Plant Biotechnology Journal (2004) **2**, pp. 517–523 doi: 10.1111/j.1467-7652.2004.00100.x

Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic *N*-glycans

Anna Koprivova[1,†], Christian Stemmer[2,†], Friedrich Altmann[3], Axel Hoffmann[1], Stanislav Kopriva[4], Gilbert Gorr[2], Ralf Reski[1] and Eva L. Decker[1,*]

[1]*University of Freiburg, Plant Biotechnology, Schaenzlestr. 1, 79104 Freiburg, Germany*
[2]*greenovation Biotech GmbH, Boetzinger Str. 29b, 79111 Freiburg, Germany*
[3]*Institute of Chemistry, University of Natural Resources and Applied Life Sciences, Muthgasse 18, A-1190 Vienna, Austria*
[4]*University of Freiburg, Department of Tree Physiology, Georges-Koehler-Allee 053, 79085 Freiburg, Germany*

Received 23 April 2004;
revised 25 June 2004;
accepted 28 June 2004.
**Correspondence* (fax (49)-761-2036945; e-mail eva.decker@biologie.uni-freiburg.de)
†These authors contributed equally to this work.

Keywords: fucosyltransferase, gene targeting, glycosylation, *Physcomitrella*, plant-made pharmaceuticals, VEGF, xylosyltransferase.

Summary

Using plants as production factories for therapeutic proteins requires modification of their *N*-glycosylation pattern because of the immunogenicity of plant-specific sugar residues. In an attempt towards such humanization, we disrupted the genes for α1,3-fucosyltransferase and β1,2-xylosyltransferase in *Physcomitrella patens* by homologous recombination. The single Δ*fuc-t* and Δ*xyl-t* plants, as well as the double knockout, lacked transcripts of the corresponding genes, but did not differ from the wild-type moss in morphology, growth, development, and ability to secrete a recombinant protein, the human vascular endothelial growth factor $VEGF_{121}$, into the culture medium. *N*-Glycan analysis, however, revealed the absence of 1,3-fucosyl and/or 1,2-xylosyl residues, respectively. Therefore, the modifications described here represent the key step towards the generation of moss lines suitable for the production of plant-made glycosylated biopharmaceuticals with nonallergenic *N*-glycans.

Introduction

Plants have great potential as biofactories for the production of therapeutic proteins. Consequently, the number of recombinant proteins successfully produced in plants is rapidly increasing (Dieryck *et al*., 1997; Ko *et al*., 2003; Ma *et al*., 2003). The advantages offered by plants compared with other production systems, such as bacteria, yeast, animals, and mammalian cell lines, include low production costs and excellent safety through minimized risks of contamination (Raskin *et al*., 2002; Fischer *et al*., 2004). Although plants perform eukaryotic post-translational modifications and protein processing, i.e. glycosylation, folding, and multimeric assembly (Raskin *et al*., 2002; Gomord and Faye, 2004), plant *N*-glycans differ considerably from the mammalian type and may cause allergic reactions (van Ree *et al*., 2000; Foetisch *et al*., 2003; Westphal *et al*., 2003). These differences represent a serious drawback for the production of glycosylated pharmaceutical proteins. The immunogenicity of complex plant *N*-glycans in animals and humans is well documented, and is related to the presence of a β1,2-xylose and an α1,3-fucose linked to the glycan core (Figure 1) (van Ree *et al*., 2000; Wilson *et al*., 2001; Bardor *et al*., 2003). Therefore, to overcome this limitation of plants as production factories for therapeutic proteins, a change in their *N*-glycosylation pattern, i.e. by knocking out the corresponding plant-specific genes, is required.

So far, the moss, *Physcomitrella patens*, represents the only feasible plant system for targeted gene knockouts because of its high rate of homologous recombination, which is unique among plants (Strepp *et al*., 1998; Imaizumi *et al*., 2002; Koprivova *et al*., 2002; Lorenz *et al*., 2003). *Physcomitrella* offers several additional advantages with respect to the production of recombinant proteins: (i) photoautotrophic cultivation in bioreactors under controlled conditions, providing strict biological containment (Hohe *et al*., 2002; Decker and Reski, 2004); (ii) the possibility of the secretion of recombinant proteins into a simple medium, thus lowering the costs for downstream processing (Reski and Gorr, 2001); and (iii) a simple transformation procedure and short time from transformation to the characterization of transgenic plants (Schaefer, 2002).

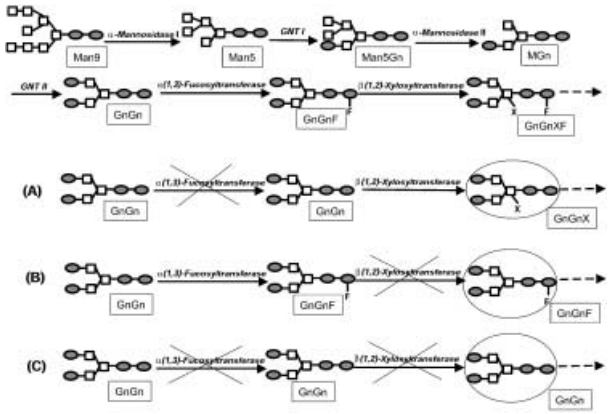

Figure 1 Processing of plant *N*-linked glycans in the Golgi apparatus. ⬭, *N*-acetylglucosamine; □, mannosyl residue; F, α1,3-fucosyl residue; X, β1,2-xylosyl residue; GNTI, *N*-acetylglucosaminyl transferase I; GNTII, *N*-acetylglucosaminyl transferase II. (A) Predicted schematic structure of *N*-glycans in Δ*fuc-t* plants. (B) Predicted schematic structure of *N*-glycans in Δ*xyl-t* plants. (C) Predicted schematic structure of *N*-glycans in Δ*fuc-t* + Δ*xyl-t* plants. Glycan structures are named by the 'proglycan' system (www.proglycan.at).

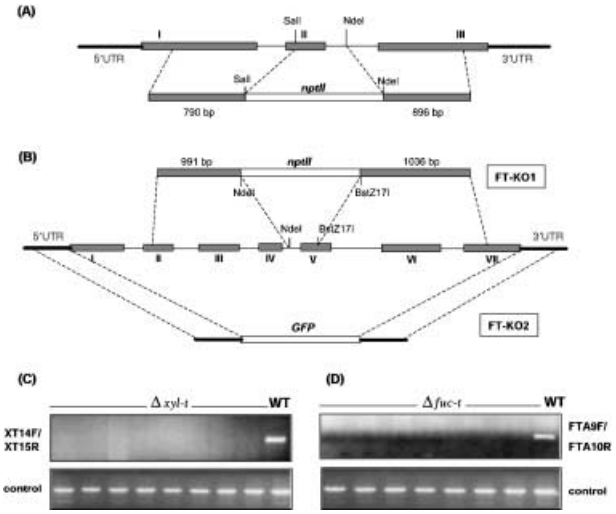

Figure 2 Schematic representation of the *fuc-t* and *xyl-t* genes, corresponding knockout constructs and molecular analysis of the knockout mutants. (A) Gene structure and knockout construct of the *xyl-t* gene. (B) Gene structure and knockout constructs (FT-KO1 and FT-KO2) of the *fuc-t* gene. Rectangles represent exons; introns are presented as lines. Grey rectangles represent the regions of the genes used for the constructs; black rectangles are untranslated gene regions. (C, D) Expression analysis of the Δ*xyl-t* and Δ*fuc-t* plants. The indicated primers span the *nptII* cassette. As a control, reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers C45F and C45R, corresponding to the constitutively expressed gene for the ribosomal protein L21. WT, wild-type.

It has been shown recently that the *N*-glycosylation pathway in *Physcomitrella patens* is similar to that in higher plants (Koprivova *et al*., 2003; Vietor *et al*., 2003). In this paper, we describe the successful production and molecular analysis of single and double knockout mutants for α1,3-fucosyltransferase (Fuc-T) and β1,2-xylosyltransferase (Xyl-T) in *Physcomitrella patens*. We demonstrate the disappearance of allergenic sugar residues from glycans in these plants, thus opening up the possibility of the production of safe proteins for therapeutic applications.

A preliminary account of these results was given at the Conference on Plant-Made Pharmaceuticals, March 16–19, 2003, Quebec City, Canada.

Results

Generation and molecular characterization of the knockout plants for 1,3-Fuc-T and 1,2-Xyl-T

The cloning of cDNA and genes for Xyl-T and Fuc-T has been described recently (Koprivova *et al*., 2003). Disruption constructs for *xyl-t* and *fuc-t* (FT-KO1) were created by exchanging small fragments of the genes by the *nptII* selection cassette (Figure 2A,B). Based on polymerase chain reaction (PCR) analysis, detecting the *nptII* cassette, disruption of the original genes, as well as correct integration of the transgenes on the 5′- and 3′-ends, eight plants were identified as putative Xyl-T knockouts (Δ*xyl-t*) and seven plants as Fuc-T knockouts (Δ*fuc-t*) (data not shown). Southern analysis confirmed disruption of the wild-type (WT) loci in these plants (data not shown). Expression analysis based on reverse transcription-polymerase chain reaction (RT-PCR) with the primers flanking the *nptII* cassette proved the absence of the transcripts in both groups of transformants, identifying them as true Δ*xyl-t* and Δ*fuc-t* plants (Figure 2C,D).

To analyse the influence of the loss of *xyl-t* and *fuc-t* genes on protein secretion in *Physcomitrella patens*, recombinant human vascular endothelial growth factor $VEGF_{121}$ protein was produced in transient expression assays and detected by enzyme-linked immunosorbent assay (ELISA) and Western blot with anti-VEGF antibodies (Table 1, Figure 3). The WT as well as the two knockouts secreted the protein in similar quantities. Thus, the disruption of *xyl-t* and *fuc-t* did not affect the secretion of the recombinant human glycoprotein.

Production of the double (Δ1,3-*fuc-t*/Δ1,2-*xyl-t*) knockout

As the single knockout lines did not show any alterations in morphology and development, we decided to produce double knockout mutants by subsequent transformation of an existing Δ*xyl-t* mutant with the new construct FT-KO2 (Figure 2B).

Table 1 Transient secretion of the human vascular endothelial growth factor $VEGF_{121}$ protein

	Concentration (ng/mL)	*n* (experiments)
Wild-type	26.8 ± 5.4	4
XT knockout	22.5 ± 9.3	3
FT knockout	27.2 ± 10.0	7

Expressed human $VEGF_{121}$ was used as the reference in the secretion assays. Transformed protoplasts were cultivated in 96-well plates and the culture medium was collected 5 days after transformation. Recombinant human $VEGF_{121}$ expressed and secreted by transiently transformed moss protoplasts into the culture medium was quantified by enzyme-linked immunosorbent assay (ELISA). The concentration measured in one experiment was an average of at least eight independent transformations.

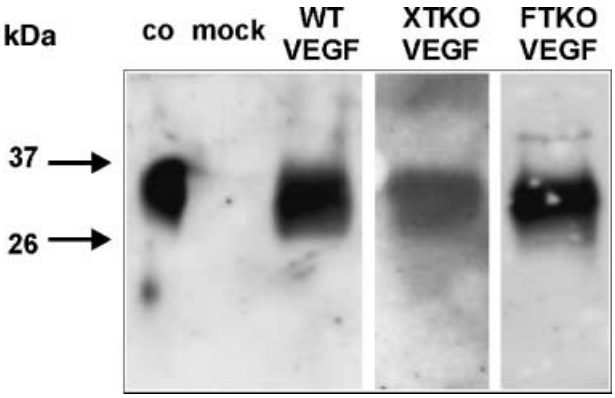

Figure 3 Secretion analysis of transiently expressed recombinant human vascular endothelial growth factor $VEGF_{121}$. $VEGF_{121}$ was expressed by transiently transformed protoplasts. The protein was secreted into the culture medium and quantified by ELISA. co, purchased VEGF (R&D Systems); mock, wild-type protoplasts transformed with an empty vector; WT VEGF, wild-type transiently expressing VEGF; XTKO VEGF, *xyl-t* knockout transiently expressing VEGF; FTKO VEGF, *fuc-t* knockout transiently expressing VEGF.

For this construct, 5′- and 3′-untranslated regions of the *fuc-t* gene were used, resulting in the replacement of the whole *fuc-t* coding sequence by a GFP cassette. PCR analysis of the transgenic plants revealed integration of the construct on both 5′- and 3′-ends of the original *fuc-t* locus (data not shown), thus indicating their double knockout nature. Like the single knockout plants, the double mutant did not show any phenotypic alterations compared with the WT (Figure 4A–D).

The knockouts exhibit alterations in their glycosylation pattern

To explore whether the disruption of the *xyl-t* and *fuc-t* genes indeed resulted in changes in the glycosylation pattern, *N*-glycans of the *Physcomitrella* WT, as well as the different

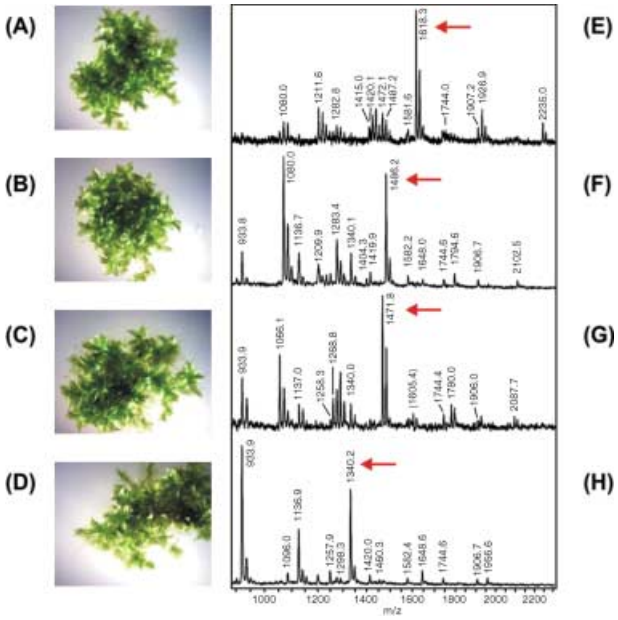

Figure 4 Analysis of knockout mutants. (A–D) Phenotypes of wild-type (WT), Δ*xyl-t*, Δ*fuc-t*, and Δ*xyl-t*/Δ*fuc-t* plants, respectively (sixfold magnification). Plants were grown under normal growth conditions on solid Knop medium. (E–H) *N*-Glycan analysis. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of the *N*-linked glycans from WT, Δ*xyl-t*, Δ*fuc-t*, and Δ*xyl-t*/Δ*fuc-t* plants, respectively. Arrows indicate the mass shifts of 132 and 146 mass units for the loss of xylose and fucose, respectively. A mass shift of 278 units is indicative of the simultaneous loss of xylose and fucose in the double mutant.

knockout lines, were analysed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. Although the major peaks in the glycan spectrum of WT moss represented complex-type *N*-glycans with both xylose and fucose, e.g. GnGnXF, the Δ*xyl-t* plants exhibited structures with fucose only (GnGnF) and, accordingly, the Δ*fuc-t* plants presented GnGnX as the main structure (Figure 4E–G, Table 2).

In the double knockout plants, the *N*-glycans lacked both 1,3-fucosyl and xylosyl residues, and 'GnGn' represented the main glycan structure (Figure 4H, Table 2). Our analyses of intracellular *N*-glycan structures revealed the specificity of the genetic modifications, as the synthesis of a small fraction of Lewis A residues, which were added by the action of different enzymes (Wilson, 2000; Koprivova *et al*., 2003), was not affected.

Discussion

N-Glycosylation is a eukaryotic post-translational modification of proteins necessary for correct folding, protection from proteolytic degradation, thermal stability, secretion, and biological function (Lerouge *et al*., 1998; Gomord and Faye,

Table 2 Structures and corresponding calculated masses of the major *N*-glycans for the wild-type moss, Δ*fuc-t*, Δ*xyl-t* and Δ*fuc-t* + Δ*xyl-t* plants

Wild-type		Δ*xyl-t*		Δ*fuc-t*		Δ*xyl-t* + Δ*fuc-t*	
Mass	Structure	Mass	Structure	Mass	Structure	Mass	Structure
1212.1	MMXF	1080.0	MMF	1065.9	MMX	933.8	MM
1415.3	GnMXF/MGnXF	1283.2	GnMF/MGnF	1269.1	GnMX/MGnX	1137.0	GnM/MGn
1618.3	GnGnXF	1486.4	GnGnF	1472.3	GnGnX	1340.2	GnGn
1926.8	(FA)GnXF/Gn(FA)XF	1794.7	(FA)GnF/Gn(FA)F	1780.6	(FA)GnX/Gn(FA)X	1648.5	(FA)Gn/Gn(FA)
2235.0	(FA)(FA)XF	2102.9	(FA)(FA)F	2088.9	(FA)(FA)X	1956.8	(FA)(FA)

A, galactose; F, fucose; (FA), Lewis A determinant; Gn, *N*-acetylglucosamine; M, mannose; X, xylose. For the corresponding structures, refer to Figure 1. Glycan structures are named by the 'proglycan' system (http://www.proglycan.com). The name (FA)(FA)XF denotes a glycan with two Lewis A determinants and xylose as well as fucose linked to the chitobiose, whereas in (FA)(FA)X and (FA)(FA)F there is only a xylose or a fucose, respectively.

2004). It can also influence the clearance rate, specific activity, and ligand–receptor interactions of the protein. However, the structure of the *N*-glycans is highly but not completely conserved between the plant and animal kingdoms which, consequently, leads to the immunogenicity of plant-specific glycans in animals (van Ree *et al*., 2000; Wilson *et al*., 2001; Bardor *et al*., 2003). To use recombinant proteins in human medicine, their glycosylation pattern needs to be human-like in order to avoid immunogenicity issues and to optimize pharmacokinetic parameters. This is reflected, for example, by the recent attempts to humanize the *N*-glycosylation pathway in the yeast *Pichia pastoris* (Hamilton *et al*., 2003). Plants possess several advantages over yeasts as production factories of therapeutic proteins but, clearly, only plants with humanized glycosylation can be useful for large-scale production.

Therefore, different strategies for the humanization of *N*-glycosylation have been explored in plants (Gomord *et al*., 2004). One drastic approach is to prevent binding of the immunogenic *N*-glycans on the recombinant protein by mutation of the *N*-glycosylation sites. However, this strategy cannot be used for heterologous proteins which require *N*-glycosylation for their stability and biological activity. Recombinant proteins may be retained in the endoplasmic reticulum, thus avoiding plant-specific modifications taking place in the Golgi apparatus (Ko *et al*., 2003). However, such proteins possess exclusively high-mannose-type *N*-glycans and have poor stability after injection into mice (Ko *et al*., 2003). Alternatively, plant-specific glycosyltransferases can be disrupted and/or human ones introduced. The first plant deficient in any glycosyltransferase was the *Arabidopsis thaliana cgl* mutant lacking GlcNAc transferase I (GNTI), which cannot produce complex-type glycans (von Schaewen *et al*., 1993). In contrast with mice, where *gntI* deficiency has been shown to be lethal (Ioffe and Stanley, 1994), the *cgl* plants completed their generation cycle normally. Complex glycans thus do not seem to be essential for the normal development of plants, and modifications in this pathway most probably would not interfere with their life cycle. In addition, human glycosyltransferases, such as GNTI (Gomez and Chrispeels, 1994) and β1,4-galactosyltransferase (Palacpac *et al*., 1999; Bakker *et al*., 2001; Misaki *et al*., 2003), are functionally expressed in plants, showing that human enzymes can be targeted to the Golgi apparatus. However, *N*-glycans of the proteins produced in these studies are highly heterogeneous and only partially humanized (Bakker *et al*., 2001).

The best solution for the alteration of plant glycosylation in this way would therefore be to knock out the genes for enzymes linking plant-specific sugar residues. Recently, Strasser and coworkers have characterized Xyl-T- and Fuc-T-deficient mutants of the model plant *Arabidopsis thaliana* (Strasser *et al*., 2004).

To produce plants with humanized complex-type *N*-glycans, we chose the moss *Physcomitrella patens*, which allows efficient gene targeting not found in higher plants and, moreover, is a potent system for the production of recombinant proteins (Decker and Reski, 2004). In the first approach, we disrupted the *gntI* gene, expecting to change the structures of *N*-glycans from complex to oligomannosidic type (Koprivova *et al*., 2003). However, no alteration in the glycan structure was observed, which might be caused by an additional isoform of *gntI* in *Physcomitrella,* indicating that the downstream enzymes in the pathway may be better targets (Koprivova *et al*., 2003). Therefore, in the present study, we knocked out the genes for Xyl-T and Fuc-T. The knockouts did not show any alterations in phenotype and morphological development when compared with WT moss under standard conditions. On the other hand, we expected the *N*-glycans of the Δ*fuc-t* and Δ*xyl-t* plants to lack the 1,3-fucose and 1,2-xylose, respectively (Figure 1). Indeed, MALDI spectra clearly proved the disappearance of these residues from the *N*-glycan

structures of the corresponding knockouts. Obviously, Fuc-T and Xyl-T are encoded by single copy genes and no other moss enzyme can substitute them. Despite the conserved character of these two sugar residues in the whole plant kingdom, the 1,3-fucose and 1,2-xylose of plant *N*-glycans are dispensable under our growth conditions, as plants lacking Fuc-T or Xyl-T activity were not impaired in growth and morphology. Moreover, the human glycosylated growth factor $VEGF_{121}$ was secreted from the knockout plants as effectively as from WT. Based on these results, we also produced a double knockout which lacked both fucosyl and xylosyl residues. As in the case of the single knockouts, this plant did not show any obvious morphological or physiological differences. The structures containing core-fucosyl and xylosyl residues, however, were completely absent from the *N*-glycan spectrum of this mutant. This is a very important finding, as 1,3-fucose and 1,2-xylose residues, being potent immunogens and even allergens (Wilson *et al*., 2001), represent a major drawback for the use of plants for the production of therapeutic proteins.

Thus, the moss strains generated in this study open up the possibility of using *Physcomitrella patens* as a safe production system for biopharmaceuticals with human-like glycosylation patterns.

Experimental procedures

Plant material and transformation procedure

Physcomitrella patens (Hedw.) B.S.G. was cultivated as described previously (Reski and Abel, 1985). Protoplasts were isolated from semicontinuous bioreactor cultures (Hohe *et al*., 2002) and transformed as described previously (Strepp *et al*., 1998). For selection, Knop media were supplemented with 50 μg/mL G418.

Creating knockout constructs for Fuc-T and Xyl-T

The cloning of cDNAs and genes for Fuc-T and Xyl-T (Accession Nos. AJ429145 and AJ429144, respectively) has been described previously (Koprivova *et al*., 2003). Knockout construction was performed by PCR amplification of a 2156-bp genomic *fuc-t* fragment (FT-KO1) (nucleotides (nt) 116–1034 of the cDNA) and a 2066-bp *xyl-t* fragment (nt 271–1750 of the cDNA). The *nptII* cartridge was inserted into the *fuc-t* fragment via PCR-introduced *Nde*I and BstZ17I restriction sites excising a 195-bp fragment. In the *xyl-t* construct, the *nptII* cassette was exchanged against a 380-bp genomic fragment via restriction digest with *Sal*I and *Nde*I.

For the FT-KO2 construct used for subsequent transformation of Δ*xyl-t* plants, the *fuc-t* coding sequence was replaced by the GFP coding sequence with the chloroplast transit sequence from *P. patens ftsZ1* (Kiessling *et al*., 2000) under control of a 35S promoter and *nos* terminator. 5′- and 3′-untransformed region (UTR) sequences of the *fuc-t* gene were used in this plasmid for homologous recombination (Figure 2B). As a selection marker in the subsequent transformation procedure, a plasmid containing the gene responsible for hygromycin resistance was used. Transformations were performed with 25 μg of linearized DNA.

Nucleic acid procedures

Pre-screening of about 100 transgenic plants for each construct was performed as described previously (Koprivova *et al*., 2002). Plants that gave the expected fragments after all four PCR reactions (confirmation of the presence of the *nptII* cassette, disruption of the original locus and correct integration on the 5′- and 3′-ends) were considered as putative knockouts and selected for further analysis.

Total RNA and DNA were isolated from moss tissue as described previously (Bierfreund *et al*., 2003). RT-PCR with primers FTA9F (ATGCTCCCAGCCCAAGAC)/FTA10R (TGTCTACTAGAGCTAGAAACG) and XT14F (TTACGAAGCACACCATGC)/XT15R (GTCCTGTTAAATGCCTTGC) for *fuc-t* and *xyl-t,* respectively, was performed according to standard protocols. As a control, RT-PCR was performed with primers C45F (GGTTGGTCATGGGTTGCG) and C45R (GAGGTCAACTGTCTCGCC) corresponding to the constitutively expressed gene for the ribosomal protein L21.

Analysis of protein secretion in transiently transformed moss protoplasts

A human blood protein, the angiogenic $VEGF_{121}$ (kindly provided by Dr Weich, GBF, Germany), was used as the reference in the secretion assays. The cDNA coding for the VEGF signal peptide and $VEGF_{121}$ with the His-Tag sequence was cloned into the vector pRT101 (Töpfer *et al*., 1987) under the control of the 35S promoter. For protoplast isolation, moss protonema was grown as described previously (Rother *et al*., 1994). Transformed protoplasts were further cultivated in 96-well plates (Nunc, Wiesbaden, Germany) containing 0.01% bovine serum albumin (BSA) (Serva, Heidelberg, Germany) in dim light (4.6 μmol/s/m^2) at 25 °C. The culture medium was replaced by fresh medium at day 1 and carefully collected 5 days after transformation. Recombinant $VEGF_{121}$ expressed by transiently transformed moss protoplasts and secreted

into the culture medium was quantified by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Western blot analysis

Recombinant $VEGF_{121}$ was collected using Ni-NTA Magnetic Agarose Beads (Qiagen, Hilden, Germany). Equal volumes of all samples were separated by 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and transferred on to an Immobilon-P membrane (Millipore, Schwalbach, Germany). The membrane was blocked overnight at 4 °C with 5% skimmed milk powder (Fluka, Steinheim, Germany) in Tris-buffered saline containing 0.05% Tween 20 (TBST), and subsequently incubated for 1.5 h with VEGF-specific goat antihuman antibody (R&D Systems) diluted 1 : 1250 in TBST containing 2.5% skimmed milk powder. After washing in TBST, rabbit antigoat immunoglobulin G (IgG) conjugated to horseradish peroxidase (Sigma-Aldrich, Munich, Germany), diluted 1 : 125 000, was added, and membranes were stained for 5 min with the ECL Advance Western Blotting Detection Kit (Amersham, Freiburg, Germany). $rhVEGF_{121}$ (R&D Systems) was used as a positive control.

Analysis of *N*-linked glycans

Structural analysis of *N*-glycans was performed with a modification of the previously described procedure (Fabini *et al*., 2001; Wilson *et al*., 2001). A portion of 300–500 mg of frozen moss tissue was homogenized in 2.5 mL of 5% formic acid and digested overnight with 0.5 mg pepsin. Glycopeptides were isolated from the digest by binding to Dowex 50W2 and by gel filtration. The glycopeptides were then digested with peptide:*N*-glycosidase A (Roche, Mannheim, Germany). The resultant oligosaccharides were isolated by a second passage over Dowex 50W2, whereby the non-binding fraction was further purified by passage over a 25 mg Zorbax SPE C18 cartridge (Agilent, Waldbronn, Germany). The aqueous flow through was lyophilized, dissolved in 20 μL of water and subjected to MALDI mass spectrometry on a Dynamo instrument (ThermoBioAnalysis, Santa Fe, NM, USA) using 2,5-dihydroxybenzoic acid (2% in 30% acetonitrile) as the matrix.

Acknowledgements

We thank Melanie Schneider, Agnes Kinal, Anja Koch, Carla Hehn, and Karin Polacsek for excellent technical support.

This work was supported by a grant from the German Federal Ministry of Education and Research (BMBF) no. 0312624.

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.

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-(1,3)-fucose and core xylose. *Glycobiology*, **13**, 427–434.

Bierfreund, N.M., Reski, R. and Decker, E.L. (2003) Use of an inducible reporter gene system for the analysis of auxin distribution in the moss *Physcomitrella patens*. *Plant Cell Report*, **21**, 1143–1152.

Decker, E.L. and Reski, R. (2004) The moss bioreactor. *Curr. Opin. Plant Biol*. **7**, 166–170.

Dieryck, W., Pagnier, J., Poyart, C., Marden, M.C., Gruber, V., Bournat, P., Baudino, S. and Merot, B. (1997) Human haemoglobin from transgenic tobacco. *Nature*, **386**, 29–30.

Fabini, G., Freilinger, A., Altmann, F. and Wilson, I.B. (2001) Identification of core α1,3-fucosylated glycans and cloning of the requisite fucosyltransferase cDNA from *Drosophila melanogaster*: Potential basis of the neural anti-horseradish peroxidase epitope. *J. Biol. Chem*. **276**, 28 058–28 067.

Fischer, R., Stoger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol*. **7**, 152–158.

Foetisch, K., Westphal, S., Lauer, I., Retzek, M., Altmann, F., Kolarich, D., Scheurer, S. and Vieths, S. (2003) Biological activity of IgE specific for cross-reactive carbohydrate determinants. *J. Allergy Clin. Immunol*. **111**, 889–896.

Gomez, L. and Chrispeels, M.J. (1994) Complementation of an *Arabidopsis thaliana* mutant that lacks complex asparagine-linked glycans with the human cDNA encoding N-acetylglucosaminyltransferase I. *Proc. Natl. Acad. Sci. USA*, **91**, 1829–1833.

Gomord, V. and Faye, L. (2004) Posttranslational modification of therapeutic proteins in plants. *Curr. Opin. Plant Biol*. **7**, 171–181.

Gomord, V., Sourrouille, C., Fitchette, A.-C., Bardor, M., Pagny, S., Lerouge, P. and Faye, L. (2004) Production and glycosylation of plant-made pharmaceuticals: the antibodies as a challenge. *Plant Biotechnol. J*. **2**, 83–100.

Hamilton, S.R., Bobrowicz, P., Bobrowicz, B., Davidson, R.C., Li, H., Mitchell, T., Nett, J.H., Rausch, S., Stadheim, T.A., Wischnewski, H., Wildt, S. and Gerngross, T.U. (2003) Production of complex human glycoproteins in yeast. *Science*, **301**, 1244–1246.

Hohe, A., Decker, E.L., Gorr, G., Schween, G. and Reski, R. (2002) Tight control of growth and cell differentiation in photoautotrophically growing moss (*Physcomitrella patens*) bioreactor cultures. *Plant Cell Report*, **20**, 1135–1140.

Imaizumi, T., Kadota, A., Hasebe, M. and Wada, M. (2002) Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*. *Plant Cell*, **14**, 373–386.

Ioffe, E. and Stanley, P. (1994) Mice lacking N-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential

role for complex or hybrid N-linked carbohydrates. *Proc. Natl. Acad. Sci. USA*, **91**, 728–732.

Kiessling, J., Kruse, S., Rensing, S.A., Harter, K., Decker, E.L. and Reski, R. (2000) Visualization of a cytoskeleton-like FtsZ network in chloroplasts. *J. Cell Biol*. **151**, 945–950.

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.

Koprivova, A., Altmann, F., Gorr, G., Kopriva, S., Reski, R. and Decker, E.L. (2003) N-glycosylation in the moss *Physcomitrella patens* is organized similarly to that in higher plants. *Plant Biol*. **5**, 582–591.

Koprivova, A., Meyer, A.J., Schween, G., Herschbach, C., Reski, R. and Kopriva, S. (2002) Functional knockout of the adenosine 5′-phosphosulfate reductase gene in *Physcomitrella patens* revives an old route of sulfate assimilation. *J. Biol. Chem*. **277**, 32 195–32 201.

Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fischette-Laine, A.C., Gomord, V. and Faye, L. (1998) N-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol. Biol*. **38**, 31–48.

Lorenz, S., Tintelnot, S., Reski, R. and Decker, E.L. (2003) Cyclin D-knockout uncouples developmental progression from sugar availability. *Plant Mol. Biol*. **53**, 227–236.

Ma, J.K.C., Drake, P.M.W. and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet*. **4**, 794–805.

Misaki, R., Kimura, Y., Palacpac, N.Q., Yoshida, S., Fujiyama, K. and Seki, T. (2003) Plant cultured cells expressing human beta1,4-galactosyltransferase secrete glycoproteins with galactose-extended N-linked glycans. *Glycobiology*, **13**, 199–205.

Palacpac, N.Q., Yoshida, S., Sakai, H., Kimura, Y., Fujiyama, K., Yoshida, T. and Seki, T. (1999) Stable expression of human β1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. *Proc. Natl. Acad. Sci. USA*, **96**, 4692–4697.

Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Neal, J.M., Cornwell, T., Pastor, I. and Fridlender, B. (2002) Plants and human health in the twenty-first century. *Trends Biotechnol*. **20**, 522–531.

van Ree, R., Cabanes-Macheteau, M., Akkerdaas, J., Milazzo, J.P., Loutelier-Bourhis, C., Rayon, C., Villalba, M., Koppelman, S., Aalberse, R., Rodriguez, R., Faye, L. and Lerouge, P. (2000) β(1,2)-xylose and α(1,3)–fucose residues have a strong contribution in IgE binding to plant glycoallergens. *J. Biol. Chem*. **275**, 11 451–11 458.

Reski, R. and Abel, W. (1985) Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. *Planta*, **165**, 354–358.

Reski, R. and Gorr, G. (2001) *Method for Production of Proteinaceous Substances*. Patent: WO 01/25456 A2.

Rother, S., Hadeler, B., Orsini, J.M., Abel, W.O. and Reski, R. (1994) Fate of a mutant macrochloroplast in somatic hybrids. *J. Plant Physiol*. **143**, 72–77.

Schaefer, D.G. (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. *Annu. Rev. Plant. Biol*. **53**, 477–501.

von Schaewen, A., Sturm, A., O'Neill, J. and Chrispeels, M.J. (1993) Isolation of a mutant *Arabidopsis* plant that lacks N-acetyl glucosaminyl transferase I and is unable to synthesize Golgi-modified complex N-linked glycans. *Plant Physiol*. **102**, 1109–1118.

Strasser, R., Altmann, F., Mach, L., Glössl, J. and Steinkellner, H. (2004) Generation of *Arabidopsis thaliana* plants with complex N-glycans lacking β1,2-linked xylose and core α1,3-linked fucose. *FEBS Lett*. **561**, 132–136.

Strepp, R., Scholz, S., Kruse, S., Speth, V. and Reski, R. (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. *Proc. Natl. Acad. Sci. USA*, **95**, 4368–4373.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H.-H. (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res*. **15**, 5890.

Vietor, R., Loutelier-Bourhis, C., Fitchette, A.C., Margerie, P., Gonneau, M., Faye, L. and Lerouge, P. (2003) Protein N-glycosylation is similar in the moss *Physcomitrella patens* and in higher plants. *Planta*, **218**, 269–275.

Westphal, S., Kolarich, D., Foetisch, K., Lauer, I., Altmann, F., Conti, A., Crespo, J.F., Rodriguez, J., Enrique, E., Vieths, S. and Scheurer, S. (2003) Molecular characterization and allergenic activity of Lyc e 2 (beta-fructofuranosidase), a glycosylated allergen of tomato. *Eur. J. Biochem*. **270**, 1327–1337.

Wilson, I.B. (2000) Identification of a cDNA encoding a plant Lewis-type alpha1,4-fucosyltransferase. *Glycoconj. J*. **18**, 439–447.

Wilson, I.B., Zeleny, R., Kolarich, D., Staudacher, E., Stroop, C.J., Kamerling, J.P. and Altmann, F. (2001) Analysis of Asn-linked glycans from vegetable foodstuffs: widespread occurrence of Lewis a, core alpha1,3-linked fucose and xylose substitutions. *Glycobiology*, **11**, 261–274.