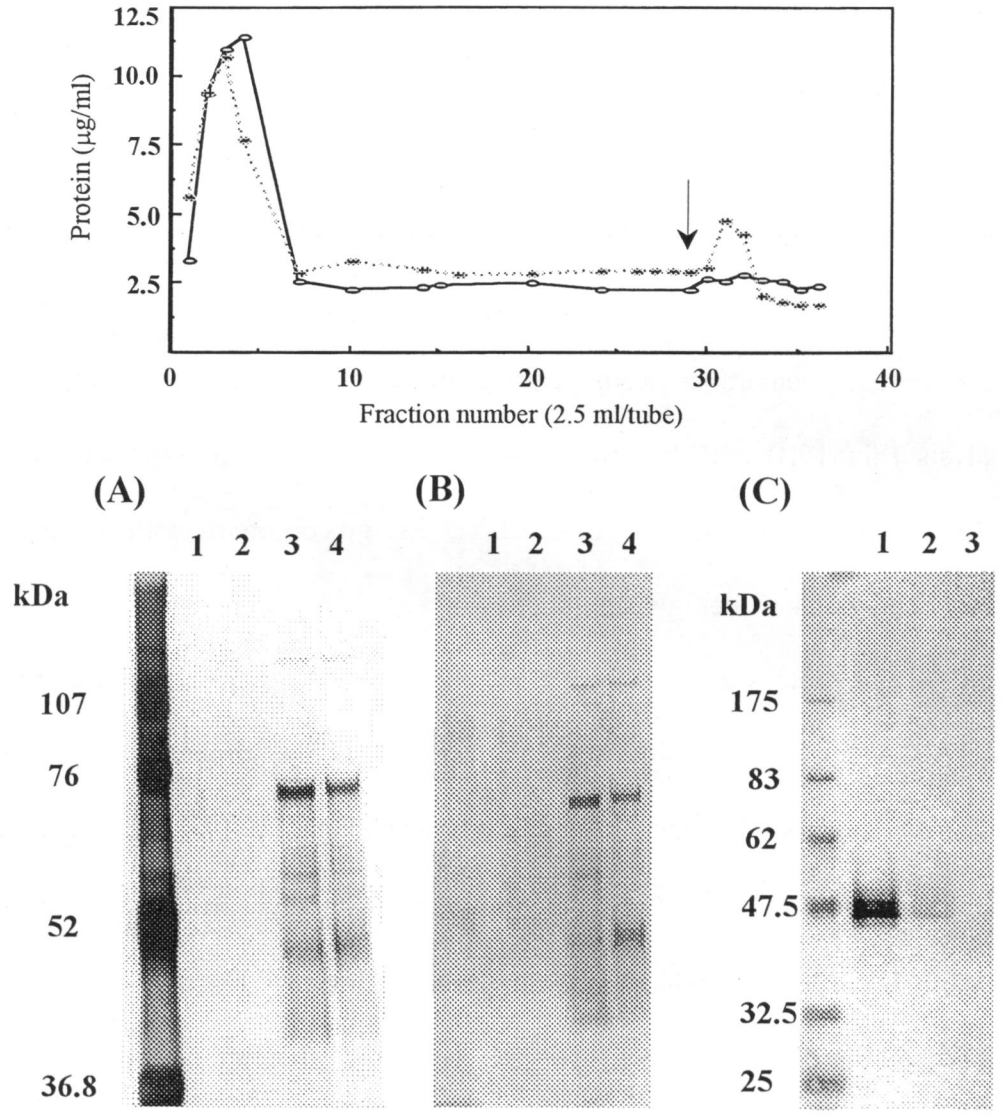


Fig. 15. Detection of galactosylated glycoproteins using *Ricinus communis* (RCA_{120}) affinity chromatography. Microsome fractions from BY2 (—) and GT6 (·····) were applied to identical small columns (5 ml) of RCA_{120}-agarose. The arrow indicates the starting point of elution with 0.2M lactose. Eluted fractions were subjected to SDS-PAGE and the gel was visualized by **(A)** silver staining, or **(B)** blotted onto nitrocellulose membrane and lectin (RCA_{120}) stained. Lanes 1 and 2, represent proteins from wild type BY2 (WT); 3 and 4, are proteins from transformed GT6. **(C).** Blots probed with a xylose-specific antiserum for plant complex glycans. Lanes 1 and 2 represent total protein extracts from BY2 and GT6, respectively; Lane 3, is the eluted glycoprotein from GT6 after RCA_{120} affinity chromatography. Molecular mass markers are in kDa.

phase (RP-) and size-fractionation (SF-) HPLC. PA-derivatives obtained from RP-HPLC (Fig. 16) were collected, and each collected fraction (I-XI) was re-chromatographed in SF-HPLC (Fig. 17).

No *N*-linked oligosaccharides were eluted from fraction I in SF-HPLC (between 10-25 min) suggesting that this is a run-through fraction containing by-products during hydrazinolysis (Fig. 16, 17). MS/MS analyses of these peaks gave no fragment ions with mass-to-charge (*m/z*) value of 300 (PA-GlcNAc). Similarly, fraction XI (50-60 min) did not contain any *N*-glycans because no peaks were observed in SF-HPLC. On the contrary, fractions II to X gave 17 peaks upon purification in SF-HPLC (Fig. 17). The elution position and molecular mass of oligosaccharides -A, -E, -H, -I, -M, -O, -P, and -Q (Fig. 17) did not correspond to standard PA-sugar chains. In addition, although these fractions gave signals at *m/z* 300 and 503 ($GlcNAc_2$-PA), no fragment ion at *m/z* 665 ($ManGlcNAc_2$; M1) was observed (data not shown). Other peaks, -B, -D and –N, were confirmed to be non *N*-glycans since no fragment ions were obtained at *m/z* 300. IS-MS/MS analyses indicate the remaining seven peaks as *N*-linked sugar chains.

The elution position and molecular mass of peaks -C (*m/z* 1637.5; 9.3% as molar ratio), -F (*m/z* 819.5 for $[M+2H]^{2+}$, 1639.0 for $[M+H]^{+}$; 15.9%), and -G (*m/z* 1475.5; 19.5%) corresponded to high mannose type sugar chains $Man_7GlcNAc_2$ isomers M7A and M7B, and $Man_6GlcNAc_2$ (M6B), respectively. Jackbean mannosidase digestion trimmed each *N*-glycan to $ManGlcNAc_2$ (M1) as analyzed by SF-HPLC (Table 5). IS-MS analysis of the resulting digests gave an ion at *m/z* 665.0, in accord with the calculated mass of 664.66 for M1, confirming the structural identity to reference PA-oligosaccharides (Fig. 18).

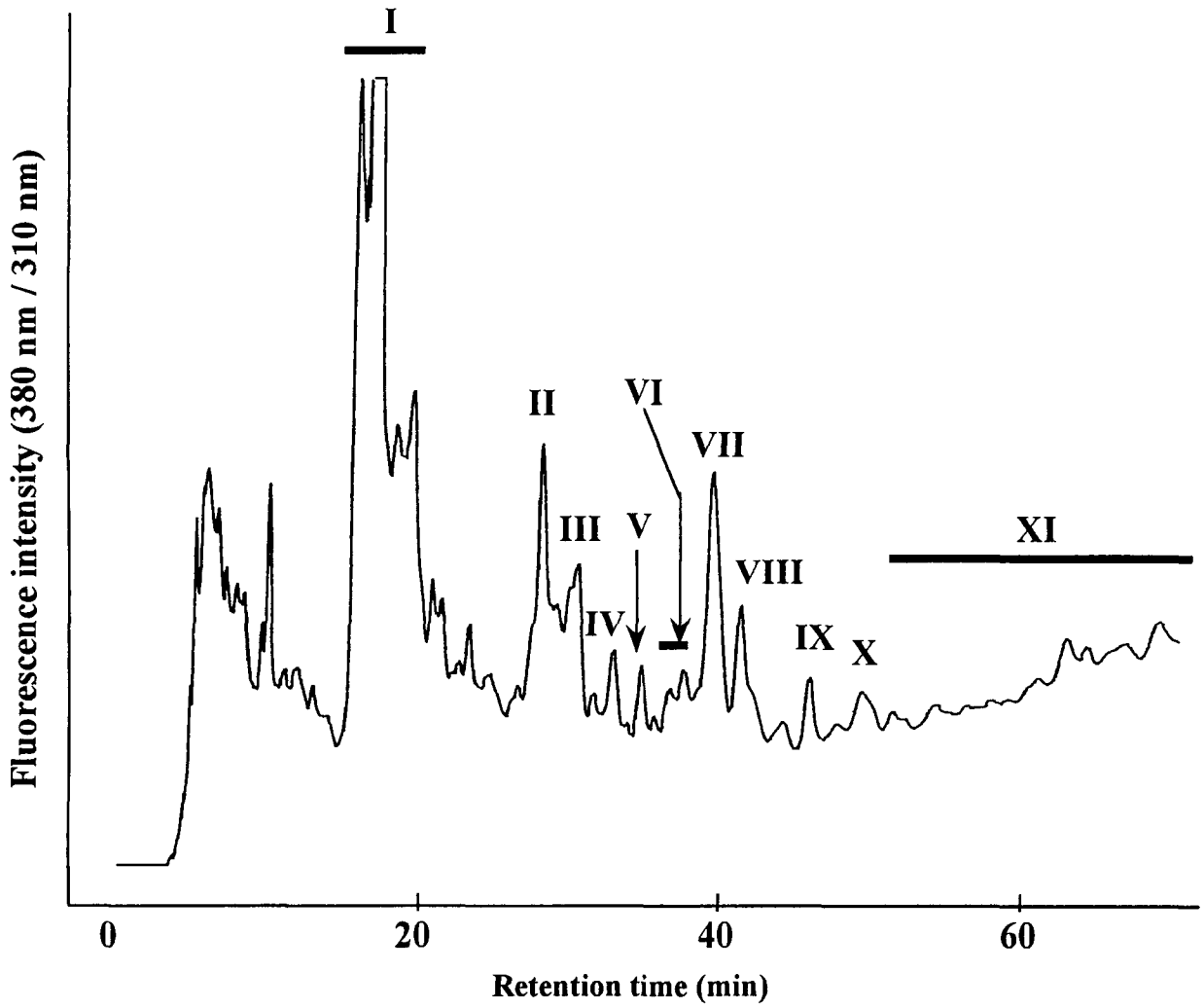


Fig. 16. Reversed-phase HPLC pattern of PA-derivatives prepared from transformed cells. PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 15% for 60 min at a flow rate of 1.2 ml/min. I-XI indicate individual fractions collected and purified for size-fractionation HPLC.

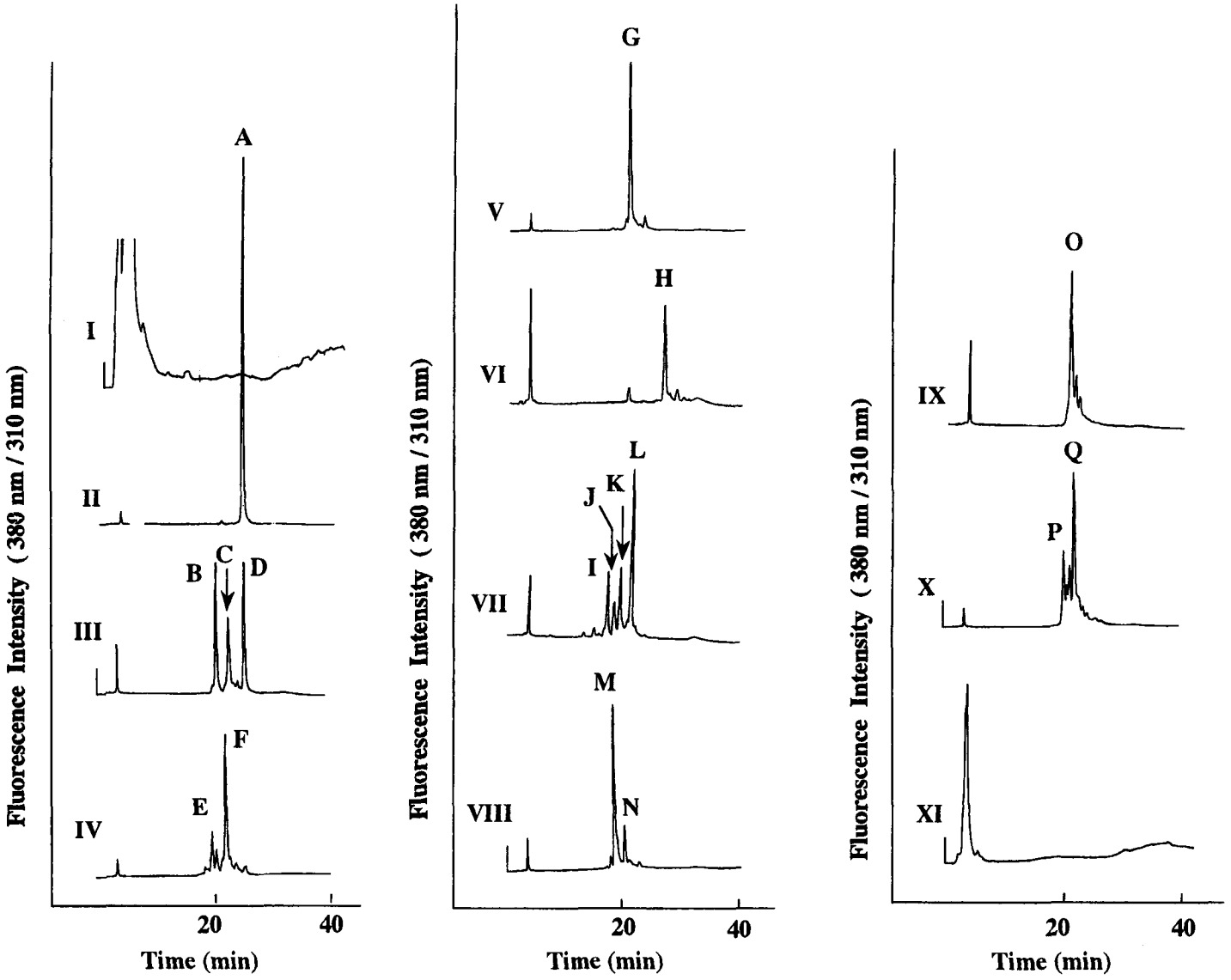
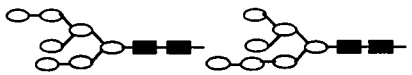
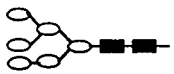
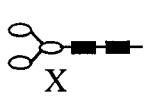
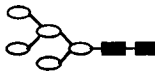
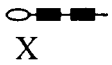
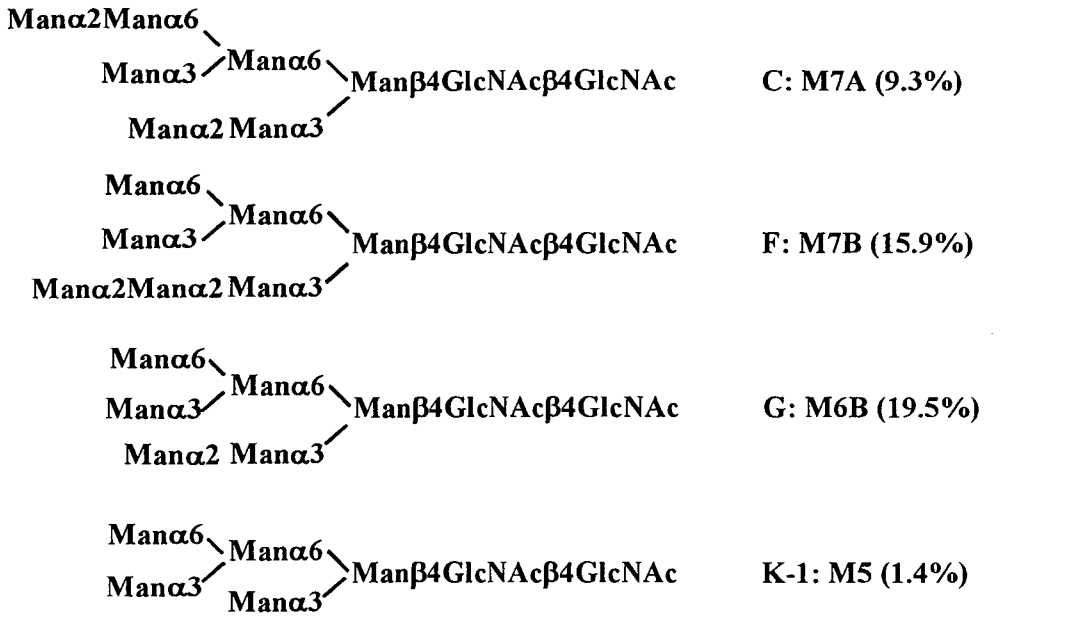


Fig. 17. Size-fractionation HPLC of PA-sugar chains from Fig. 15. PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 30% to 50% for 40 min at a flow rate of 0.8 ml/min.

Table 5. Characterization of PA-sugar chains from transformed GT6 cells.

Peak name	C	F	G	J	K-1	K-2	L
Expected structure based on elution position with standards: **RP-/SF-HPLC**	**M7A**	**M7B**	**M6B**	**M3X**	**M5A**		
IS-MS $[M+H]^+$ (Obtained vs. Calculated Mass)	**1637.5** (1637.5)	**1639** (1637.5)	**1475.5** (1475.4)	**1121.5** (1121.0)	**1314.0** (1313.2)	**1354.5** (1354.3)	**1680.0** (1678.55)
Deduced structures ■, GlcNAc; ○, mannose; X, xylose							
Glycosidase digestion Jackbean mannosidase							
SF-HPLC	**M1**	**M1**	**M1**	**MX**	**M1**		
IS-MS $[M+H]^+$	**665**	**665**	**665**	**797.0**	**665.0**		
							

High mannose type glycans:



Complex type glycans:

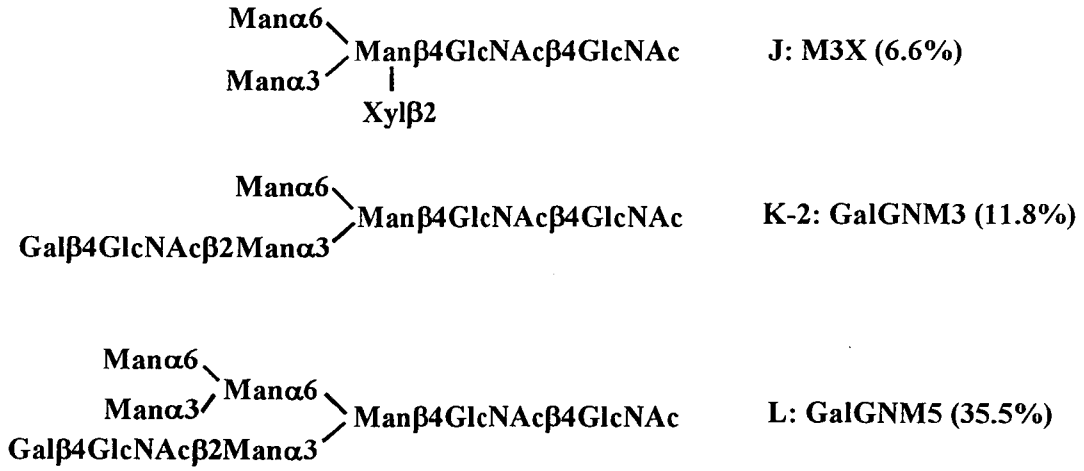


Fig. 18. Proposed structures of *N*-linked glycans obtained from transformed cells. Enclosed numbers represent molar ratios.

Peak -J (6.6%) with a molecular mass of 1121.5, agreed well with the calculated mass for $Man_3Xyl_1GlcNAc_2$-PA (M3X; *m/z* 1121.05). The location of the fragment ions at *m/z* 989.5, 827.5, 665.5, 503.3, and 300.0 was consistent with the serial loss of each monosaccharide from the parent molecule in the order of Xyl, Man, Man, Man, and GlcNAc. α-Mannosidase (jackbean) digestion of the sugar chain resulted in a change in elution position equivalent to $ManXylGlcNAc_2$-PA (Table 5), suggesting the trimming of two non-reducing terminal mannose residues. The deduced structure of the PA-sugar chain is shown in Fig. 18.

IS-MS analysis of peak -K (13.2%) showed that the fraction contained two *N*-glycans, one at a molecular mass of 1314.0 and the other at 1354.5. Peak -K was thus re-injected in reversed-phase HPLC, resulting to two peaks, -K-1 (1.4%) and -K-2 (11.8%). The sugar chain at *m/z* 1314.0 (-K-1) corresponded exactly to authentic $Man_5GlcNAc_2$ (M5), and subsequent α-mannosidase (jackbean) digestion removed four mannose residues as indicated by the elution shift that matched the elution position of M1 (Table 5, Fig. 18). Two peaks, -K-2 and L (35.5%), that did not correspond to the standard PA glycans are discussed in detail below.

3.3.5 N-glycans wih galactose residues in GT6 cells

The sugar chain at *m/z* 1354.5 (peak -K-2) is in good agreement with the expected mass (*m/z* 1354.3) for $Gal_1GlcNAc_1Man_3GlcNAc_2$-PA (GalGNM3). Relevant signals observed were assigned as fragment ions derived from the parent: *m/z* 1193.5 ($GlcNAcMan_3GlcNAc_2$-PA), 989.5 ($Man_3GlcNAc_2$-PA), 827.5 ($Man_2GlcNAc_2$-PA), 665.0 ($ManGlcNAc_2$-PA), 503.0 ($GlcNAc_2$-PA), 336.0 (ManGlcNAc), 300.0 (GlcNAc-PA), and 204.0 (GlcNAc) (Fig. 19). Based on the

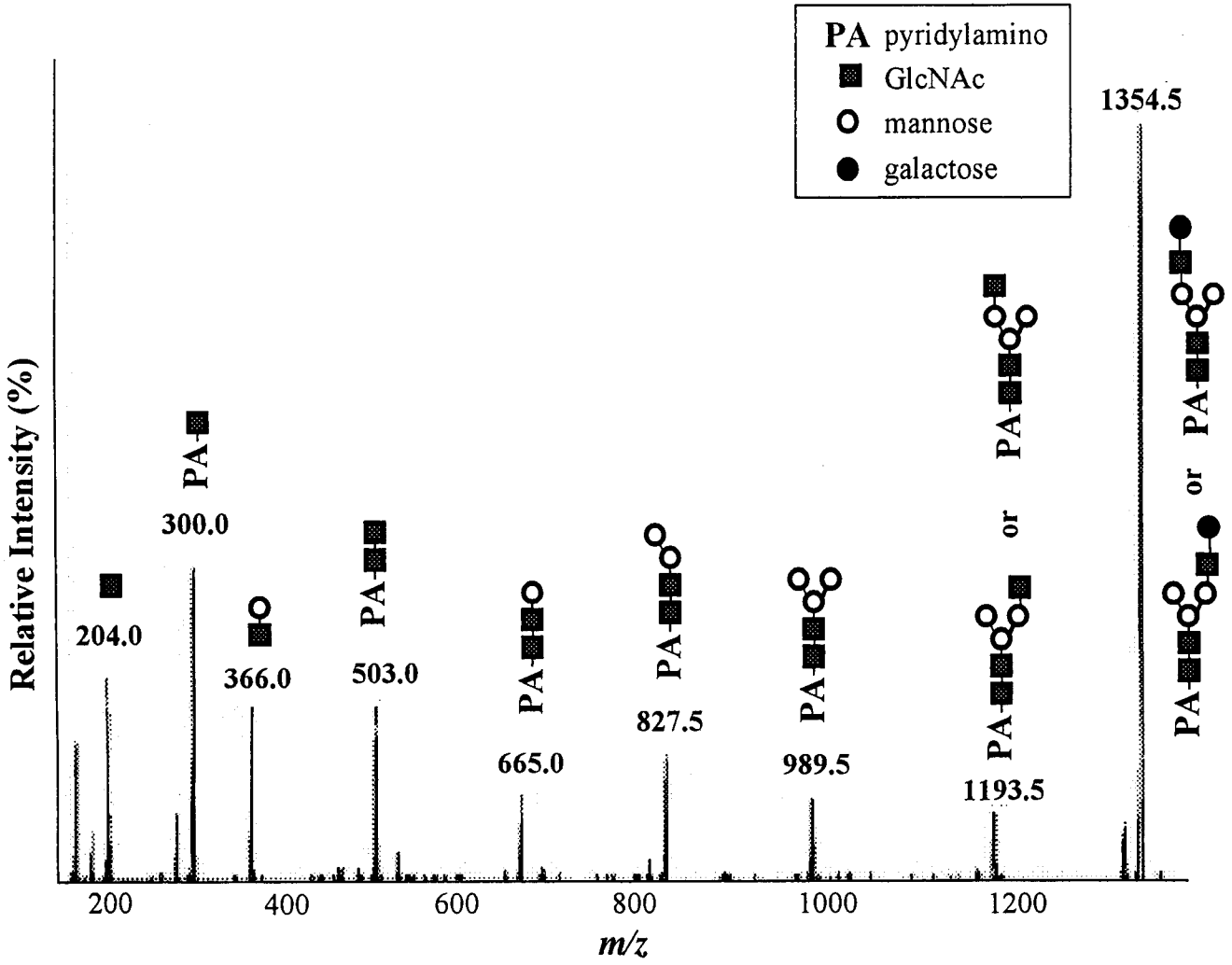
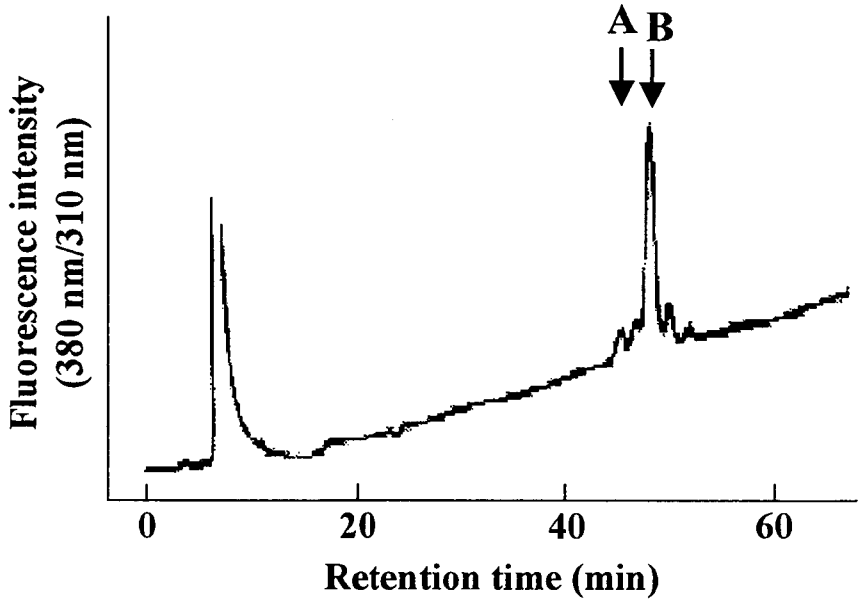


Fig. 18. MS/MS spectrum of PA-sugar chain -K-2 (*m/z* 1354.5).

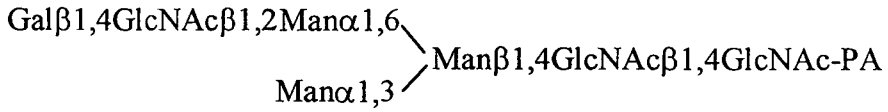
deduced *N*-glycan structure, there are two possible isomers for GalGNM3, *i.e.*, Galβ1,4GlcNAcβ1,2Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-PA and Man α1,6(Galβ1,4GlcNAcβ1,2Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-PA. The elution position of the purified PA-sugar chain matched with that of the latter (Fig. 20).

Ensuing exoglycosidase digestions confirmed the structural identity as shown in Fig. 21. A product derived from *D. pneumoniae* β-galactosidase, which is specific for Galβ1,4GlcNAc linkage (Twining, 1984), has similar elution position with $GlcNAcMan_3GlcNAc_2$-PA (Fig. 21-II). IS-MS/MS analysis of the product gave a signal at *m/z* 1192.0, corresponding to the loss of one hexose unit. These results suggest that one β1,4-linked galactose residue was removed from one non-reducing end GlcNAc. Digestion of $GlcNAcMan_3GlcNAc_2$-PA with β-*N*-acetylglucosaminidase (*D. pneumoniae*), that specifically hydrolyzes β1,2-GlcNAc linkages (Yamashita et al., 1983), resulted to a peak that co-eluted with $Man_3GlcNAc_2$-PA (Fig. 21-III), which in turn was further trimmed to $ManGlcNAc_2$-PA when digested with jackbean α-mannosidase (Fig. 21-IV). The deduced structure of peak -K-2 is shown in Fig. 18.

The molecular mass of peak –L (840.0 for $[M+2H]^{2+}$, 1680.0 for $[M+H]^{+}$) is comparable to the expected mass of $Gal_1GlcNAc_1Man_5GlcNAc_2$-PA (GalGNM5; *m/z* 1678.55). The locations of the fragment ions at *m/z* 1476.0, 1313.5, 1152.0, 989.5, 827.5, 665.5, 503.0, and 300.0 were consistent with the serial loss of each monosaccharide from the parent molecule (Fig. 22). *D. pneumoniae* β-galactosidase digestion suggested the presence of galactose β1,4-linked to one non-reducing end GlcNAc residue (Fig. 23-II). The removal of the galactose residue was also confirmed



A:



B:

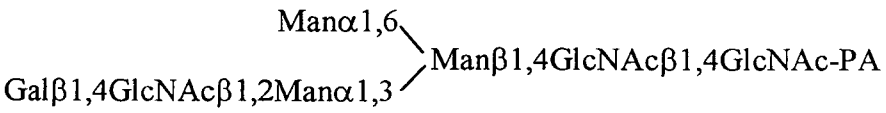


Fig. 20. Elution position of peak -K-2 in reversed-phase HPLC compared to two authentic sugar chains A and B. Elution conditions are described as in Fig. 16.

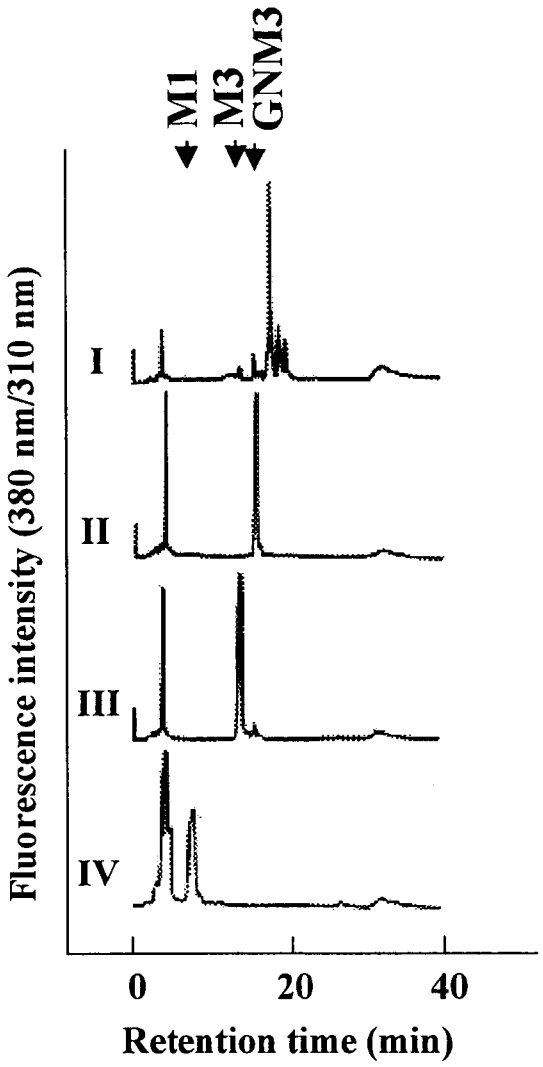


Fig. 21. SF-HPLC profiles of PA-sugar chain K-2 obtained after exoglycosidase digestions. PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 18% to 40% for 25 min at a flow rate of 0.8 ml/min. **I.** Elution position of purified K-2; **II.** β-galactosidase digest of I; **III.** β-*N*-acetylglucosaminidase digest of II; **IV.** jackbean α-mannosidase digest of III.

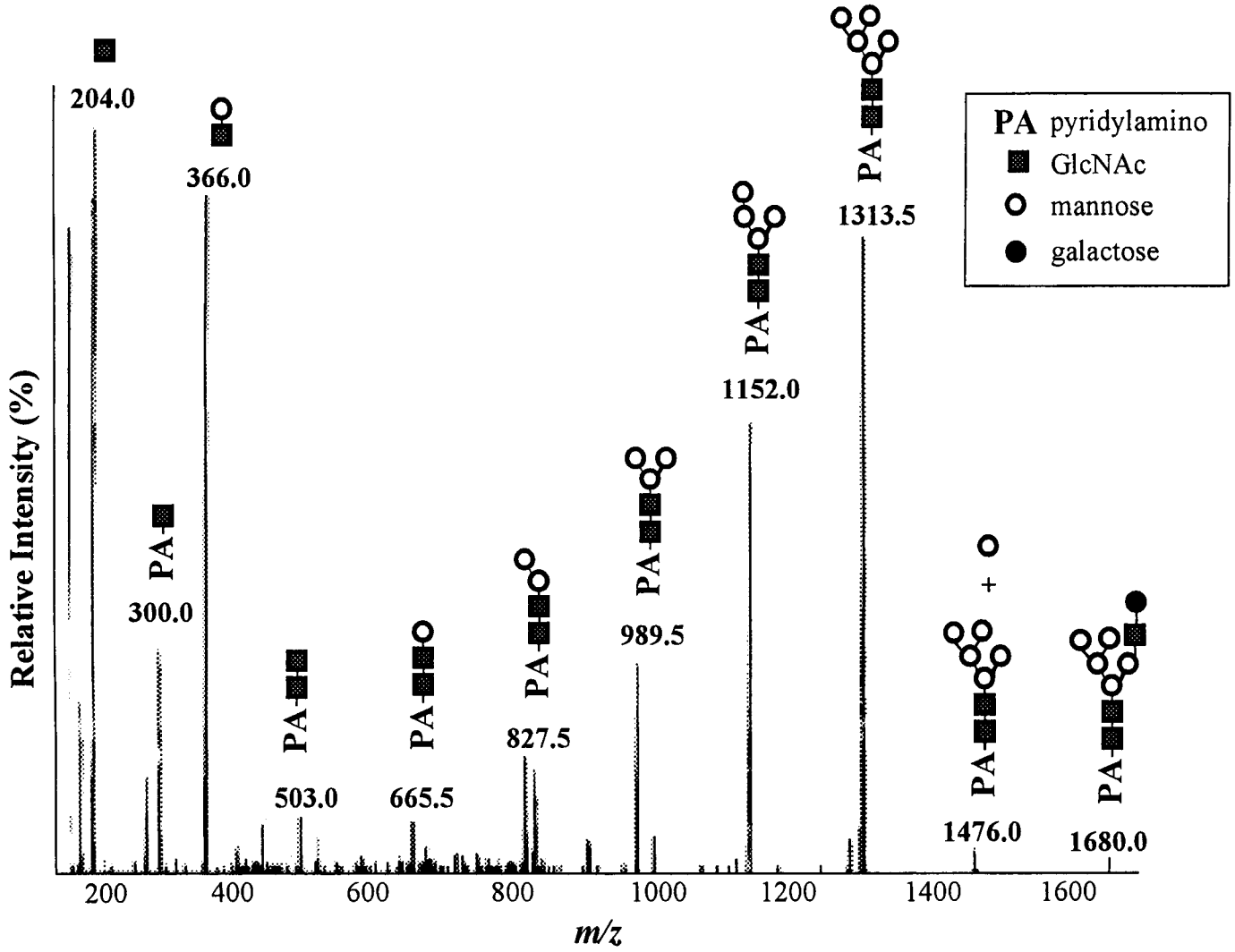


Fig. 22. MS/MS spectrum of PA-sugar chain -L (*m/z* 1680.0).

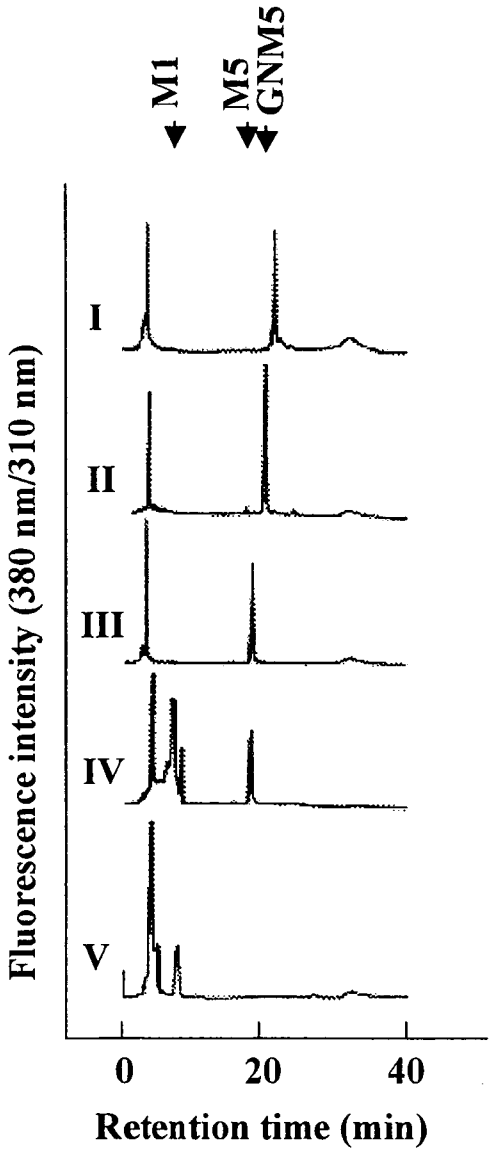


Fig. 23. SF-HPLC profiles of PA-sugar chain -L obtained after exoglycosidase digestions. PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 18% to 40% for 25 min at a flow rate of 0.8 ml/min. **I.** Elution position of purified -L; **II.** β-galactosidase digest of I; **III.** β-*N*-acetylglucosaminidase digest of II; **IV.** α1,2 mannosidase digestion of III; **V.** jackbean α-mannosidase digest of III.

by IS-MS/MS analysis, with the resulting molecular mass of 759.0 for $[M+2H]^{2+}$ or 1518.0 for $[M+H]^{+}$. From the parent signal at *m/z* 1518.0 ($GlcNAc_1Man_5GlcNAc_2$-PA), the fragment ions at 1314.0, 1152.0, 990.0, 827.5, 665.5, 503.5 and 300.5 corresponded to $Man_5GlcNAc_2$-PA, $Man_4GlcNAc_2$-PA, $Man_3GlcNAc_2$-PA, $Man_2GlcNAc_2$-PA, $Man_1GlcNAc_2$-PA, $GlcNAc_2$-PA and GlcNAc-PA, respectively. Digestion of $GlcNAc_1Man_5GlcNAc_2$-PA with *diplococcal* β-*N*-acetylglucosaminidase, yielded a product with an elution position similar to $Man_5GlcNAc_2$-PA (Fig. 23-III). No elution shift was observed when the PA-sugar chain was treated further with α1,2-mannosidase (Fig. 23-IV). Treatment with jackbean mannosidase (Fig. 23-V), however, resulted in an elution position corresponding to M1, suggesting the removal of four non-reducing terminal mannose residues. Results of mannosidase digestions indicate that the PA-sugar chain consists of five alpha-mannosyl residues, whose structure is similar to the standard M5A [Manα1,6(Manα1,3)Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-PA]. Based on exoglycosidase digestions, 2D sugar chain mapping, and IS-MS/MS, the structure of peak -L was identified as GalGNM5 (Fig. 18).

3.4 Discussion

The human β1,4-galactosyltransferase gene was successfully cloned and expressed in transgenic tobacco cells. As a consequence, the β1,4-galactosyltransferase gene in transformed tobacco cells altered the glycosylation pattern of endogenous proteins.

High activity in the Triton-X 100 solubilized 100,000 x*g* fraction suggests that the catalytic form of the enzyme is oriented towards the luminal side of the Golgi (data not shown), similar to that of the mammalian glycosyltransferase (Narimatsu, 1994). The affinity of glycoproteins to RCA_{120} suggested that the human glycosyltansferase in transformed BY2 cells was able to convert some complex-type sugar chains from non-galactose into galactose containing types. Moreover, it is remarkable that the galactosylated *N*-glycans do not contain any β1,2-xylose residues. Interestingly, there were no significant differences in the *in vitro* growth properties of wild type BY2 or transformed GT6 cells. The transformed GT6 cells were maintained continuously for over 150 weekly transfers in the laboratory.

Structural studies of *N*-glycans showed the diversity of oligosaccharide structures in transformed cells. In GT6 cells, almost half of all sugar chains have been galactosylated (47.3% as molar ratio), and the normally robust fucosylation and xylosylation activites were greatly reduced. The complex sugar chains represent only 53.9% of the total sugar chain. The lower conversion efficiency of oligomannose type sugar chains to complex glycans can also be inferred from the amount of high mannose type sugars containing α1,2-linkages (44.7%). Clearly, the expression of human β1,4-galactosyltransferase extended and modified the *N*-linked glycosylation pattern in BY2 (Fig. 24).

Based on the structural diversity of the *N*-linked sugar chains, a possible processing scheme for the biosynthesis of complex oligosaccharides containing a galactose residue is deduced (Fig. 25). The presence of GalGNM5, a hybrid *N*-glycan, may infer that once $Man_5GlcNAc_2$ (M5) is formed, GlcNAc transferase I acts immediately to transfer GlcNAc residue to M5 (Szumilo, et al., 1986; Johnson and

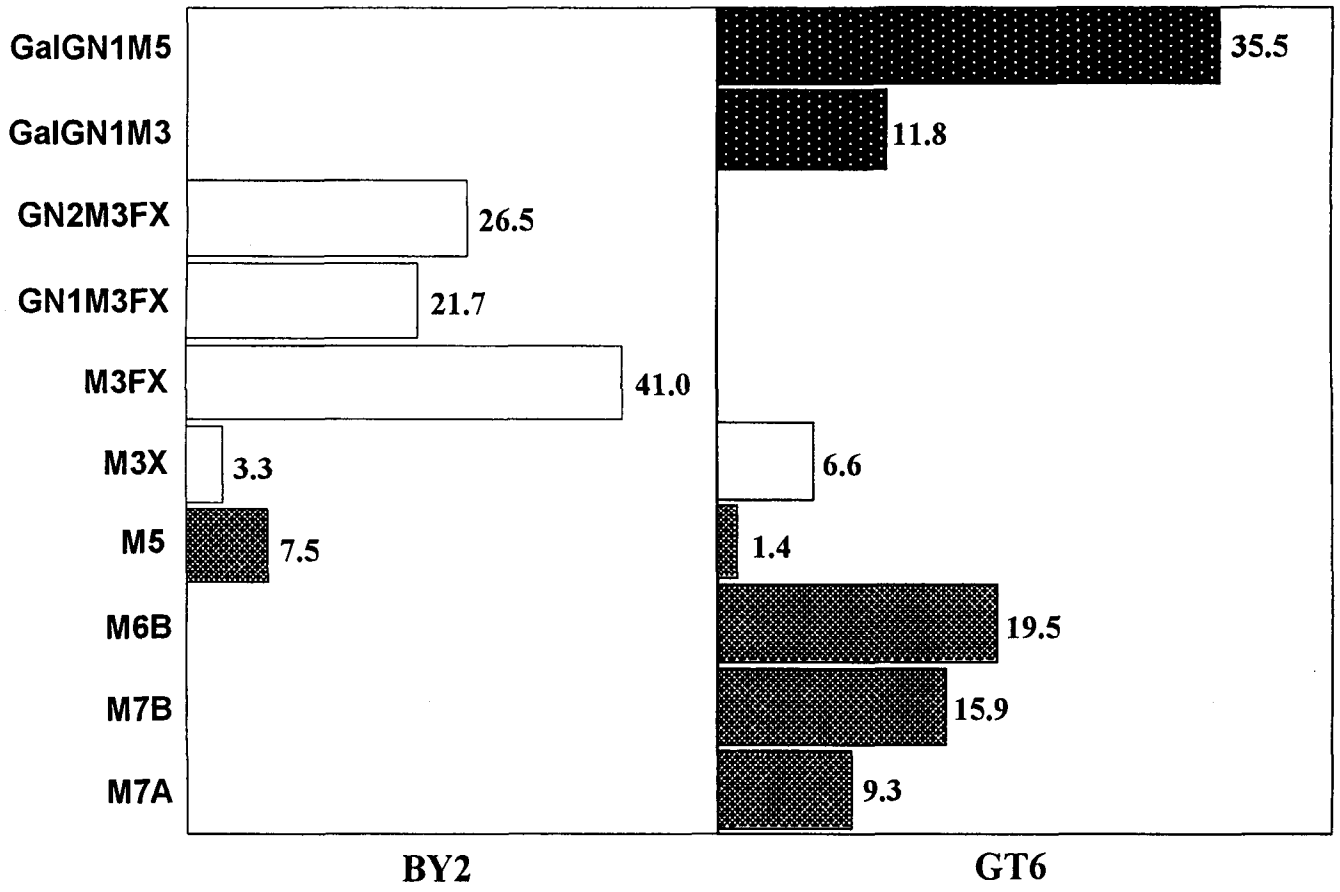


Fig. 24. A schematic representation of the differences in the glycan structures obtained from glycoproteins of wild type (BY2) and transformed cells (GT6). Glycans bearing galactose residues at the end of their sugar moities are represented by blocked bars. Clear bars represent glycans with fucose and xylose residues. Notched bars are high mannose type glycans. Numbers indicate the relative molar ratios.

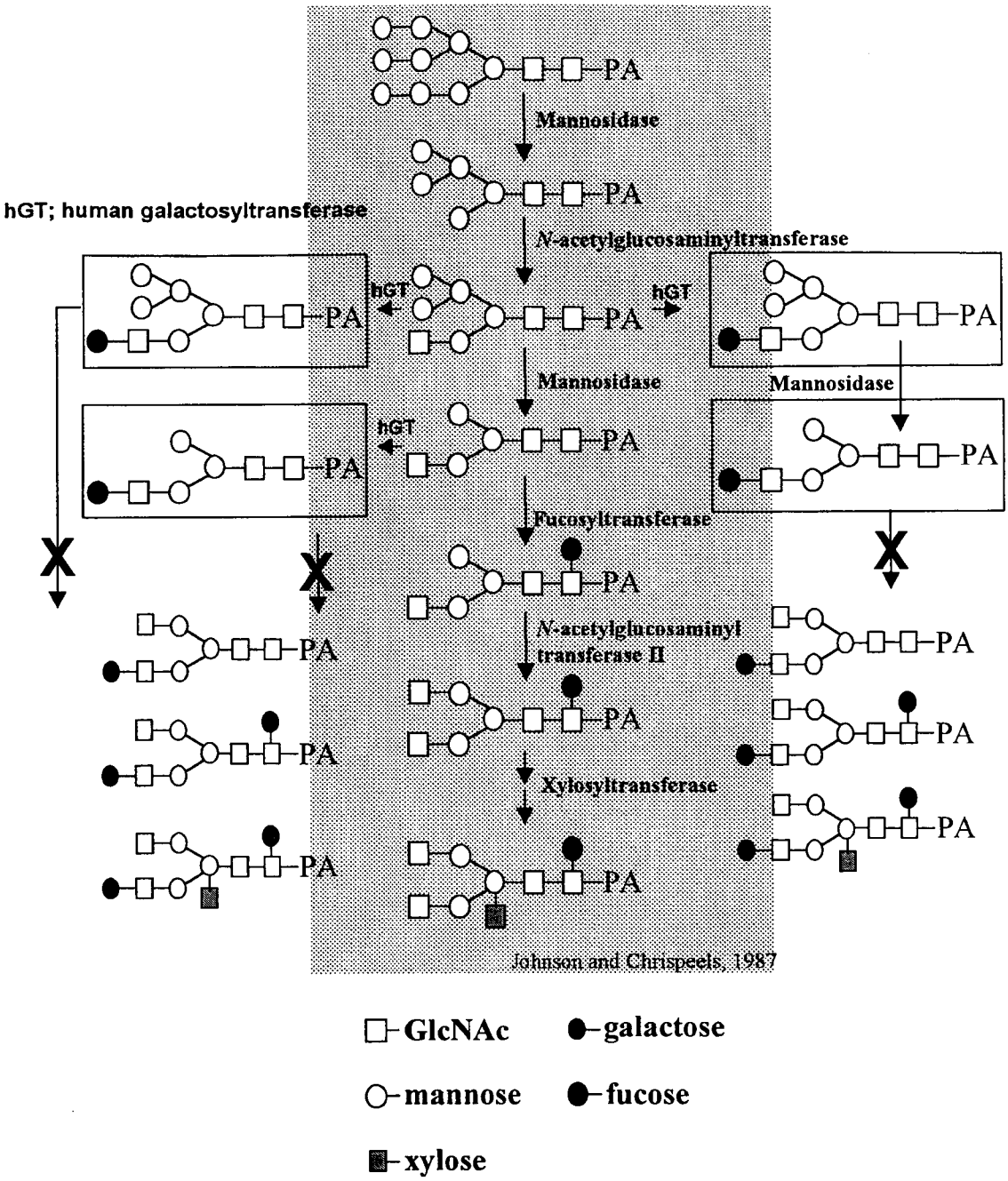


Fig. 25. The deduced processing pathway for the biosynthesis of *N*-linked glycans containing a galactose residue. The scheme is based from the structural diversity of sugar chains obtained and the proposed pathway of Johnson and Chrispeels (1987).

Chrispeels, 1987), from which the resulting hybrid glycan will be acted upon by galactosyltransferase. The modification of M5 to GalGNM5 may be the preferred route for the biosynthesis of galactosylated sugar chains since GalGNM5 represents 35.5% of the total sugar chain obtained. Whether further trimming by mannosidase II (Man II) is possible after galactose addition to yield GalGNM3 remains to be investigated. To date, plant Man II substrate specificity studies inferred that the purified Man II from mungbean seedlings is in most respect similar to the mammalian Man II (Kaushal et al., 1990), and thus the enzyme activity can be greatly reduced with the presence of additional residues in the GlcNAcβ1,2Manα1,3 branch. Likewise, GalGNM5 acted as a poor substrate for jackbean mannosidase when assayed *in vitro* (data not shown).

According to substrate specificity studies of mungbean Man II, Man II may cleave GNM5 to GNM3 yielding another possible substrate for galactosyltransferase (Kaushal et al., 1990). The absence of *N*-glycans bearing GlcNAc residues at the α1,6-linked mannose may suggest that the sugar chains with GlcNAc residues at the α1,3-mannose branch were processed by galactosyltransferase before GlcNAc transferase II. The galactosylated glycans are poor substrates for GlcNAc transferase II (Szumilo et al., 1987; Bendiak and Schachther, 1987), fucosyl- and xylosyltransferases (Staudacher et al., 1995; Zeng et al., 1997).

The significant reduction in plant-specific oligosaccharides bearing xylosyl and fucosyl residues in GT6 cell lines may not only provide a system where issues on immunogenicity of various glycans can be tackled but also suggest alternatives for the production of recombinant proteins with more of mammalian-like *N*-glycans in plants. By enlarging the spectrum of glycosyltransferases in plant suspension cultured cells, it was thus possible to produce endogenous glycoproteins with modified *N*-glycans. It

is then of interest to ascertain the efficiency of GT6 as a host for the production of foreign proteins with modified and defined glycosylation status.

3.5 Summary

To determine the extent of modification a human glycosyltransferase can render in the plant *N*-glycosylation machinery, tobacco BY2 suspension cultured cells were transformed with the full-length human galactosyltransferase gene placed under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter. The expression of β1,4-galactosyltransferase was confirmed by enzymatic activity assay, as well as by Southern and Western blotting. Structural analysis of glycans in transformed cells (GT6) proved the presence of galactose residues at the terminal non-reducing ends, indicating the successful modification of the plant cell *N*-glycosylation pathway. The galactosylated *N*-glycans account for 47.3 % of the total sugar chains, while high mannose type glycans bearing α1,2-linkages account for 44.7%, implying a lower efficiency in the conversion of high mannose type glycans to complex type. The dominant fucosylated and xylosidated type sugar chains in BY2 was absent in GT6, suggesting that GT6 cells could be used to produce glycoproteins without the highly immunogenic glycans typically found in plants. Results demonstrate the synthesis in plants of *N*-linked glycoproteins with modified and defined sugar chain structures similar to mammalian glycoproteins.

CHAPTER 4

STRUCTURAL MODIFICATION OF THE HORSERADISH PEROXIDASE (HRP) ISOZYME C PROTEIN IN GT6 CELLS

4.1 Introduction

The successful modification of the *N*-glycosylation pathway in tobacco cultured cells transformed with human β1,4-galactosyltransferase suggests that the introduced mammalian glycosyltransferase could function as part of the plant cell *N*-linked processing pathway. However, the efficiency of the GT6 cell line to modify the glycan structure of a foreign glycoprotein remains to be elucidated. Tobacco GT6 cells were, thus, transformed with the gene coding for the horseradish peroxidase isozyme C (HRP) protein, a glycoprotein whose glycan structures is well studied.

Horseradish peroxidase isozyme C (HRP, E.C. 1.11.17) was used as a model glycoprotein. Previous analyses of *N*-linked glycans in HRP showed eight *N*-linked oligosaccharides that accounts for 22-27% of its molecular mass (Smith et al., 1990; Kurosaka et al., 1991; Hiner et al., 1995; Tams and Welinder, 1995; Yang et al., 1996, Gray et al., 1998; Takahashi et al., 1998). The glycans were reported to be highly heterogeneous, but four glycans, namely $Man_3Fuc_1Xyl_1GlcNAc_2$, $Man_2Fuc_1Xyl_1GlcNAc_2$, $Man_3Fuc_1GlcNAc_2$, and $Man_3Xyl_1GlcNAc_2$, represent greater than 95% of the carbohydrate moiety with the most abundant glycan (60-80%) being $Man_3Fuc_1Xyl_1GlcNAc_2$ [M3FX] (Kurosaka et al., 1991; Tam and Welinder, 1995; Yang et al., 1996). HRP contains no detectable β1,4-linked galactose. The most extensively branched chain contains only one terminal GlcNAc residue linked to

the trimannosyl core. The relative molar ratio of this extended glycan structure varies from less than 1.0 to 2.7% (Harthill and Ashford, 1992; Takahashi et al., 1998).

4.2 Materials and Methods

4.2.1 Plant transformation

The HRP expression plasmid, pBIHm-HRP, was a kind gift of Dr. A. Shinmyo (Nara Institute of Science and Technology). The binary vector carries a kanamycin (npt II) and a hygromycin (hpt) resistant gene (Katsuya et al., unpublished results). HRP cDNA was placed under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter and the nopaline synthase terminator (nos-t; Fig. 26). Plasmid pBIHm-HRP was mobilized into *Agrobacterium tumefaciens* strain EHA101 by triparental mating (Bevan, 1984). Wild type tobacco BY2 suspension cultured cells (*Nicotiana tabacum* L. *cv.* Bright Yellow 2) and transgenic GT6 cells with the β1,4-galactosyltransferase gene were transformed with the HRP gene as described by Rempel and Nelson (1995). Transformants were selected on modified Linsmaier and Skoog medium (LS, see Chapter 3) supplemented with 20 μg/ml hygromycin.

4.2.2 Preparation of crude cell extracts for isoelectric focusing, peroxidase activity assays, and column chromatography

Tobacco suspension cultured cells were homogenized, and the homogenate centrifuged at 12,000 rpm, for 20 min, 4°C. The resulting supernatant was used as crude enzyme solution. Protein concentrations were determined according to the method of Lowry et al. (1951).

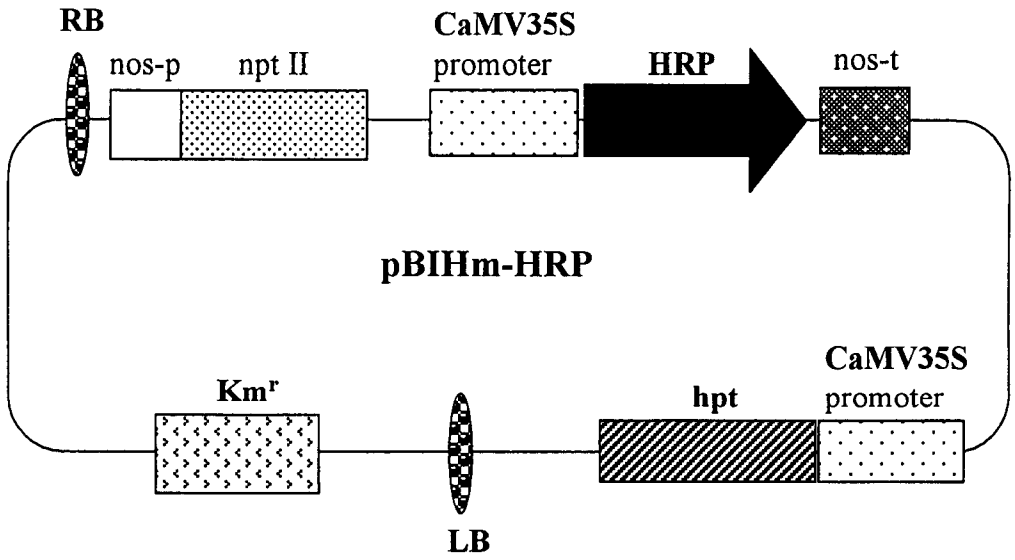


Fig. 26. The binary vector, pBIHm-HRP, carrying the horseradish peroxidase isozyme C (HRP) cDNA under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter and the nopaline synthase terminator (nos-t). The vector has a kanamycin resistant gene (npt II), and a hygromycin resistant gene (hpt). The neomycin phosphotransferase II (npt II) gene is under the control of the nopaline synthase promoter (nos-p). RB/LB, denotes right and left border, respectively.

4.2.3 Isoelectric focusing and peroxidase activity staining

Isoelectric focusing (IEF) was performed as described (BioRad Model 111 mini IEF cell) and electrophoresed with the corresponding standard and pI values: pycocyanin (4.45, 4.65, 4.75), equine myoglobin (7.00 and 7.10), and hemoglobin C (7.50). For peroxidase activity staining, IEF gels were incubated in a 4:1 solution of buffer A and B. Buffer A consists of 150 mM NaCl in 50 mM Tris-HCl (pH 7.5). Solution B consists of 0.3% 4-chloro-1-naphthol, 0.1% H_2O_2 and 85% methanol. Stained gels were visualized at room temperature and air-dried.

4.2.4 Immunoblotting and peroxidase activity assay

Cell homogenates (crude enzyme) or purified HRP were solubilized in Laemmli sample buffer (Laemmli, 1970), subjected to SDS-PAGE (10%), blotted onto nitrocellulose (Schleicher and Schuell) or PVDF membranes (BioRad), cut into strips and probed with several anti-HRP antiserum or an HRP-conjugated RCA_{120} lectin. The rabbit anti-HRP antibody and alkaline-phosphatase conjugated anti-rabbit IgG (Dako) were used for Western blotting. Rabbit polyclonal antiserum against HRP and goat horseradish-peroxidase conjugated anti-rabbit IgG (Sigma) were used for purified HRP preparations. Blots were also probed with a polyclonal antiserum raised against carrot cell wall β-fructosidase, an antiserum specific for plant complex glycans (Lauriere et al., 1989). For lectin staining, horseradish-peroxidase labeled RCA_{120} (Honen Co.) was preincubated for two hours at room temperature with or without 0.7 M galactose (Faye and Chrispeels, 1985; Hollister et al., 1998). Lectin and antibody binding was visualized using POD immunostain kit (Wako). Pretreatments of purified HRP with *Diplococcus pneumoniae* β-galactosidase

(Boehringer Mannheim) prior to lectin blotting was done as described (Jarvis and Finn, 1996; Hollister et al., 1998).

Peroxidase enzyme reactions were carried out as reported (Kawaoka et al., 1994).

4.2.5 Purification of HRP

Crude cell lysates obtained from 50 g homogenized 7-day old cultures were applied onto CM Sepharose FF (Pharmacia) column (1 x 10 cm) equilibrated with 10 mM sodium phosphate buffer (pH 6.0), eluted and the absorbance of the eluate measured at 403 nm. Pooled fractions were concentrated by ultrafiltration (MW cut-off: 10,000, Advantec), dialyzed in 50 mM sodium phosphate buffer (pH 7.0) and applied onto an equilibrated affinity column (1 x 10 cm) of benzhydroxamic acid-agarose (Kem EmTec). Columns were washed with 15 bed volumes of 50 mM sodium phosphate buffer, pH 7.0 and the bound HRP was eluted with 0.5 M boric acid. The resulting peroxidase-active fractions were pooled, dialyzed and concentrated.

4.2.6 Characterization of purified HRP

Purified enzyme solutions were incubated for 5 min at varying temperatures (25 to 95°C) and pH (4 to 10), inactivated by cooling on ice, and the enzyme activity calculated in percentage relative to the activity at pH 7.0, 25°C. The buffers used for determining the effect of pH were: 0.2 M sodium acetate (for pH 4.0-5.6), 0.2 M sodium phosphate (pH 6.0-8.0), and 0.2 M Tris-HCl (pH 8.0-10.0).

4.2.7 Affinity chromatography of purified HRP on RCA_{120}-agarose

Purified HRP from wild type and transformed cells were applied onto an RCA_{120}-agarose (Wako) column as described in Chapter 3. The bound proteins were fractionated onto 10% SDS-PAGE gels and visualized by either silver staining or blotted onto nitrocellulose membranes and probed with an anti-HRP antibody or horseradish-peroxidase labeled RCA_{120} lectin.

4.2.8 Structural analysis of galactosylated glycans

Dialyzed, purified HRP samples were hydrazinolyzed, labeled with 2-aminopyridine (PA) and applied onto Sephadex G-25 column as in the previous chapter. PA-labeled oligosaccharides were separated from free PA by fluorescence spectrometry (excitation and emission wavelengths: 310 and 400 nm, respectively; Hitachi 650-105 Fluorescence Spectrophotometer) and applied onto an RCA_{120}-agarose column (Wako). The eluates were assayed by fluorescence (Hitachi F2000 Fluorescence Spectrophotometer) and fractions containing PA-oligosaccharides were dialyzed against distilled water, concentrated and fractionated on reversed-phase (RP-) HPLC. PA-sugar chains were further purified in size-fractionation (SF-) HPLC for purification. Characterization of the PA-sugar chain was done by a combination of 2D sugar chain mapping and exoglycosidase digestions. Elution conditions in both RP- and SF- HPLC, and the preparation of standards are as described in Chapter 3.

4.3 Results

4.3.1 Production of HRP in tobacco suspension cultured cells

The plasmid pBIHm-HRP was used to transform wild type (BY2) and transgenic (GT6) tobacco suspension cultured cells. Production of HRP analyzed from cell extracts by isoelectric focusing and peroxidase activity staining revealed a band at pI 7.8 for both transformed BY2 and GT6 cells. No corresponding band was obtained from non-transformed wild type (WT) cells (Fig. 27A). The results suggested that the produced peroxidase is a neutral isoenzyme as previously reported (Fujiyama, et al., 1988). Similar results were obtained from transgenic tobacco and *Arabidopsis* plants expressing pBIHm-HRP (Katsuya, et al., and unpublished results). Western blotting further confirmed the presence of HRP as shown in Fig. 27B. Peroxidase activity in transgenic plants was four to five-times higher than that of the wild type cells (Table 6).

Table 6. Peroxidase activity from crude cell lysates of transformed tobacco suspension cultured cells.

Clone no.	Activity [U/mg-protein]
WT-HRP-1	10.3
5	11.3
7	12.6
GT-HRP-4	11.1
5	9.35
8	9.47
Wild type (BY2)	2.49

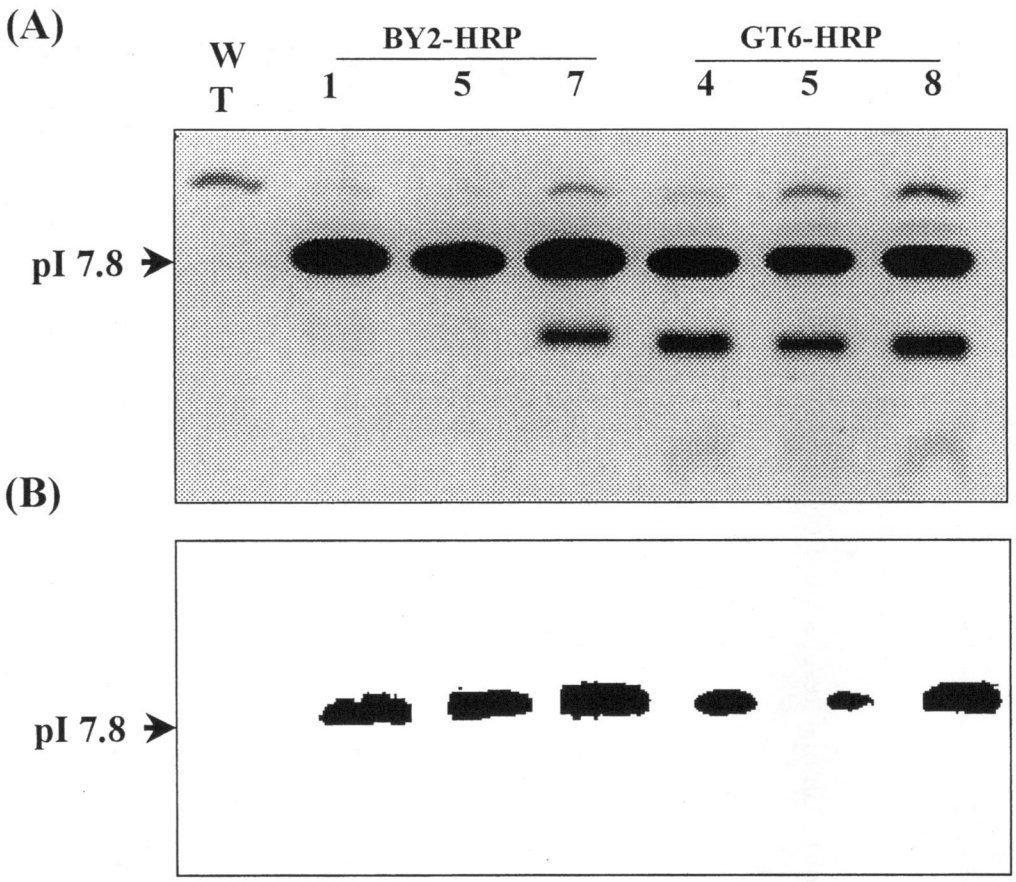


Fig. 27. Production of HRP in transgenic suspension cultured cells. Crude cell lysates were analyzed by isoelectric focusing, stained for peroxidase activity **(A)**, and transferred to PVDF membrane for Western blotting **(B)**. WT: wild type; BY2-HRP 1, 5, 7: cell line for BY2 cells transformed with HRP gene. GT6-HRP 4, 5, 8: cell line for GT6 cells transformed with HRP gene. The number and arrow to the left indicate the expected isoelectric point.

4.3.2 Isolation of oligosaccharides from HRP that bound to RCA_{120}

Affinity chromatography and lectin staining of purified HRP eluted from RCA_{120} agglutinin showed that only the purified HRP produced from GT6 transformed cells bound to the column (Fig. 28). Lectin binding was greatly reduced by pre-incubating the lectin with excess galactose (Fig. 28-III). Exoglycosidase digestion of purified HRP with *D. pneumoniae* β-galactosidase also precluded RCA_{120} binding (Fig. 28-II). These results indicated the presence of a terminal β1,4-linked galactose residue on the HRP glycoprotein from GT6 cells. The absence of RCA_{120}-bound glycoproteins from BY2-HRP cells (Fig. 28-I) suggest that the cells do not have HRP glycans with terminal β1,4-linked galactose.

4.3.2 Structural analyses of HRP sugar chains eluted from RCA_{120}

PA-derivatives obtained from reversed-phase HPLC (Fig. 29) were collected and re-chromatographed in size-fractionation HPLC (data not shown). No peaks in SF-HPLC were obtained from RP-HPLC fractions eluted earlier than 20 minutes, suggesting a run-through fraction containing by-products during hydrazinolysis. Reversed-phase HPLC of PA-labeled RCA_{120}-purified GT6-HRP, however, have a single peak at about 45 minutes, while no detectable peak was obtained from BY2-HRP fractions (Fig. 29). The oligosaccharide from GT6-HRP was found to be homogenous on size-fractionation HPLC. Two dimensional mapping analysis, and co-chromatography with standard sugar chains, showed that the oligosaccharide corresponded with the previously identified $Gal_1GlcNAc_1Man_5GlcNAc_2$. Further confirmation of the structure was done by sequential exoglycosidase digestions (Fig. 30).

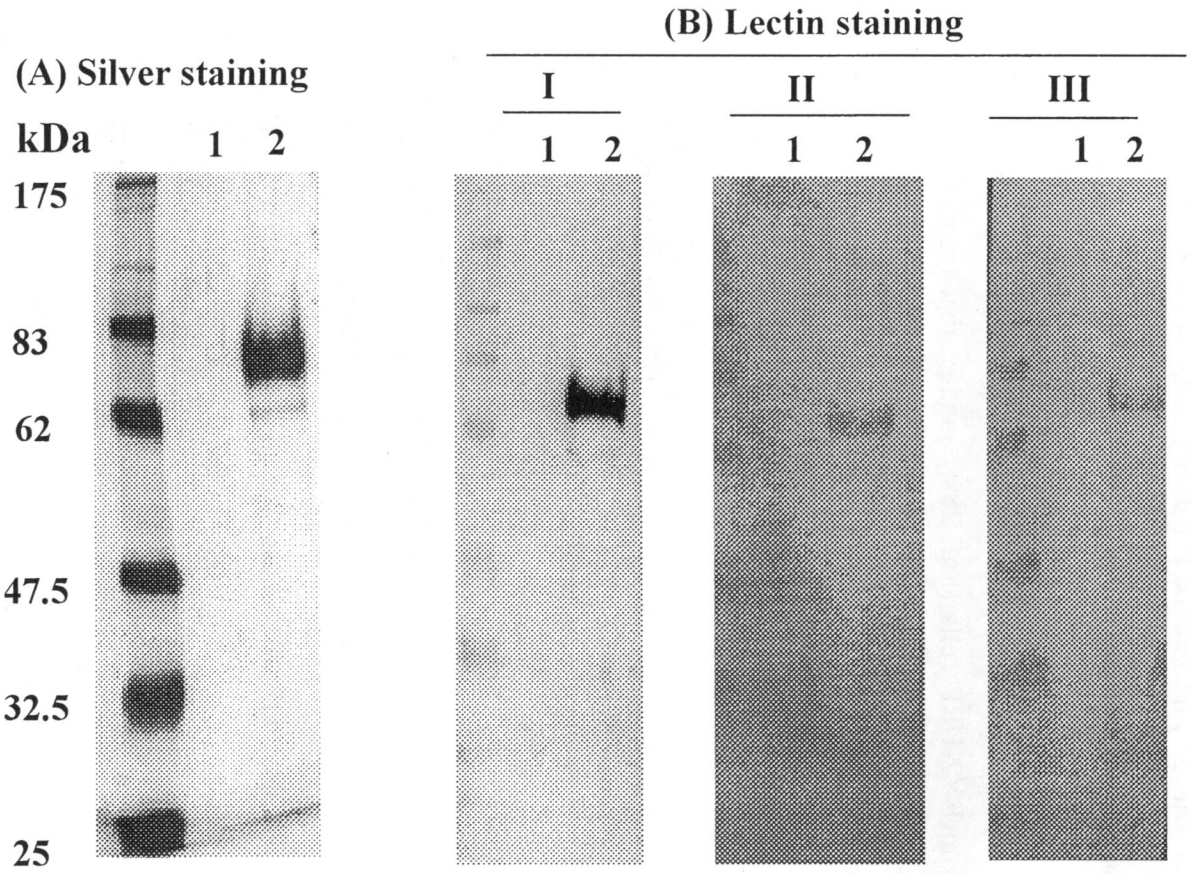


Fig. 28. Detection of galactosylated HRP using *Ricinus communis* 120 (RCA_{120}) affinity chromatography. Purified peroxidase from BY2-HRP and GT6-HRP were loaded onto an RCA_{120} column, eluted with 0.2M lactose and fractionated on a 10% SDS/PAGE. Proteins were either visualized by silver staining **(A)** or lectin (RCA_{120}) staining **(B)**. The filters for lectin staining were cut into strips and probed with either RCA_{120} preincubated in buffer alone (I and II) or buffer containing excess galactose (III). Alternatively, the HRP may have also been treated with *Diplococcus pneumoniae* β-galactosidase prior to SDS-PAGE (II). Lane 1, collected fraction from BY2-HRP; Lane 2, collected fraction from GT6-HRP. Numbers on the left indicate the position and sizes (in kDa) of protein standards.

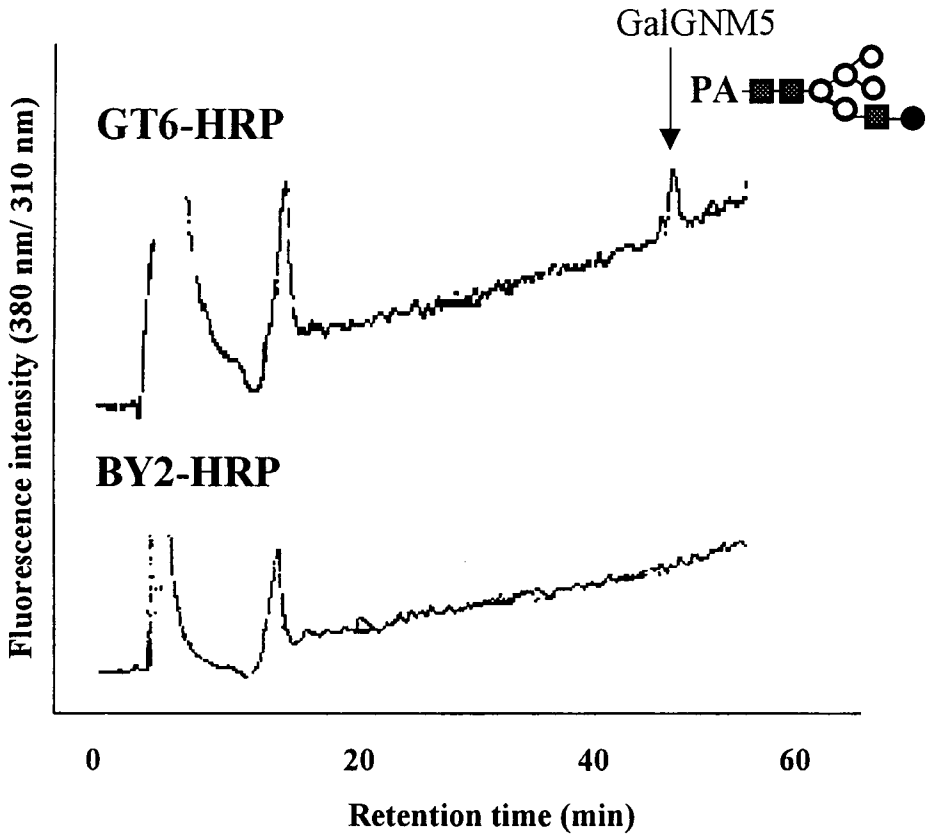


Fig. 29. Reversed-phase HPLC of PA-sugar chains from purified HRP after RCA_{120} affinity chromatography. PA-sugar chains were eluted from Cosmosil 5C18-P column (6 x 250 mm) by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 15% for 60 min at a flow rate of 1.2 ml/min. Excitation and emission wavelengths were 310 nm and 380 nm, respectively. Only one major peak was observed from GT6-HRP. The PA-labeled sample corresponded to the expected elution position of $Gal_1GlcNAc_1Man_5GlcNAc_2$ (GalGNM5).

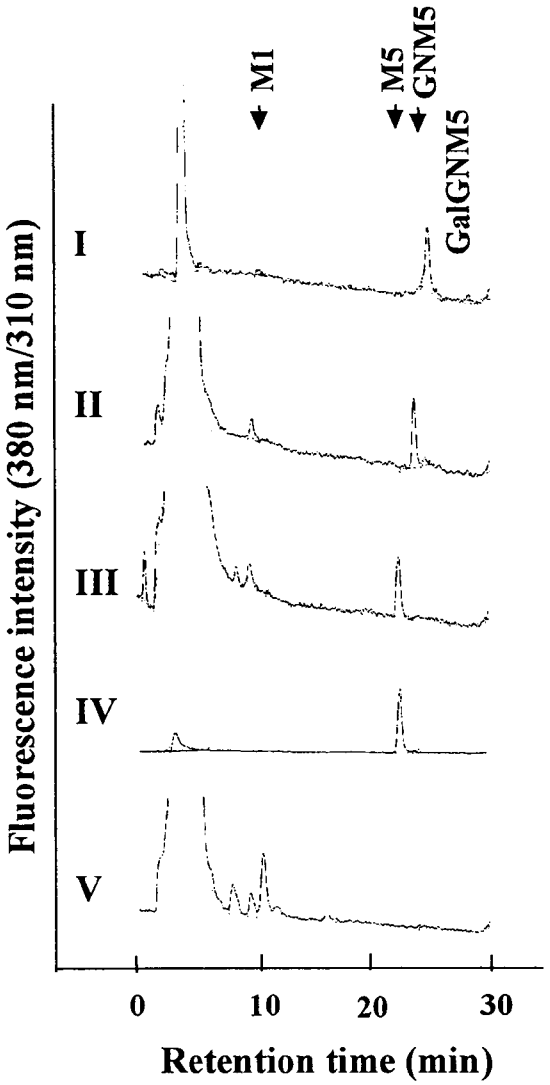


Fig. 30. SF-HPLC profiles of the major peak obtained in Fig. 28. PA-sugar chains were eluted from Asahipak NH2P-50 column (4.6 x 250 mm) by increasing the water content in the water-acetonitrile mixture from 30% to 50% for 25 min at a flow rate of 0.8 ml/min. **I**. Elution position of the native PA-sugar chain; **II**. β-galactosidase digest of I; **III**. β-*N*-acetylglucosaminidase digest of II; **IV**. α1,2 mannosidase digest of III; **V**. jackbean α-mannosidase digest of III.

β-Galactosidase digestion (*D. pneumoniae*) of the PA sugar chain resulted to a change in elution position which corresponded to the standard $GlcNAc_1Man_5GlcNAc_2$ (Fig. 30-II), suggesting the removal of one β1,4-linked galactose residue. Further digestion of the product with *diplococcal* β-*N*-acetylglucosaminidase yielded a sugar chain that matched the elution position of $Man_5GlcNAc_2$ (Fig. 30-III), hereby confirming the loss of a GlcNAc residue β1,2-linked to one non-reducing end mannose residue. Based on the *N*-linked processing pathway (Lerouge et al., 1998; Rayon et al., 1998) the removed GlcNAc residue is expected to be attached to the α1,3-mannose linked to the β-mannosyl residue. The linkage position of the GlcNAc residue was verified by α1,2 mannosidase digestion of the resulting $Man_5GlcNAc_2$ (M5) sugar chain. As expected, there was no shift in elution position (Fig. 30-IV) confirming that the obtained M5 has the expected structure of Manα1,6(Manα1,3)Manα1,6(Manα1,3)Manβ1,4GlcNAcβ1,4GlcNAc-PA. The sugar chain when trimmed with jackbean mannosidase corresponded to the known elution position of $Man_1GlcNAc_2$ (Fig. 30-V). Thus, the structure of the sugar chain corresponded to Manα1,6(Manα1,3)Manα1,6(Galβ1,4GlcNAcβ1,2Manα1,3)Man β1,4GlcNAcβ1,4GlcNAc ($Gal_1GlcNAc_1Man_5GlcNAc_2$). The structure of the galactosylated HRP *N*-linked glycan was expected based on the analysis of glycan structures in GT6 and the inferred influence of β1,4 galactosylation on the endogenous *N*-linked processing pathway.

The difference in glycosylation between BY2-HRP and GT6-HRP was also implied by immunodetection studies using rabbit anti-HRP (Fig. 31-A). HRP has an expected molecular mass of 42.2-44.0 kDa as determined by sequence analysis and

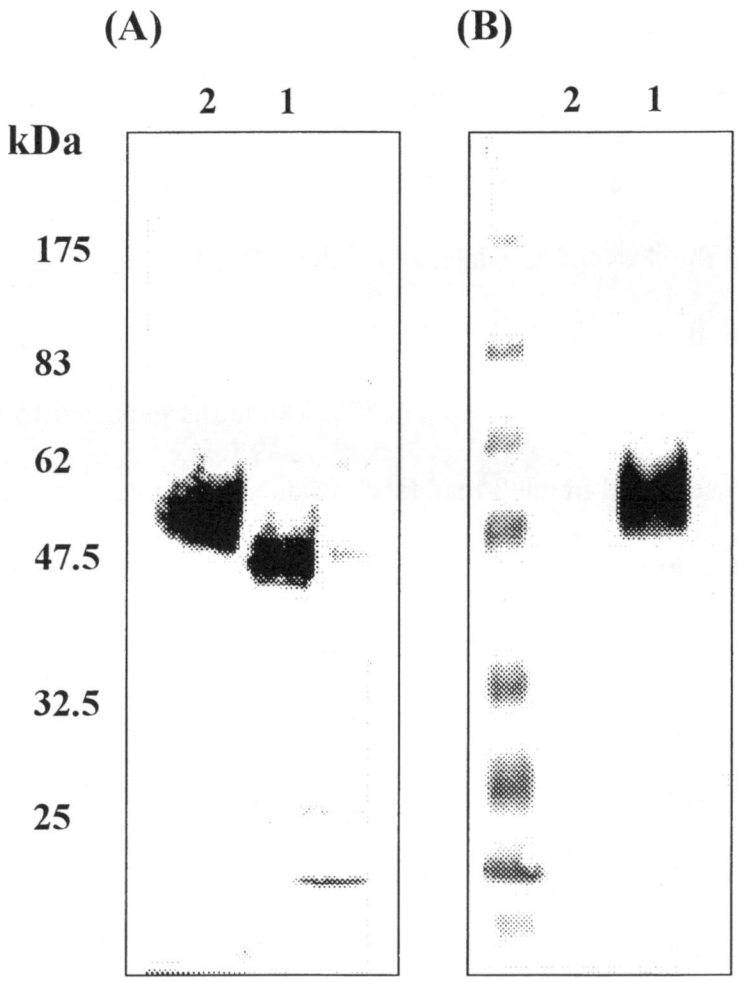


Fig. 31. Immunodetection of plant-specific complex glycans. Purified HRP was fractionated on SDS-PAGE, transferred to nitrocellulose and probed with rabbit anti-HRP **(A)** or with an antiserum specific for plant complex glycans **(B).** Lane 1, purified HRP protein extracted from BY2-HRP cells; Lane 2, RCA_{120}-bound HRP from GT6-HRP cells obtained by affinity chromatography. The position of molecular size markers (kDa) are indicated on the left.

electron spray spectrometry (Tams and Welinder, 1995). By SDS-PAGE, the purified HRP obtained from BY2 cells was estimated to be 47.5 kDa., whereas that from GT6 cells has an apparent molecular mobility corresponding to about 52 kDa., denoting differences in the extent of glycosylation.

The galactosylated *N*-glycans of HRP, analogous to the galactosylated glycans from GT6, did not react with the antiserum directed against the β1,2-xylose residue characteristic of plant *N*-glycans (Fig. 31-B), suggesting the absence of xylose residue. Xylose residues are implicated to be the likely cause of the unusually immunogenic nature of plant complex type glycans (Garcia-Casado et al., 1996).

4.4 Discusssion

The above results had substantiated that the HRP produced from GT6 was differentially glycosylated with resulting *N*-linked glycans containing terminal β1,4-linked galactose residue at one non-reducing end GlcNAc. The structure of the galactosylated *N*-glycan in HRP is reflective of the proposed alteration in the *N*-linked glycosylation pathway in tobacco cells brought about by the complementation of the human β1,4-galactosyltransferase. By analogy with the results obtained from GT6 endogenous glycoproteins, the galactosylated HRP *N*-glycans did not bind to the antiserum against β1,2-xylose residues, suggesting the absence of xylose. The ability of GT6 to synthesize foreign proteins with predicted and defined glycan structure establish definitively that GT6 cells can serve as an efficient production system for foreign glycoproteins bearing glycan structures more akin to mammalian type. In

this respect, the feasibility to enlarge the spectrum of glycosyltransferases in plant cells can augment the *N*-glycosylation machinery in plants.

Interestingly, differences in the *N*-glycan structures between BY2 and GT6 produced HRP did not affect the specific enzymatic activity of HRP (data not shown). Likewise, characterization of purified HRP from both cells revealed similar pH and broad temperature optima. These findings lend credence to previous studies of Tam and Welinder (1995) and Gajhede et al (1997) that glycosylation is not critical for HRP enzymatic activity. However, the specific activity of HRP from GT6 at 90°C was 40% of the original activity while that from BY2 was 60%. These results suggest that the glycan modification in GT6-HRP may have affected enzyme stability at higher temperature. Further studies using BY2 and GT6 to produce other galactosylated and non-galactosylated versions of recombinant glycoproteins may unravel the role of terminal galactose residues in mammalian glycoproteins and/or the significance of *N*-glycans in plant glycoproteins.

4.5 Summary

Tobacco cells harboring the human β1,4-galactosyltransferase gene (GT6) were transformed with the gene coding for the horseradish peroxidase isozyme C. Analysis of the HRP protein by activity staining, Western blotting and enzymatic assay indicated that the introduced HRP gene was expressed and translated into a mature protein. The presence of HRP *N*-glycans with terminal galactose residue in GT6-HRP transformed cells was inferred from lectin staining, affinity chromatography and structural analyses of PA-labeled RCA_{120} bound sugar chains. From exoglycosidase digestions and 2D sugar chain mapping, the deduced structure

of the galactosylated sugar chain was $Gal_1GlcNAc_1Man_5GlcNAc_2$. Thus, GT6 cells was proven to be efficient host for producing of foreign glycoproteins with extended glycan structure.

CHAPTER 5

GENERAL CONCLUSION AND FUTURE PROSPECTS

Glycosylation, one of the key steps in the production of functional glycoproteins, is receiving considerable attention for the production of beneficial therapeutic glycoproteins in heterologous hosts. To date, several *N*-glycosylated proteins from mammals have been expressed in transgenic plants, though issues on *in vivo* biological activity and immunological properties still remain (Owen and Pen, 1996; Kusnadi et al., 1997). Mutants created through knocking out genes involved in glycosylation have blocked the extension of *N*-glycans with undesirable residues, such as xylose, albeit the glycoproteins produced lack carbohydrate moieties as well as linkages found in complex *N*-glycans of mammalian cells (Lerouge et al., 1998; Rayon et al., 1998; Rayon et al., 1999). Thus, new strategies for an effective modification of *N*-glycosylation in plants is deemed essential to achieve an appropriate control of the glycosylation profile towards tailoring glycan structures akin to mammalian type.

A model host system where targeted *N*-glycosylation modification can be effective should be able to provide ample material for structural analyses and have glycan structures which can be further extended with mammalian glycosyltransferases (Lerouge et al., 1998). The *N*-glycans in the glycoproteins of wild type tobacco BY2 cells offers just the right prerequisite for a model system. Of the total sugar chains, 48% have glycan structures with GlcNAc residues at the non-reducing ends and 41% are comprised of M3FX motif, a probable derivative of GN1M3FX and GN2M3FX (Gomord and Faye, 1996; Lerouge et al., 1998; Rayon et al., 1998). The studies reflect the activity of *N*-acetylglucosaminyl transferases I and II, and the amount of

sugar chains bearing terminal GlcNAc residues permissible for further extension of *N*-linked oligosaccharides.

The elaboration of the *N*-linked oligosaccharides with terminal GlcNAc residues in mammalian cells is achieved by galactosyltransferases, fucosyltransferases and sialyltransferases forming mature complex oligosaccharides (Kornfeld and Kornfeld, 1985; Kukuruzinska and Lennon, 1998). Thus, the consequence of complementing one of the respective enzymes to the *N*-glycosylation system of BY2 should yield structurally similar mature complex oligosaccharides, if the ordered and well-orchestrated manner of processing in the synthesis of *N*-linked glycans is to be considered. Indeed, transformed BY2 cell (GT6), harboring human β1,4-galactosyltransferase gene, produce glycoproteins with *N*-glycans bearing β1,4-linked galactose residues accounting for 47.3% of the total sugar chains. The absence of the dominant M3FX plant glycan and the reduction of the normally robust fucosylation and xylosylation (92.5% to 6.6%) events presuppose that human β1,4-galactosyltransferase may competitively inhibit the actions of the other enzymes involved in the maturation of typical plant complex type *N*-glycans. Thus, the presence of the hybrid glycan, GalGNM5; and the absence of GlcNAc residues at the Manα1,6 arm in the galactosylated GalGNM3, may reflect the low activity of Man II and GnT-II; and/or the inability of these glycosidase and glycosyltransferase to compete with galactosyltransferase. In addition, the α1,3-fucose and β1,2-xylose residues characteristic of plant *N*-glycans were not found on the identified galactosylated complex type glycans, suggesting the capability of the galactosyltransferase to compete with xylosyl- and fucosyl transferases.

Given these possibilities, it is of interest to speculate on the localization of the galactosyltransferase in the plant Golgi. Moreover, it might not be possible to conclude outright the possible conservation of glycosyltransferases targeting mechanisms between plant and mammalian cells as proposed by Wee et al (1998). In mammalian cells, β1,4-galactosyltransferase is retained in the trans-Golgi (Shaper and Shaper, 1992; Schwientek et al., 1996). Ideally, β1,4-galactosyltransferase is expected to extend sugar chains only after the trimming by mannosidase II, the action of *N*-acetylglucosaminyl transferase II, and xylosyltransferase or fucosyltransferase, as glimpsed from the inferred processing of large complex-type plant *N*-glycans (Lerouge et al., 1998); or only after the action of *N*-acetylglucosaminyl transferase II in mammalian cells (Kukuruzinska and Lennon, 1998). However, *N*-glycans in transformed cells seems to be acted upon directly by β1,4-galactosyltransferase after the action of GnT-I and to a lesser extent after trimming with mannosidase II, suggesting perhaps a different Golgi subcompartment retention of the glycosyltransferase. Direct evidence of a specific subcompartmentalization or retention in the Golgi apparatus of the glycosyltransferase, however, was not obtained. Further investigations will be required to understand in depth the targeting mechanisms, the specificity and the regulation of the glycosyltransferase in the transformed tobacco cells. Nevertheless, the strategy rendered successful modification of the tobacco *N*-glycosylation, with resultant complex type glycans similar to that of mammalian type and devoid of the undesirable β1,2-xylose and α1,3-fucose residues.

So far, few studies have conducted structural analysis of glycoproteins produced in transgenic plants and no definitive judgement can be made on how accurately the transgenic plants can glycosylate foreign glycoproteins (Kusnadi et al.,

1997). While an authentic human glycosylation may be viewed as the ultimate goal, it may also desirable to produce foreign glycoproteins with restricted and defined glycosylation status (Jenkins and Curling, 1994; Pen, 1996). The transformation of GT6 with horseradish peroxidase isozyme C (HRP) gene yielded glycoproteins with the expected galactosylated complex type glycan, thereby validating the efficiency of GT6 as a host for the production of foreign glycoproteins with defined glycan structures that differ significantly from plant complex type glycans. The fact that introduction of HRP gene to wild type BY2 and GT6 cells did not cause any significant differences in the growth rate, and that both transformed cell lines produce non-galactosylated and galactosylated HRP, respectively, make BY2 and GT6 competent hosts for studies dealing with functional comparisons of galactosylated *N*-glycans. Moreover, the absence of xylose and fucose residues on galactosylated sugar chains provides valuable insights on further manipulation of the plant glycosylation pathway.

The present study had provided a detailed description on the structural changes in the *N*-glycan structures and the effect of human β1,4-galactosyltransferase on the *N*-glycosylation pathway in tobacco cells. The glycoproteins, produced in the transformed cells, has the defined intended glycosylation status, though issues on compartmentation of glycosyltransferases and *N*-glycans still remains to be investigated. Nonetheless, the modification of *N*-glycosylation proved worthy for the production of glycoproteins not only devoid of undesirable sugar residues implicated to allergenic reactions in human but also possessing more extensively branched complex *N*-glycans analogous to mammalian type. Glycoproteins produced from GT6 cells would not need *in vitro* modification in eliminating the xylose and fucose residues, the glycosidases of which are not commercially available. Furthermore, the

availability of GalGNM5 and GalGNM3 can be a milestone on the production of glycoproteins with galactosylated and sialylated complex *N*-glycans typical to mammalian cells. The synthesis of sialylated proteins can be achieved either by further complementation of at least three enzymes into the *N*-glycosylation system of tobacco cells, or by synthetic addition (Wee et al., 1998). The galactose residue could readily serve as a basis for the *in vitro* synthesis of a mammalian-like glycan having sialic acid residues. The *in vivo* synthesis of sialylated proteins will require in addition to sialyltransferase, enzymes that can synthesize sialic acid, convert it to CMP-sialic acid and transfer the nucleotide sugar across the Golgi cisternal membrane (Chrispeels and Faye, 1996; Wee et al., 1998). Certainly, the expression system may also be broadly applicable to the production of other high-value proteins for veterinary and industrial use. This thesis, thereby, can be an initiative for the production of desired therapeutic glycoproteins and the heuristic approach for a thorough understanding of the biological functions of glycan structures.

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RELATED PUBLICATIONS

Palacpac, N.Q., Kimura, Y., Fujiyama, K., Yoshida, T., and Seki, T. 1999. Structures of *N*-linked oligosaccharides of glycoproteins from tobacco BY2 suspension cultured cells. *Biosci. Biotech. Biochem.* 63(1): 35-39.

Palacpac, N.Q., Yoshida, S., Sakai, H., Kimura, Y., Fujiyama, K., Yoshida, T., and Seki, T. Stable expression of human β1,4-galactosyltransferase in plant cells modifies *N*-linked glycosylation patterns. (accepted, *Proc. Natl. Acad. Sci. USA*)

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