

Production of a monoclonal antibody in plants with a humanized *N*-glycosylation pattern

Matthias Schöhs¹, Richard Strasser¹, Johannes Stadlmann², Renate Kunert³, Thomas Rademacher⁴ and Herta Steinkellner^{1,*}

¹Institute of Applied Genetics and Cell Biology, BOKU-Wien, Austria

²Department of Chemistry, BOKU-Wien, Austria

³Institute of Applied Microbiology, BOKU-Wien, Austria

⁴Rheinisch-Westfälische Technische Hochschule (RWTH), Aachen, Germany

Received 2 March 2007;

revised 5 May 2007;

accepted 10 May 2007.

*Correspondence (fax 43 1 36006 6392;

e-mail herta.steinkellner@boku.ac.at)

Summary

In recent years, plants have become an attractive alternative for the production of recombinant proteins. However, their inability to perform authentic mammalian *N*-glycosylation may cause limitations for the production of therapeutics. A major concern is the presence of β 1,2-xylose and core α 1,3-fucose residues on complex *N*-linked glycans, as these *N*-glycan epitopes are immunogenic in mammals. In our attempts towards the humanization of plant *N*-glycans, we have generated an *Arabidopsis thaliana* knockout line that synthesizes complex *N*-glycans lacking immunogenic xylose and fucose epitopes. Here, we report the expression of a monoclonal antibody in these glycan-engineered plants that carry a homogeneous mammalian-like complex *N*-glycan pattern without β 1,2-xylose and core α 1,3-fucose. Plant and Chinese hamster ovary (CHO)-derived immunoglobulins (IgGs) exhibited no differences in electrophoretic mobility and enzyme-linked immunosorbent specificity assays. Our results demonstrate the feasibility of a knockout strategy for *N*-glycan engineering of plants towards mammalian-like structures, thus providing a significant improvement in the use of plants as an expression platform.

Keywords: antibodies, β 1,2-xylose and core α 1,3-fucose, plant glycan engineering, recombinant proteins.

Introduction

Although the number of interesting recombinant glycoproteins for human therapy is steadily increasing, the current production capacities act as a bottleneck. Therefore, alternative production systems are currently the subject of intense research. Over recent years, plants have been considered as an attractive vehicle for the expression of recombinant pharmaceutical proteins as they are inexpensive and versatile systems, amenable to rapid and economical scale-up. Production levels of up to 60% of total soluble protein (TSP) have been reported recently, which makes the system of economic interest (De Jaeger *et al.*, 2002; Marillonnet *et al.*, 2004, 2005). A major advantage of plants is that they are higher eukaryotes that possess an endomembrane system and a secretory pathway similar to that of mammalian cells. Therefore, complex proteins are correctly folded and assembled, which has been demonstrated very impressively for a secre-

tory immunoglobulin A (IgA) produced in tobacco (Ma *et al.*, 1995). In addition, the increasing awareness of regulatory agencies of correct protein glycosylation requires a controlled glycosylation machinery. In this respect, plants are interesting, as they synthesize a core complex *N*-glycan structure similar to that in mammalian cells (Figure 1). However, by contrast with the common core structure, the terminal residues of plant and mammalian complex *N*-glycans differ; plant *N*-glycans are generally much smaller, lack β 1,4-galactose and sialic acid, and, instead, carry plant-specific β 1,2-xylose and core α 1,3-fucose residues. However, the immunogenicity of β 1,2-xylose and core α 1,3-fucose residues in mammals is well documented (Kurosaka *et al.*, 1991; Faye *et al.*, 1993; Bardor *et al.*, 2003; Bencurova *et al.*, 2004; Jin *et al.*, 2006). Furthermore, altered *N*-glycosylation may affect the function of a protein (for a recent review, see Jefferis, 2005). Thus, the unique *N*-glycans added by plants could impact on both the immunogenicity and functional activity, and, consequently,

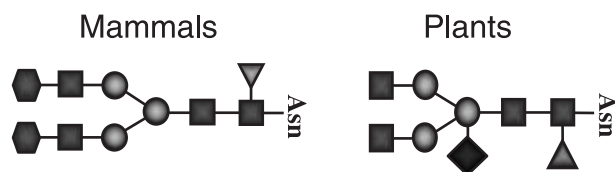


Figure 1 Schematic representation of complex *N*-glycans from mammals and plants (simplified). ■, *N*-acetylglucosamine; ●, mannose; ◆, β 1,2-xylose; ▲, α 1,3-fucose; ▼, α 1,6-fucose; ●, α 1,4-galactose.

may represent a limitation for plants to be used as a production platform.

Various strategies have been applied to avoid plant-specific *N*-glycosylation. In this respect, targeting of proteins to specific subcellular compartments with defined *N*-glycan structures has been reported (Schouten *et al.*, 1996). For example, retention of recombinant proteins in the endoplasmic reticulum has resulted in their accumulation, carrying mainly oligo-mannosidic *N*-glycans, typical of endoplasmic reticulum-resident proteins. However, these structures may lead to a dramatic decrease in the *in vivo* half-life of the target protein, as recently reported for a plant-produced antibody (Ko *et al.*, 2003). Another promising strategy is based on the specific manipulation of the *N*-glycosylation pathway in host plants. The over-expression of human β 1,4-galactosyltransferase, which competes for the same acceptor substrate as β 1,2-xylosyltransferase (XT) and core α 1,3-fucosyltransferase (FT), resulted in a significant decrease in β 1,2-xylose and core α 1,3-fucose (Palacpac *et al.*, 1999; Bakker *et al.*, 2001, 2006). However, the complete elimination of these glycan epitopes was not achieved. Nevertheless, it is possible to partially elongate plant *N*-glycans with β 1,4-galactose, a terminal residue present on many mammalian *N*-linked glycans, but absent in plants. The importance of this glycan residue is twofold: first, it may affect the stability and/or *in vivo* activity of a protein and, second, it is the prerequisite structure for the attachment of sialic acid.

Recently, a knockout (k.o.) line from the model plant *Arabidopsis thaliana* was generated in our laboratory, with deficiency of active XT and core FT, the enzymes responsible for the transfer of β 1,2-xylose and core α 1,3-fucose (Strasser *et al.*, 2004). This line (XT/FT k.o. line) lacks immunogenic β 1,2-xylose and core α 1,3-fucose residues and, instead, synthesizes predominantly humanized structures with terminal *N*-acetylglucosamine (GlcNAc) residues (GnGn). Interestingly, XT/FT k.o. plants are viable and reveal no obvious morphological phenotype under standard growth conditions. In addition, an RNA interference (RNAi) strategy has been applied to eliminate xylose and fucose residues in the aquatic plant

Lemna minor (Cox *et al.*, 2006). An IgG produced in these 'glycan-optimized' plants exhibited a homogeneous complex *N*-glycan (GnGn) structure which showed an up to 50-fold increase in antibody-dependent cellular cytotoxicity (ADCC). Core α 1,3-fucose seems to have a similar effect to mammalian α 1,6-fucose, as the removal of this residue in Chinese hamster ovary (CHO)-derived antibodies dramatically increases Fc γ receptor binding and ADCC activity (Shields *et al.*, 2001).

Here, we describe the feasibility of *A. thaliana* XT/FT k.o. lines for the production of recombinant proteins, with a monoclonal antibody as a model glycoprotein. K.o. lines were transformed with genes from heavy and light chains, and selected according to their IgG expression. As expected, antibodies purified from the mutant line carry complex *N*-glycan structures, but completely lack xylose and fucose, as determined by immunoblotting and mass spectrometry (MS). The IgG obtained assembled correctly and exhibited an unaltered antigen-binding affinity compared with CHO-derived IgG.

Results and discussion

Expression and characterization of 2G12 in *A. thaliana* wild-type (wt) and XT/FT k.o. plants

We have recently generated an *A. thaliana* triple k.o. line (XT/FT k.o.) that synthesizes complex *N*-glycans completely devoid of immunogenic β 1,2-xylose and core α 1,3-fucose (Strasser *et al.*, 2004). A monoclonal antibody of the class IgG1 against human immunodeficiency virus (HIV) (2G12; Trkola *et al.*, 1995) was chosen to investigate the feasibility of these k.o. lines for the production of recombinant proteins. The two complementary DNAs (cDNAs) were cloned in tandem into a binary vector (Figure 2a) that also contained DsRed, a fluorescent protein, as selection marker. *A. thaliana* wt and XT/FT k.o. mutants were transformed, and the resulting seeds were monitored according to their DsRed expression using fluorescence microscopy. Seeds that exhibited a strong DsRed signal were chosen for further propagation (Figure 2b). To test whether 2G12 antibodies were actually expressed in these plants, soluble proteins were extracted from leaves and subjected to Western blot analysis using antibodies against heavy and light chains (C_H , C_L). The expression of both chains was clearly visible in wt and XT/FT k.o. plants (Figure 3a). Subsequently, 2G12 antibodies were purified from leaves via protein A affinity chromatography, and subsequent sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis under reducing conditions revealed the presence of two bands that correlated with the expected molecular masses of C_H and C_L (55 kDa and 25 kDa, respectively; Figure 3b). No

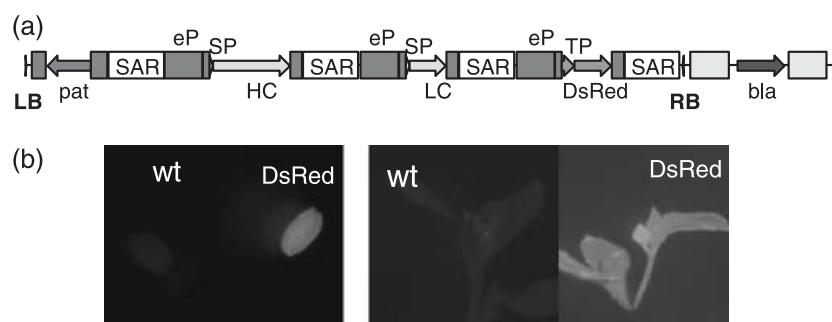


Figure 2 (a) Plant expression vector used for the expression of immunoglobulin G (IgG) antibodies: bla, ampicillin resistance gene; DsRed, red fluorescent protein; eP, enhanced cauliflower mosaic virus (CaMV)-35S promoter; HC, LC, heavy and light chain; LB, RB, left and right border; pat, basta resistance gene with promoter and terminator of the nopaline synthase gene; SAR, scaffold attachment region; SP, signal peptide; TP, transit peptide. (b) Seeds and seedlings examined under fluorescence microscopy: DsRed, seed transformed with the binary vector described above; wt, non-transformed seed.

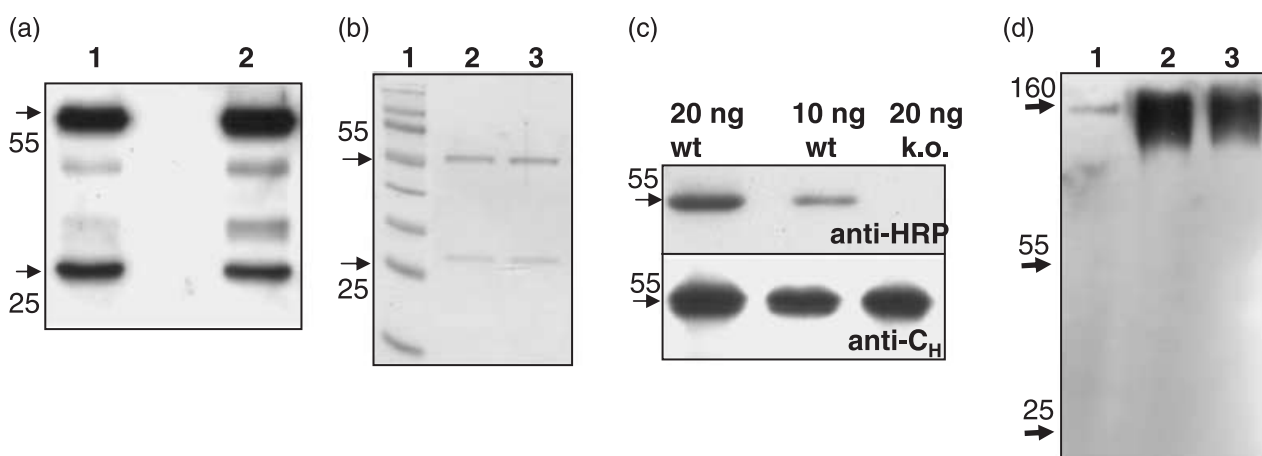


Figure 3 (a) Western blot analysis of proteins extracted from 2G12-transformed wild-type (wt) (lane 1) and β 1,2-xylosyltransferase and α 1,3-fucosyltransferase knockout (XT/FT k.o.) (lane 2) plants probed with rabbit anti-human immunoglobulin G (IgG) (C_H and C_L -specific). (b) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Coomassie Blue stained): lane 1, protein marker; 2G12 purified from wt *Arabidopsis thaliana* (lane 2) and k.o. line (lane 3). (c) Western blot analysis of purified 2G12 using anti-horseradish peroxidase (anti-HRP) antibodies (recognizing xylose and fucose) and goat anti-human IgG (γ -chain-specific, anti- C_H): wt, 2G12 derived from *A. thaliana* wt; k.o., 2G12 derived from XT/FT k.o. line. (d) Western blot analysis of purified 2G12 after native PAGE using rabbit anti-human IgG (C_H and C_L -specific): lane 1, Chinese hamster ovary (CHO)-derived 2G12; 2G12 derived from wt *A. thaliana* (lane 2) and k.o. line (lane 3). Numbers next to the gels indicate the protein masses in kilodaltons.

further bands, degradation products or impaired heavy or light chains in PAGE under native conditions were visible, indicating the purity and high rate of antibody assembly. The weak extra bands between C_H and C_L of 2G12 that were visible when TSP extracts were used for immunoblotting (Figure 3a) may represent degradation products from C_H within the plant cell; however, they disappeared completely after 2G12 purification. In addition, purified CHO- and plant-derived IgGs were subjected to immunoblotting using antibodies that recognized C_H - and plant-specific glycan epitopes [anti-horseradish peroxidase (anti-HRP) antibody], respectively

(Figure 3c). As expected, a single band with a molecular mass of about 55 kDa was detected in all samples using C_H antibodies. By contrast, no signal was obtained in XT/FT k.o.-derived IgG using anti-HRP anti-serum, indicating the absence of immunogenic glycan epitopes.

The expression level of 2G12 in *A. thaliana* wt and k.o. lines varied between 0.2 and 0.05% of TSP in young plants (up to the six-leaf stage). As plants aged, the expression level dropped below 0.05% TSP. 2G12-expressing plants, both wt and k.o. lines, were normal and viable and did not show any obvious morphological phenotype under standard growth conditions.

In previous studies, it has been shown that 2G12 recognizes a specific epitope at the HIV envelope protein gp120 (Trkola *et al.*, 1996). In order to test the antigen-binding specificities of plant-produced 2G12, a specificity enzyme-linked immunosorbent assay (ELISA) coated with HIV gp160, which encompasses gp120, was carried out. Compared with CHO-derived 2G12, the binding capacity of the plant-derived counterpart was 100% or slightly above, demonstrating unaltered antigen-binding specificity.

Determination of *N*-glycan composition of 2G12 from *A. thaliana* wt and XT/FT k.o. lines

To determine the exact *N*-glycan composition of 2G12, the corresponding heavy chains were subjected to tryptic digestion and MS. Owing to incomplete digestion of C_H, two glycopeptides, which contain the carbohydrate at asparagine-297 but differ in four amino acids (482.3 Da), are present. The absence of xylose and fucose residues on *N*-glycans can be monitored by the decrease in mass of the respective peaks (132 mass units for xylose and 146 mass units for fucose).

The mass spectrum of wt-derived C_H exhibited three major peaks for both glycopeptides (Figure 4a). The major peak was derived from the complex *N*-glycan GnGnXF with two terminal GlcNAc residues, followed by two minor peaks with one GlcNAc residue (GnMXF/MGnXF) and without any terminal GlcNAc residues (MMXF). All of these detected glycoforms contained immunogenic β 1,2-xylose and core α 1,3-fucose residues. By contrast, XT/FT k.o.-derived 2G12 lacked the three major peaks carrying β 1,2-xylose and core α 1,3-fucose (Figure 4b). Instead, GnGn was the most abundant *N*-glycan structure. Apart from the complex *N*-glycans, two minor glycopeptides with oligomannosidic structures (Man7, Man8) were identified in both wt- and XT/FT k.o.-derived IgG, which indicates that not all glycans were completely processed to complex-type forms. *N*-Glycan analysis of CHO-derived 2G12 revealed a mixture of structures containing either no (GnGnF), one (AGnF, GnAF) or two (AAF) terminal galactose residues and, in addition, minor amounts of glycopeptides with sialic acid (NaAF, NaNaF). Interestingly, all detected glycoforms contained a fucose residue linked α 1,6 to the innermost GlcNAc. Importantly, it has been shown that the absence of this fucose residue significantly increases Fc receptor-mediated ADCC (Shields *et al.*, 2001).

Our results clearly demonstrate that engineering of complex plant *N*-glycans towards a mammalian type is possible without any deleterious effects in growth behaviour. We have shown that glyco-engineered plants are able to produce monoclonal antibodies with a defined humanized *N*-glycan

profile, not only lacking plant-specific glycan epitopes, but also α 1,6-fucose, a glycan residue that seems to negatively influence the ADCC potency of antibodies (Shields *et al.*, 2001). Indeed, recently, it has been demonstrated very impressively that such 'glycan-optimized' plant-derived antibodies carrying a homogeneous GnGn structure exhibit a significant ADCC enhancement compared with the CHO-produced counterpart (Cox *et al.*, 2006). These results and recent improvements in plant expression systems using *A. thaliana* seeds and *Nicotiana* species, where IgG and antibody fragment levels of up to 0.5 g/kg leaf material were obtained (Giritch *et al.*, 2006; Van Droogenbroeck *et al.*, 2007), demonstrate the great potential of plants as an alternative expression system for therapeutics, not only in terms of quantity, but also in quality improvement.

Experimental procedures

Expression of 2G12 antibody in *A. thaliana* plants

The cDNAs of 2G12 heavy and light chains were isolated, together with the sequence for the signal peptide, from their eukaryotic expression vectors (Trkola *et al.*, 1995). Each chain was subcloned into an expression cassette consisting of the duplicated cauliflower mosaic virus (CaMV)-35S promoter, the tobacco etch virus (TEV) 5'-untranslated region and the CaMV-35S transcriptional terminator. For co-expression of heavy and light chains, the two cassettes were inserted head-to-tail into the binary vector pTRAP, a derivative of pPAM (GENBANK: AY027531). The tandem expression units were separated by scaffold attachment regions (SARs) of the tobacco RB7 gene (GENBANK: U67919). In addition, an expression cassette for DsRed (Jach *et al.*, 2001), fused to a plastid transit peptide, was cloned behind the antibody chain cassettes to create the final construct pTRAP-2G12-Ds (Figure 2a). The plasmid was transformed into *Agrobacterium* strain GV3101::pMP90RK.

Transgenic *A. thaliana* plants were generated by floral dipping of Col-0 wt and XT/FT k.o. lines (Strasser *et al.*, 2004). A Leica MZ FLIII stereomicroscope (Leica, Wetzlar, Germany), equipped with a Leica DC 500 digital camera and a DsRed filter set (No. 10447079), was used to detect DsRed fluorescence in seeds and young seedlings.

Purification of recombinant 2G12 antibody from *A. thaliana* leaves

Leaves (200 mg) were frozen in liquid nitrogen and ground in a mixer mill (Retsch MM2000, Haan, Germany). The powder was dissolved in 400 μ L of 1 $\times$ phosphate-buffered saline (PBS) (pH 6) containing 1% protease (v/v) inhibitor cocktail (Sigma, P9599, St. Louis, MO, USA), and incubated at 4 °C for 15 min. After centrifugation for 30 min at 16 000 *g*, the supernatant (SN1) was incubated at 4 °C for 90 min with 15 μ L of rProteinA Sepharose Fast Flow (GE Healthcare, Chalfont, UK) using an orbital shaker. The incubated slurry was transferred into a Micro Bio-Spin chromatography column (Biorad, Hercules, CA, USA) and washed three times with 250 μ L of 1 $\times$ PBS. Elution was performed with 15 μ L of 0.1 M glycine-HCl buffer (pH 3.0).

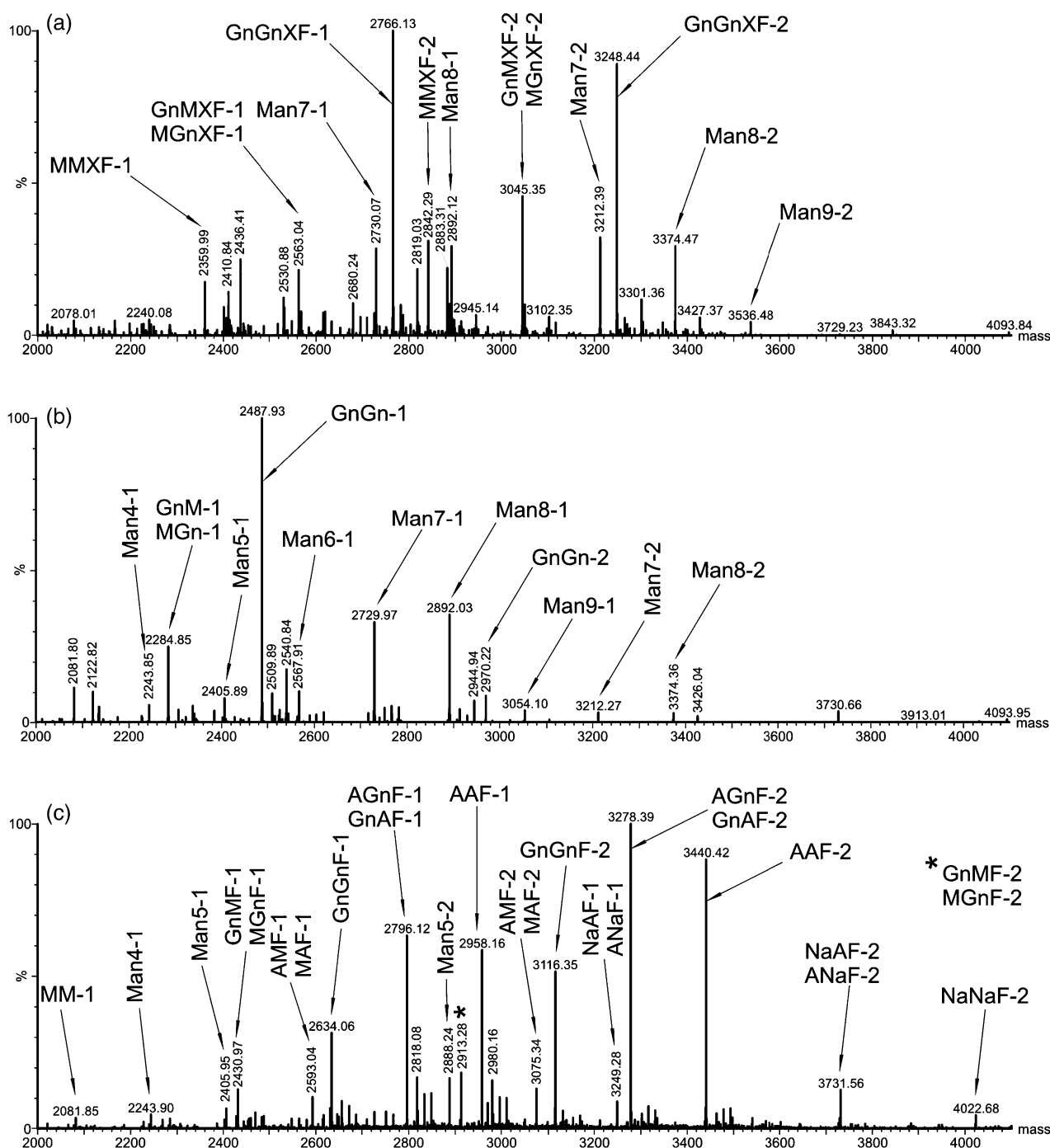


Figure 4 Liquid chromatography-mass spectrometry (LC-MS) of glycosylated peptides (-1, EEQYNSTYR; -2, TKPREEQYNSTYR) containing asparagine-297 from the 2G12 Fc region: (a) glycopeptides derived from wild-type (wt)-produced 2G12; (b) glycopeptides derived from β 1,2-xylosyltransferase and α 1,3-fucosyltransferase knockout (XT/FT k.o.) plant-produced 2G12; (c) glycopeptides derived from Chinese hamster ovary (CHO)-produced 2G12. See <http://www.proglycan.com> for an explanation of *N*-glycan abbreviations.

Immunoblot analysis

SN1 and purified IgG (100 ng) samples were subjected to SDS-PAGE (12.5% polyacrylamide) under reducing and non-reducing conditions. The separated proteins were blotted to Hybond-ECL membranes (GE Healthcare) and detected with either a goat anti-human IgG

($C_H + C_L$ -specific) antibody conjugated to peroxidase (Promega, Madison, WI, USA) or a goat anti-human IgG (γ -chain-specific) antibody conjugated to peroxidase (Sigma, A8775), both diluted 1 : 5000 in 1 $\times$ PBS (pH 7.4) containing 1% (w/v) bovine serum albumin (BSA). For the detection of N-linked glycans with β 1,2-xylose and core α 1,3-fucose, rabbit anti-HRP antibody was used as described

previously (Strasser *et al.*, 2004). The detection of bound antibodies was performed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

2G12 quantification and antigen-binding assay

To quantify and determine the antigen-binding specificity of plant-derived 2G12, a Standard Operating Procedure (SOP) developed by Polymun Scientific was carried out. The IgG concentration and gp160 binding were determined by coating ELISA plates with a polyclonal antibody 'goat anti-human IgG' (γ -chain, Sigma; for quantification) and with 100 ng purified gp160 [provided by Polymun Scientific (Vienna, Austria); for antigen-binding assays]. After washing, 2G12 samples were diluted to a concentration of about 200 ng/mL. Subsequently, a 1 : 2 serial dilution was carried out (eight dilutions) and 50 μ L was transferred per well. Recombinant 2G12 purified from CHO culture supernatants (provided by Polymun Scientific) was used as standard, and applied identically to plant-derived 2G12. Plates were incubated for 1 h at room temperature (gentle shaking) and, after washing, a polyclonal antibody 'goat anti-human IgG' (κ -chain) conjugated to alkaline phosphatase (1 : 1000, Sigma) was incubated. After intensive washing, alkaline phosphatase was detected by *p*-nitro-phenylphosphate substrate solution using an ELISA reader at 405 nm, with 620 nm as reference wavelength. All analyses were carried out in duplicate, and a four-parameter polynomial was applied for curve fitting and data analysis.

N-Glycan analysis

Purified 2G12 IgG1 (0.5 μ g) was separated by SDS-PAGE (12.5% polyacrylamide) analysis under reducing conditions, and polypeptides were detected by Coomassie Blue staining. The heavy chain was excised from the gel, destained, carbamidomethylated and in-gel trypsin digested as described previously (Kolarich and Altmann, 2000). Tryptic peptides were dried in a Speed Vac concentrator and reconstituted with water containing 0.1% (v/v) formic acid. Mass spectrometric analysis was performed on a quadrupole time-of-flight (Q-TOF) Ultima Global (Waters Micromass, Milford, MA, USA) equipped with a standard electro-spray unit, a Cap-LC system (Waters Micromass) and a 10-port solvent switch module (Rheodyne, Rohnert Park, MA, USA). Samples were first captured by an Aquasil C18 precolumn (30 $\times$ 0.32 mm, Thermo Electron, Waltham, MA, USA) using water as the solvent. The analytical column was held at 5% acetonitrile before solvent switching, and then a linear gradient from 5 to 50% acetonitrile was applied at a flow rate of 2 μ L/min. All eluents contained 0.1% formic acid. The mass spectrometer had been previously tuned with [Glu1]-fibrinopeptide B to give the highest possible sensitivity and a resolution of c. 10 000 (full width at half-maximum, FWHM). Mass tuning of the TOF analyser was performed in the tandem MS mode using, again, [Glu1]-fibrinopeptide B. Samples were analysed in the MS mode. Because no switching between MS and tandem MS mode was performed, no loss of signal, especially for the analysis of the glycopeptides, occurred. Data analysis was performed with MassLynx 4.0 SP4 Software (Waters Micromass).

The mass spectrometric data of tryptic peptides were analysed against the *in silico*-generated tryptic digestion of the 2G12 C_H amino acid sequence, employing the program 'PeptideMass' (<http://www.expasy.org/tools/peptide-mass.html>). Based on the tryptic

peptide data set, the tryptic glycopeptide data sets ('glycopeptide 1' and 'glycopeptide 2', representing the 'perfectly' cleaved tryptic glycopeptide 'EEQYNSTYR', and the tryptic glycopeptide bearing one missed cleavage site 'TKPREEQYNSTYR', respectively) were generated by the addition of the respective glycan masses to the tryptic peptide masses of glycopeptide 1 and glycopeptide 2.

Acknowledgements

We thank Chunsheng Jin and Robert Weik (BOKU-Wien, Austria) for assistance in antibody purification, and Ali Assadian and Gabriele Stiegler (BOKU-Wien, Austria) for IgG quantification and gp160-binding assays. This work was supported by a Wiener Wirtschafts-, Forschungs-, und Technologiefonds-funded project (www.wwf.at) and the European Community (www.pharma-planta.com).

References

- Bakker, H., Bardor, M., Molthoff, J.W., Gomord, V., Elbers, I., Stevens, L.H., Jordi, W., Lommen, A., Faye, L., Lerouge, P. and Bosch, D. (2001) Galactose-extended glycans of antibodies produced by transgenic plants. *Proc. Natl. Acad. Sci. USA*, **98**, 2899–2904.
- Bakker, H., Rouwendal, G.J., Karnoup, A.S., Florack, D.E., Stoope, G.M., van Helsper, J.P., van Ree, R., Die, I. and Bosch, D. (2006) An antibody produced in tobacco expressing a hybrid beta-1,4-galactosyltransferase is essentially devoid of plant carbohydrate epitopes. *Proc. Natl. Acad. Sci. USA*, **103**, 7577–7582.
- Bardor, M., Faveeuw, C., Fitchette, A.C., Gilbert, D., Galas, L., Trottein, F., Faye, L. and Lerouge, P. (2003) Immunoreactivity in mammals of two typical plant glyco-epitopes, core alpha (1,3)-fucose and core xylose. *Glycobiology*, **13**, 427–434.
- Bencurova, M., Hemmer, W., Focke-Tejkl, M., Wilson, I.B. and Altmann, F. (2004) Specificity of IgG and IgE antibodies against plant and insect glycoprotein glycans determined with artificial glycoforms of human transferrin. *Glycobiology*, **14**, 457–466.
- Cox, K.M., Sterling, J.D., Regan, J.T., Gasdaska, J.R., Frantz, K.K., Peele, C.G., Black, A., Passmore, D., Moldovan-Loomis, C., Srinivasan, M., Cuisson, S., Cardarelli, P.M. and Dickey, L.F. (2006) Glycan optimization of a human monoclonal antibody in the aquatic plant *Lemna minor*. *Nat. Biotechnol.*, **24**, 1591–1597.
- De Jaeger, G., Scheffer, S., Jacobs, A., Zambre, M., Zobell, O., Goossens, A., Depicker, A. and Angenon, G. (2002) Boosting heterologous protein production in transgenic dicotyledonous seeds using *Phaseolus vulgaris* regulatory sequences. *Nat. Biotechnol.*, **20**, 1265–1268.
- Faye, L., Gomord, V., Fitchette-Laine, A.C., and Chrispeels, M.J. (1993) Affinity purification of antibodies specific for Asn-linked glycans containing alpha 1 $\rightarrow$ 3 fucose or beta 1 $\rightarrow$ 2 xylose. *Anal. Biochem.*, **209**, 104–108.
- Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V. and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfecting with noncompeting viral vectors. *Proc. Natl. Acad. Sci. USA*, **103**, 14 701–14 706.
- Jach, G., Binot, E., Frings, S., Luxa, K. and Schell, J. (2001) Use of red fluorescent protein from *Discosoma* sp. (dsRED) as a reporter for plant gene expression. *Plant J.*, **28**, 483–491.

- Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog.* **21**, 11–16.
- Jin, C., Bencurova, M., Borth, N., Ferko, B., Jensen-Jarolim, E., Altmann, F. and Hantusch, B. (2006) Immunoglobulin G specifically binding plant N-glycans with high affinity could be generated in rabbits but not in mice. *Glycobiology*, **16**, 349–357.
- Ko, K., Tekoah, Y., Rudd, P.M., Harvey, D.J., Dwek, R.A., Spitsin, S., Hanlon, C.A., Rupprecht, C., Dietzschold, B., Golovkin, M. and Koprowski, H. (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. *Proc. Natl. Acad. Sci. USA*, **100**, 8013–8018.
- Kolarich, D. and Altmann, F. (2000) N-Glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1. *Anal. Biochem.* **285**, 64–75.
- Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T. and Kawasaki, T. (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.* **266**, 4168–4172.
- Leonard, R., Rendic, D., Wilson, I.B.H. and Altmann, F. (2005) Adaptation of the 'in gel release method' to glycom studies from a very little amount of material. *Glycoconjugate J.* **22**, 238.
- Ma, J.K., Hiatt, A., Hein, M., Vine, N.D., Wang, F., van Stabila, P., Dolleved, C., Mostov, K. and Lehner, T. (1995) Generation and assembly of secretory antibodies in plants. *Science*, **268**, 716–719.
- Marillonnet, S., Giritch, A., Gils, M., Kandzia, R., Klimyuk, V. and Gleba, Y. (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc. Natl. Acad. Sci. USA*, **101**, 6852–6857.
- Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V. and Gleba, Y. (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotechnol.* **23**, 718–723.
- Palacpac, N.Q., Yoshida, S., Sakai, H., Kimura, Y., Fujiyama, K., Yoshida, T. and Seki, T. (1999) Stable expression of human beta1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. *Proc. Natl. Acad. Sci. USA*, **96**, 4692–4697.
- Schouten, A., Roosien, J., van Engelen, F.A., de Jong, G.A., Borst-Vrens, A.W., Zilverentant, J.F., Bosch, D., Stiekema, W.J., Gommers, F.J., Schots, A. and Bakker, J. (1996) The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* **30**, 781–793.
- Shields, R.L., Lai, J., Keck, R., O'Connell, L.Y., Hong, K., Meng, Y.G., Weikert, S.H. and Presta, L.G. (2001) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fc gamma RIII and antibody-dependent cellular toxicity. *J. Biol. Chem.* **277**, 26 733–26 740.
- Strasser, R., Altmann, F., Mach, L., Glossl, J. and Steinkellner, H. (2004) Generation of *Arabidopsis thaliana* plants with complex N-glycans lacking beta 1,2-linked xylose and core alpha 1,3-linked fucose. *FEBS Lett.* **561**, 132–136.
- Trkola, A., Pomales, A.B., Yuan, H., Korber, B., Maddon, P.J., Allaway, G.P., Katinger, H., Barbas, C.F., Burton, D.R., Ho, D.D. and Moore, J.P. (1995) Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. *J. Virol.* **69**, 6609–6617.
- Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., Srinivasan, K., Sodroski, J., Moore, J.P. and Katinger, H. (1996) Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J. Virol.* **70**, 1100–1108.
- Van Droogenbroeck, B., Cao, J., Stadlmann, J., Altmann, F., Colanesi, S., Hillmer, S., Robinson, D.G., Van Lerberge, E., Terryn, N., Van Montagu, M., Liang, M., Depicker, A. and De Jaeger, G. (2007) Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic *Arabidopsis* seeds. *Proc. Natl. Acad. Sci. USA*, **104**, 1430–1435.