Influence of Growth Conditions and Developmental Stage on *N*-Glycan Heterogeneity of Transgenic Immunoglobulin G and Endogenous Proteins in Tobacco Leaves

Ingrid J.W. Elbers, Geert M. Stoopen, Hans Bakker, Lucas H. Stevens, Muriel Bardor, Jos W. Molthoff, Wilco J.R.M. Jordi, Dirk Bosch, and Arjen Lommen*

State Institute for Quality Control of Agricultural Products (RIKILT), P.O. Box 230, NL–6700 AE Wageningen, The Netherlands (I.J.W.E., A.L.); Plant Research International, P.O. Box 16, NL–6700 AA Wageningen, The Netherlands (G.M.S., H.B., L.H.S., J.W.M., W.J.R.M.J., D.B.); and Laboratoire des Transports Intracellulaires-Centre National de la Recherche Scientifique 6037, IFRMP 23, Université de Rouen, 76821 Mont Saint Aignan, France (M.B.)

Plants are regarded as a promising system for the production of heterologous proteins. However, little is known about the influence of plant development and growth conditions on *N*-linked glycosylation. To investigate this, transgenic tobacco (*Nicotiana tabacum* cv Samsun NN) plants expressing a mouse immunoglobulin G antibody (MGR48) were grown in climate rooms under four different climate conditions, i.e. at 15°C and 25°C and at either low or high light conditions. *N*-glycans on plantibodies and soluble endogenous proteins were analyzed with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS). Antibodies isolated from young leaves have a relatively high amount of high-mannose glycans compared with antibodies from older leaves, which contain more terminal *N*-acetylglucosamine. Senescence was shown to affect the glycosylation profile of endogenous proteins. The relative amount of *N*-glycans without terminal *N*-acetylglucosamine increased with leaf age. Major differences were observed between glycan structures on endogenous proteins versus those on antibodies, probably to be attributed to their subcellular localization. The relatively high percentage of antibody *N*-glycan lacking both xylose and fucose is interesting.

N-Linked glycans are sugar structures, which are covalently attached to asparagin residues of glycoproteins. Biogenesis of *N*-linked glycans (Schachter, 1991) begins with the synthesis of a lipid-linked oligosaccharide moiety ($Glc_3Man_9GlcNAc_2$-) that is transferred en bloc to the nascent polypeptide chain in the endoplasmatic reticulum (ER). Through a series of trimming reactions by exoglycosidases in the ER and cis-Golgi compartments, the so-called "high-Man" ($Man_9GlcNAc_2$ to $Man_5GlcNAc_2$) glycans are formed. The formation of complex type glycans subsequently starts with the transfer of the first GlcNAc onto $Man_5GlcNAc_2$ and further trimming by mannosidases to form GlcNAc-$Man_3GlcNAc_2$. Complex glycan biosynthesis continues while the glycoprotein is progressing through the secretory pathway with the transfer in the Golgi apparatus of the second GlcNAc residue as well as other monosaccharide residues onto the GlcNAc-$Man_3GlcNAc_2$ under the action of several glycosyl transferases. Plants and mammals differ with respect to the formation of complex glycans. In plants, complex glycans are characterized by the presence of β(1,2)-Xyl residues linked to the Man-3 and/or an α(1,3)-Fuc residue linked to GlcNAc-1, instead of an α(1,6)-Fuc residue linked to the GlcNAc-1 (Lerouge et al., 1998). Plant glycans lack the β(1,4)-Gal and terminal α(2,6) NeuAc residues often found on mammalian glycans. Exoglycosidases are known to rapidly trim glycoproteins stored in plant vacuoles leading to truncated $Man_3XylFuc$-$GlcNAc_2$-type complex glycans (Vitale and Chrispeels, 1984; Lerouge et al., 1998). Complex glycans carrying terminal GlcNAc and possibly other sugar residues attached to GlcNAc are often found on extracellular plant glycoproteins and such residues are therefore indicative for secretion to the apoplast (Fitchette-Lainé et al., 1997; Melo et al., 1997). Due to steric constraints imposed by the three-dimensional structure of individual glycoproteins as well as to differences in routing of glycoproteins and compartmentalization of enzymes involved in *N*-glycan processing, any of the above-described conversions may not go to completion. This explains for a large part the enormous heterogeneity found in *N*-glycan structures in eukaryotic cells, even on a single polypeptide (Sturm et al., 1987).

Plants are in potential cost-efficient and contamination-safe factories for the production of recombinant proteins. Many heterologous proteins—including monoclonal antibodies—have successfully been produced in plants (Miele, 1997; Hood and Jilka,

* Corresponding author; e-mail a.lommen@rikilt.wag-ur.nl; fax 31–317–417717.

1999). The produced proteins can serve in food, industrial, and medicinal applications by using the whole plant parts, plant extracts, or the purified form. Particularly for pharmaceutical applications, the quality and product homogeneity are of major importance. A very important quality aspect concerning many commercially interesting proteins are the *N*-linked glycans, which are covalently attached to an Asn in the protein backbone. The specific composition and structures of these sugar oligomers are crucial for the biological activity, stability, solubility, immunogenicity, and plasma clearance characteristics of many glycoproteins (Varki, 1993).

It has been documented that physiological and environmental factors can have profound effects on glycosylation in mammals (Malhotra et al., 1995; van Dijk et al., 1995). In vitro cultured cell lines and tissue cultures from various origins exhibit changes in glycan formation upon changes in the culture media (Andersen and Goochee, 1994). Such effects of culture parameters on glycosylation are of biotechnological relevance when the cultures are used; for example, for mAb production (Wright and Morrison, 1997). Also, in vivo changes in humans on glycosylation are observed in association with a number of physiological states. Serum IgG of patients with rheumatoid arthritis, Crohn's disease, and tuberculosis have a higher than normal proportion of *N*-glycans that lack Gal and consequently terminate in GlcNAc residues (Axford, 1998). The distribution of these different glycoforms changes not only with disease severity but also with age. During pregnancy, the galactosylation of serum IgG glycans increases both in normal persons as well as in patients mentioned above (Pekelharing et al., 1988). Mammalian cells apparently can adapt glycosylation as a result of changes in the environment and in physiology.

Relatively little is known about the effects of environmental and developmental conditions on the quality of recombinant proteins produced by plants grown in a controlled manner. During tissue senescence and during stress, catabolic processes are induced to remobilize nutrients for transport to other plant parts or when an increased capacity for synthesis of stress gene products is required. The induction of these processes can be triggered by a number of external and internal factors (for review, see Smart, 1994; Buchanan-Wollaston, 1997; Noodén et al., 1997). We recently obtained experimental evidence that the integrity of recombinant proteins can be affected by the developmental stage of the plant tissue. The heavy chain of transgenic expressed IgG in tobacco (*Nicotiana tabacum* cv Samsun NN) was found to be degraded during leaf senescence (Stevens et al., 2000). Here, we present an extension of this study, in which we investigated whether the adaptation of plant cells to some changes in environment and physiology is reflected in *N*-glycosylation. The *N*-linked glycans of both soluble endogenous glycoproteins as well as of recombinantly mouse antibody from leaves of transgenic tobacco plants of different age and grown under different conditions were analyzed. The results are relevant from the biotechnological perspective if consistency in quality of recombinantly expressed glycoproteins in tobacco plants is required.

RESULTS

Effect of Growth Conditions and Developmental Stage on the Ratio Complex/High-Man *N*-Glycans

The ratio complex/high-Man *N*-glycans of proteins isolated from top, middle, and base leaves of the tobacco plants grown under four different conditions was calculated by using the relative peak intensities in matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) spectra. Of the total *N*-glycans exhibited by all of the endogenous protein samples, about 88% was of the complex and about 12% was of the high-Man type (Fig. 1A). The endogenous protein fraction is defined in "Materials and Methods." The consistency of these figures throughout all 12 samples implies that the growth conditions of the plants had no detectable effect on the ratio of complex and high-Man *N*-glycans present on these endogenous

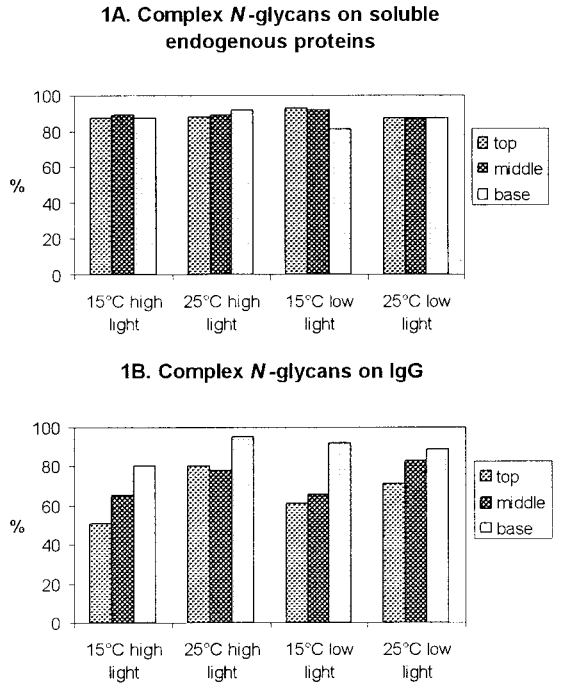


Figure 1. The relative amount of complex *N*-glycans present on soluble endogenous proteins (A) and on transgenic IgG (B) isolated from top, middle, and base leaves of tobacco plants that were grown under four climate conditions. Top leaves represent the youngest leaves at the stem top, middle leaves represent the leaves at the stem middle, and base leaves represent the oldest leaves at the stem base. The amounts are based on relative peak intensities in MALDI-TOF mass spectrometry (MS) spectra and are expressed as percentage of total *N*-glycans.

proteins. In addition, no significant differences related to growth conditions between the youngest leaves at the stem top ("top"), mature leaves at the stem middle ("middle"), and oldest leaves at the stem base ("base") could be observed other than a trend toward less terminal GlcNAc during leaf maturation and decreased fucosylation in the base leaves of plants grown at 25°C and high light intensity (Table II). The latter aspect probably represents a physiological stage beyond maturation because at these favorable growth conditions the basal leaves already started to become yellow.

In contrast to the endogenous protein samples, the IgG samples exhibited considerable differences (Fig. 1B). These differences mainly reflected a substantial effect of developmental stage of the leaves because the ratio complex/high-Man *N*-glycans of IgG showed a significant increase during senescence (alpha = 0.05 according to Friedman's statistics). The results also suggest that there may be a slightly suppressive effect of low temperature on the share of complex *N*-glycans in the total amount of *N*-glycans present on the transgenic antibody (Fig. 1B). However, it should be noted that this temperature effect, if present at all, may be due to different rates of development at different temperatures, as has previously been described in detail for these plants (Stevens et al., 2000).

N-Glycan Composition

The relative amounts of each glycan contributing to either the high-Man or the complex glycan fraction were calculated. Table I shows the *N*-glycan structures found on IgG and endogenous proteins. Figure 2 is a typical example of MALDI-TOF spectra obtained in this study. Differences in *N*-glycan compositions are given in Table II, as well as in Figures 3, 4, and 5.

The most obvious difference between the identity of *N*-glycans from IgG and from endogenous protein was the presence of the compounds 1,339 D ($GlcNAc_2Man_3GlcNAc_2$), 1,136 D (GlcNAc-$Man_3GlcNAc_2$), and 1,617 D ($GlcNAc_2$XylFuc-$Man_3GlcNAc_2$) in the IgG spectrum. These *N*-glycans were virtually absent in the spectrum of endogenous protein. The 1,339-D compound ($GlcNAc_2Man_3GlcNAc_2$) has been found previously by Bakker et al. (2000) on plant-produced IgG.

Composition of High-Man *N*-Glycans

Figure 3 shows the distribution of high-Man-type *N*-glycans of IgG and endogenous protein in top, middle, and base leaves, grown at 25°C and low light. In the top leaves there is a clear difference in relative amounts and distribution of high-Man structures on IgG as compared with endogenous protein. This manifests itself in an increased relative amount of high-Man *N*-glycans and the occurrence of Man-8 and Man-9 on IgG. Toward older leaves the differences seem to decline. The other growth conditions show comparable results, exhibiting the same trend. This suggests that the identity of the antibody glycoforms will depend on the age of the leaves from which they are isolated. It should be noted that Man-4 glycans were isolated from IgG in all leaf levels, whereas this type of glycan was not detected in the endogenous protein fraction.

Composition of Complex *N*-Glycans

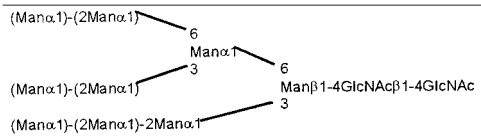
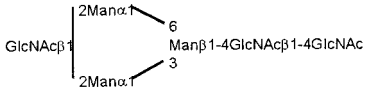
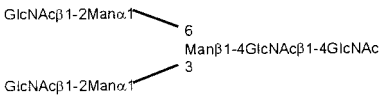
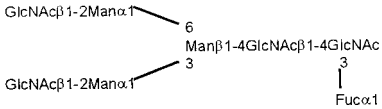
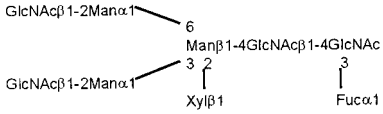
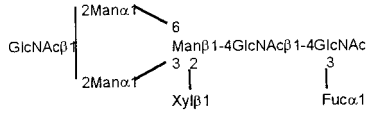
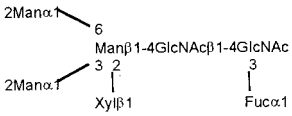
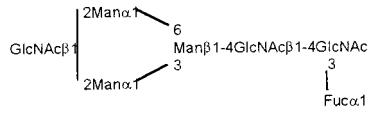
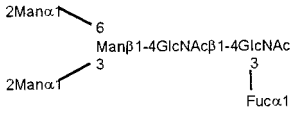
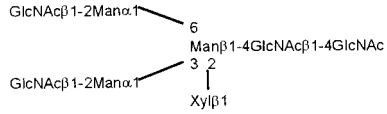
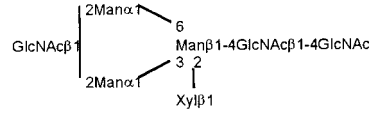
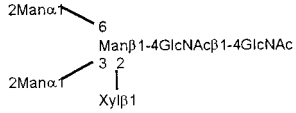
Figures 4 and 5 (top, middle, and base leaves, grown at 25°C, low light) show the differences in complex *N*-glycans between the endogenous protein fraction and IgG. Other growth conditions showed similar results. As shown in Figure 4, the complex *N*-glycan structures of IgG contain a remarkably higher level of terminal GlcNAc than the corresponding structures of endogenous protein. Compounds 1,339 D ($GlcNAc_2Man_3GlcNAc_2$), 1,136 D (GlcNAc-$Man_3GlcNAc_2$), and 1,617 D ($GlcNAc_2$XylFuc-$Man_3GlcNAc_2$) were identified in the IgG spectrum, whereas these *N*-glycans were virtually absent in the spectrum of glycans isolated from endogenous proteins. The presence of two terminal GlcNAc residues on one *N*-glycan structure of the endogenous protein was a minor fraction that was only sometimes found, whereas for IgG this type of *N*-glycan was the most abundant group in all leaves. Figure 5 indicates that in endogenous protein the majority of the complex structures bore both Xyl as well as Fuc residues, whereas structures that lack both Xyl and Fuc could not be detected. On the other hand, IgG samples clearly showed more diversity in complex *N*-glycans, which either miss Fuc, Xyl, or both sugars. In particular, the structure without Xyl and Fuc (i.e. $GlcNAc_2Man_3GlcNAc_2$) is interesting, because this demonstrates that plants—in principle—can produce antibodies that carry complex glycans (having terminal GlcNAc), which are mammalian compatible and probably nonimmunogenic.

DISCUSSION

In this study, the influence of plant development and growth conditions on *N*-linked glycosylation was examined. The possibility to draw conclusions based on the analysis of glycans isolated from total plant proteins or from pools of proteins is limited. Changes in glycosylation of individual proteins may go unobserved because they are averaged out. Furthermore, observed changes may have been caused, not by changes in the glycosylation machinery, but by the up- or down-regulation of biosynthesis of individual glycoproteins with a specific type of glycosylation (for example, in the case of vacuolar localization). Therefore, additional information may be gained by analyzing glycosylation of individual pro-

Table I. *Structures of high-Man and complex N-linked glycans isolated from soluble endogenous proteins and/or IgG*

Structures are in sequence according to the biochemical route.

Structures	Code	$(M+Na)^+$
	Man-9 Man-8 Man-7 Man-6 Man-5 Man-4	1,905 1,743 1,581 1,419 1,257 1,095
	A	1,136
	B	1,339
	C	1,485
	D	1,617
	E	1,414
	F	1,211
	G	1,282
	H	1,079
	I	1,471
	J	1,268
	K	1,065

teins. Plantibodies are a convenient tool to study such effects on individual proteins because they can be purified relatively easily in large enough quantities to allow detailed analysis from large numbers of plant samples. Our data demonstrate for the first time that developmental processes of plants, in this

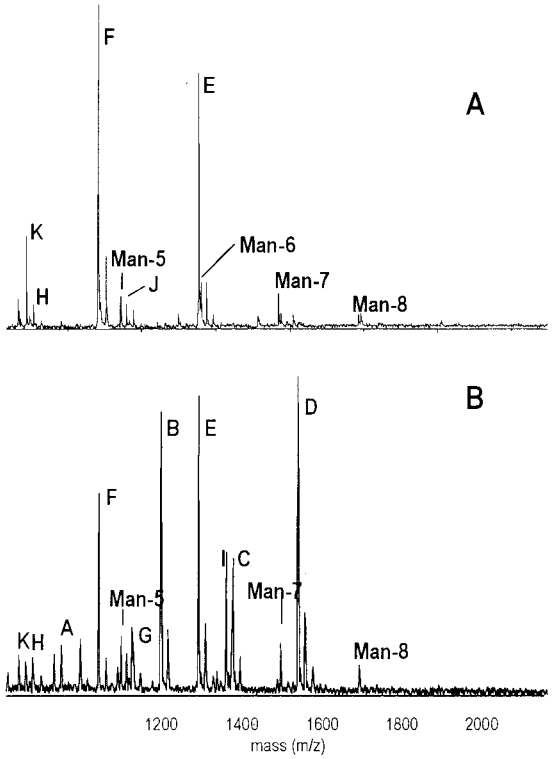


Figure 2. MALDI-TOF mass spectrum of $(M + Na)^+$ adducts of *N*-glycans isolated from soluble endogenous proteins (A) and IgG (B).

case senescence, can influence glycosylation. The different applied growth conditions did not significantly alter glycosylation.

Antibodies isolated from young, top leaves have a relatively high amount of high-Man glycans as compared with antibodies extracted from older base leaves, whereas IgG in older base leaves have more terminal GlcNAc (see Fig. 4 and Table II). This suggests that the high-Man antibodies are gradually processed to (terminal) GlcNAc-rich complex glycans during leaf maturation. Another less probable explanation could be that (terminal) GlcNAc-rich *N*-glycans provide for more stable antibodies (Deisenhofer, 1981) with regard to, for instance, protease activity in vivo during leaf maturation. The high incidence of GlcNAc residues on IgG glycans suggests that the cells excrete the majority of the antibodies. This is in agreement with localization studies that confirmed the presence of glycosylated plantibodies in the apoplastic space (Hein et al., 1991; van Engelen et al., 1994; De Wilde et al., 1998).

Senescence was shown to affect the glycosylation profile of endogenous proteins. The relative amount of *N*-glycans without terminal GlcNAc increased with leaf age (Fig. 4 and Table II; alpha = 0.05 according to Friedmans statistics). This finding indicates that *N*-glycans are probably being processed gradually during leaf maturation. The presence of complex *N*-glycans carrying xylosyl or fucosyl residues, but no terminal GlcNAc, is indicative for vac-

Table II. *Relative amounts of N-glycans isolated from soluble endogenous proteins and IgG at different growth conditions*

The amounts are based on relative peak intensities in MALDI-TOF MS spectra and are expressed as percentage of total *N*-glycans. T, Top; M, middle; B, base. EP, *N*-glycans on endogenous proteins; IgG, *N*-glycans on IgG. *, Not detectable.

%	15°C, Low Light						25°C, Low Light						15°C, High Light						25°C, High Light					
	T EP	T IgG	M EP	M IgG	B EP	B IgG	T EP	T IgG	M EP	M IgG	B EP	B IgG	T E	T IgG	M E	M IgG	B E	B IgG	T E	T IgG	M E	M IgG	B E	B IgG
Man-9	*	2	*	2	*	1	*	2	*	1	*	*	*	3	*	2	*	1	2	1	*	1	*	*
Man-8	*	9	*	8	2	2	*	5	2	4	2	2	*	11	*	10	*	4	3	4	*	4	*	*
Man-7	*	0	*	9	4	2	3	7	4	4	2	3	*	12	3	10	4	4	*	4	2	6	*	*
Man-6	6	7	6	6	7	2	5	5	5	*	5	2	6	9	7	6	6	4	4	3	5	4	*	*
Man-5	*	9	*	8	5	1	3	8	2	4	4	3	4	11	*	7	*	5	2	6	4	5	5	*
Man-4	*	2	*	2	*	1	*	2	*	2	*	1	*	3	*	*	*	2	*	3	*	1	*	*
A = $GlcNAcMan_3GlcNAc_2$	*	2	*	2	*	10	*	2	*	4	*	3	*	*	*	*	*	5	*	3	*	1	*	9
B = $GlcNAc_2Man_3GlcNAc_2$	*	19	*	11	*	14	*	10	*	10	*	16	*	17	*	5	*	8	*	13	*	8	*	14
C = $GlcNAc_2Man_3FucGlcNAc_2$	*	4	*	5	*	2	*	6	*	5	*	8	*	4	*	4	*	4	*	4	*	4	*	5
D = $GlcNAc_2Man_3XylFucGlcNAc_2$	*	10	*	17	2	19	8	19	*	23	*	18	*	13	*	16	*	20	*	23	*	20	*	32
E = $GlcNAcMan_3XylFucGlcNAc_2$	43	8	43	11	27	18	42	13	37	13	31	17	44	8	28	17	27	23	29	16	25	22	15	17
F = $Man_3XylFucGlcNAc_2$	41	10	41	11	38	14	29	12	39	14	39	11	37	5	46	17	50	14	49	13	52	15	20	14
G = $GlcNAcMan_3FucGlcNAc_2$	*	2	*	1	*	2	*	2	*	2	*	4	*	1	*	*	*	*	*	2	*	1	*	*
H = $Man_3FucGlcNAc_2$	*	*	*	2	4	*	*	1	*	1	4	2	*	*	*	*	*	*	*	*	*	*	*	*
I = $GlcNAc_2Man_3XylGlcNAc_2$	*	3	*	3	*	8	*	3	*	4	*	8	*	2	*	2	*	5	*	3	*	3	*	9
J = $GlcNAcMan_3XylGlcNAc_2$	*	2	*	1	3	2	3	1	4	3	3	2	4	1	3	*	*	*	2	2	1	1	14	*
K = $Man_3XylGlcNAc_2$	8	2	8	1	9	2	6	1	7	4	10	2	*	*	11	4	11	2	9	2	11	3	42	*
detection limit (%)	<2.8	<0.6	<4.5	<0.6	<1.1	<0.4	<2.2	<0.9	<1.8	<0.9	<1.3	<0.5	<2.2	<0.9	<2.4	<1.3	<1.1	<0.8	<0.9	<0.7	<0.8	<0.5	<3.4	<3.0

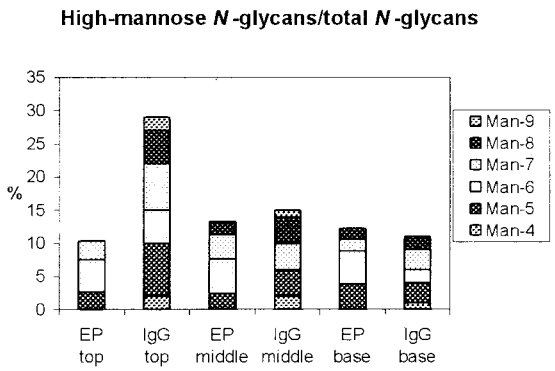


Figure 3. Distribution of high-Man *N*-glycans from soluble endogenous proteins and IgG isolated from the plants that were grown at 25°C and low light. The amounts are based on relative peak intensities in MALDI-TOF MS spectra and are expressed as percentage of total *N*-glycans.

uolar localization and suggests that the majority of the proteins in the endogenous protein fraction are vacuolar proteins. An alternative explanation to the idea of gradual processing could be that a shift in glycosylation of endogenous proteins in the older leaves toward vacuolar-type glycans with a low GlcNAc content is caused by increased induction of vacuolar proteins in these leaves.

Major differences were observed between glycan structures on endogenous proteins versus those on antibodies. These differences can be attributed to a large extent to their different subcellular localization. Particularly interesting is the relatively high percentage of antibody complex *N*-glycans that lack both Xyl and Fuc. These nonimmunogenic glycans still carry at least one terminal GlcNAc residue resulting in mammalian-like structures GlcNAc-$Man_3GlcNAc_2$ and $GlcNAc_2Man_3GlcNAc_2$. The lack of Xyl and Fuc and the preservation of terminal GlcNAc residues on antibodies may be caused by steric hindrance of xylosyltransferase, fucosyltransferase, and GlcNAcsidases due to the buried nature of the *N*-glycan as in natural IgG (Deisenhofer, 1981). The incomplete processing of antibodies alternatively may be caused by aberrant routing through the secretory pathway. These results are in line with results reported by Bakker et al. (2000). Cabanes-Macheteau et al. (1999), however, did not find structures lacking Fuc and Xyl. They defined complex glycans as the fraction not bound to concanavalin A. Non-xylosylated complex plant glycans, however, are retained by concanavilin A (Wilson and Altmann, 1998). The presence of terminal GlcNAcs on the tri-Man structure recognized by concanavilin A does not inhibit binding (Dam et al., 1998). In our study, no fractionation of *N*-glycans was undertaken. Furthermore, their antibody has an additional glycosylation site in the variable region. Although this extra site has high-Man as well as complex *N*-glycans, it still could have a different glycosylation profile.

Our observations are of biotechnological relevance because it appears to matter from which leaves the antibodies are harvested. Favorable from the biotechnological perspective is our observation that *N*-glycosylation seems to be not significantly influenced by temperature or light intensity when host plants are grown under controlled conditions. This suggests that more uncontrolled growth would give similar results.

Although we show here that the structure of *N*-linked glycans varies with different developmental stages, as yet no specific role of *N*-linked glycans in biological processes in plants have been described in literature. This is in striking contrast to the well-documented importance of *N*-linked glycans in many biological processes in mammals.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Transgenic tobacco (*Nicotiana tabacum* cv Samsun NN) plants expressing a mouse IgG antibody (MGR48; de Boer et al., 1996) against saliva proteins (sub-ventral gland proteins) of the nematode *Globodera rostochiensis* were grown in climate rooms under four different climate conditions, i.e. at 15°C and 25°C and at either low- or high-light conditions. Plant origin and exact growth conditions have previously been described by Stevens et al. (2000).

Of every group, three portions of leaves of different developmental stages were harvested (top, middle, and basal leaves, respectively) resulting in 12 different leaf samples. The top leaves are defined as the youngest leaves of at least 5 cm in length; the basal leaves are the first leaf at the bottom of the plant of at least 15 cm in length together with the first one in succession; the middle leaves are defined as the three leaves in the middle between the top and the basal leaves. The rest of the leaves were not analyzed. Immediately after harvest the plant material was frozen in liquid nitrogen and stored at −70°C.

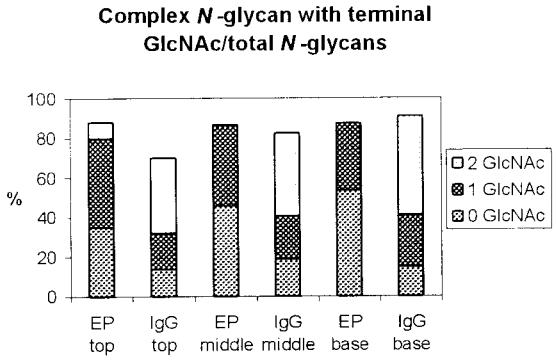


Figure 4. Distribution of complex *N*-glycans with and without terminal GlcNAc isolated from soluble endogenous proteins and IgG at 25°C and low light. The amounts are based on relative peak intensities in MALDI-TOF MS spectra and are expressed as percentage of total *N*-glycans.

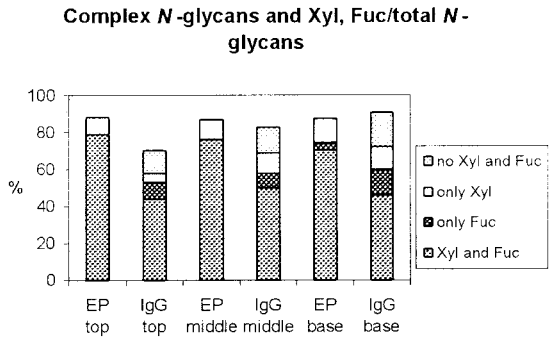


Figure 5. Distribution of complex *N*-glycans with and without Xyl and/or Fuc isolated from soluble endogenous proteins and IgG at 25°C and low light. The amounts are based on relative peak intensities in MALDI-TOF MS spectra and are expressed as percentage of total *N*-glycans.

Separation of IgG from Endogenous Glycoproteins of Tobacco Leaves

Frozen tobacco leaves were powdered in a stainless steel blender, which was cooled with liquid nitrogen. Per 10 g of powder, 30 mL of extraction buffer (5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM sodium bisulfite, and 0.5 g of polyvinylpolypyrrolidone in 150 mM sodium phosphate [pH 7.0]) were added. After 15 min of stirring at 4°C, the mixture was centrifuged (10 min, 10,000*g*, 4°C). The pellet was washed with 4.5 mL of 150 mM sodium phosphate (pH 7.0) including 5 mM EDTA and centrifuged (15 min, 10,000*g*, 4°C). Both supernatants were pooled and partially pelleted at 20% ammonium sulfate saturation (5 min, 5,000*g*, 4°C). The supernatant was submitted to a second precipitation with ammonium sulfate at 60% saturation (20 min, 10,000*g*, 4°C). The pellet was resuspended with 4.5 mL of 100 mM NaCl in 50 mM sodium phosphate (pH 7.0). After centrifugation (30 min, 20,000*g*, 4°C) the sample was applied on a Protein G Sepharose 4 Fast Flow bioaffinity column (column volume 1 mL; Amersham Pharmacia Biotech, Roosendaal, The Netherlands), which was equilibrated with 100 mM NaCl in 50 mM sodium phosphate buffer (pH 7.0). The column subsequently was washed with 10 column volumes of the same buffer. All non-binding protein was collected in one fraction ("endogenous glycoproteins") and kept for further analysis. IgG was eluted with 0.1 M Gly-HCl buffer (pH 2.7) and immediately neutralized with 50 μL of 1 M Tris (pH 9.0) per milliliter of eluate. Finally, IgG was desalted on Sephadex G25 (PD-10 columns, Amersham Pharmacia Biotech) that was equilibrated with 100 mM ammonium carbonate.

The quality of this extraction and separation procedure with respect to stability of the IgG protein as well as the IgG *N*-glycans was checked in duplicate as follows. Five grams of base leaves and 5 g of top leaves were mixed and ground to a powder. After homogenization with 30 mL of extraction buffer and centrifugation (10 min, 10,000*g*, 4°C) the supernatant was divided in three portions. Portion 1 was immediately brought in (100 mM NaCl and 50 mM sodium phosphate [pH 7.0]) by gel filtration on Sephadex G25, and within 1 h separated by Protein G bioaffinity chromatography into IgG and endogenous protein. To portion 2, a cocktail of extra protease inhibitors (Complete, Boehringer Mannheim, Almere, The Netherlands) was added as described by Boehringer Mannheim. Portions 2 and 3 were subjected to the complete separation procedure described above including the more time-consuming precipitation steps taking altogether about 8 h. The IgG sample prepared by the short procedure from portion 1 and the IgG samples prepared by the standard separation procedure in the presence and absence of extra protease inhibitors (prepared from portion 2 and 3, respectively) were quantitatively and qualitatively analyzed by SDS-PAGE and immunoblotting (as described by Stevens et al., 2000). Immunodetection of complex *N*-glycans with anti-Xyl and anti-Fuc antibodies was performed according to Faye et al. (1993). Affino-detection of high-Man *N*-glycans was performed using the concanavalin A/peroxidase method of Faye and Chrispeels (1985). Final development of the blots was performed by incubation of the blots with ECL western blotting detection reagent (Amersham Pharmacia), after which light-sensitive films were exposed to the blots. No quantitative and qualitative differences were found between the three different IgG samples, indicating that during the applied procedure for the separation of IgG and endogenous proteins no significant degradation of IgG occurred.

Isolation of *N*-Linked Glycans

After lyophilization the purified IgG was digested with 100 μg pepsin in 10 mM HCl (pH 2.2) for 48 h at 37°C. The pepsin digest was neutralized with approximately 35 μL of 1 N NH_4OH and heated at 100°C for 5 min. After lyophilization, the sample was deglycosylated with 0.5 milli-units peptide *N*-glycosidase A (PNG-ase A, Roche Chemicals, Almere, The Netherlands) in 0.1 M sodium acetate buffer (pH 5.0) overnight at 37°C. The *N*-glycans were purified by passing through a cation exchange column (1.6 mL 50W-X2, Bio-Rad, Veenendaal, The Netherlands), which was equilibrated with water and a C_{18} Bond Elut SPE column (Varian, Middleburg, The Netherlands), which was equilibrated with acetonitril. For MALDI-TOF analysis, the *N*-glycans were further purified with a Carbograph Ultra Clean SPE column (Alltech, Breda, The Netherlands) as described by Packer et al. (1998) and subsequently lyophilized.

The endogenous protein fraction was brought to pH 2.2 with 37% (w/v) HCl after which 2 mg pepsin was added. Further purification of *N*-glycans of endogenous proteins was performed in the same way, except for the use of 1 milli-unit PNG-ase A and a 2-fold extra purification over C_{18} Bond Elut SPE.

MALDI-TOF MS

The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in 1 mL of acetonitrile:water

(7:3, v/v). Lyophilized *N*-glycans originating from IgG were dissolved in 5 μL of water; lyophilized *N*-glycans originating from the endogenous glycoproteins were dissolved in 1 mL water. On the MALDI-TOF plate, 1 μL of this *N*-glycan solution was mixed with 1 μL of matrix solution. The plate was dried in a gentle stream of air at room temperature. MALDI-TOF mass spectra were measured on a Voyager-DE RP reflectron apparatus. Typically, a 3-ns pulse width at 337 nm was applied using a nitrogen laser. The mass spectrometer was operating in the positive-ion mode. Ions were accelerated to an energy of 12 kV before entering the TOF mass spectrometer. Fifty to 80 laser shots were accumulated to obtain an acceptable signal to noise ratio. All data presented are based on relative peak intensities in MALDI-TOF spectra.

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