Glyco-Engineering of Moss Lacking Plant-Specific Sugar Residues

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Abstract: **The commercial production of complex pharmaceutical proteins from human origin in plants is currently limited through differences in protein N-glycosylation pattern between plants and humans. On the one hand, plant-specific α(1,3)-fucose and β(1,2)-xylose residues were shown to bear strong immunogenic potential. On the other hand, terminal β(1,4)-galactose, a sugar common on N-glycans of pharmaceutically relevant proteins, e.g., antibodies, is missing in plant N-glycan structures. For safe and flexible production of pharmaceutical proteins, the humanisation of plant protein N-glycosylation is essential. Here, we present an approach that combines avoidance of plant-specific and introduction of human glycan structures. Transgenic strains of the moss *Physcomitrella patens* were created in which the α(1,3)-fucosyltransferase and β(1,2)-xylosyltransferase genes were knocked out by targeted insertion of the human β(1,4)-galactosyltransferase coding sequence in both of the plant genes (knockin). The transgenics lacked α(1,3)-fucose and β(1,2)-xylose residues, whereas β(1,4)-galactose residues appeared on protein N-glycans. Despite these significant biochemical changes, the plants did not differ from wild type with regard to overall morphology under standard cultivation conditions. Furthermore, the glyco-engineered plants secreted a transiently expressed recombinant human protein, the vascular endothelial growth factor, in the same concentration as unmodified moss, indicating that the performed changes in glycosylation did not impair the secretory pathway of the moss. The combined knockout/knockin approach presented here, leads to a new generation of engineered moss and towards the safe and flexible production of correctly processed pharmaceutical proteins with humanised N-glycosylation profiles.**

Key words: **N-glycosylation, gene knockout, glyco-engineering, *Physcomitrella patens*, complex biopharmaceuticals, VEGF.**

Abbreviations:

FT:	α(1,3)-fucosyltransferase
XT:	β(1,2)-xylosyltransferase
GT:	β(1,4)-galactosyltransferase
ko:	knockout
ki:	knockin
dokoki:	double knockout/knockin
VEGF:	vascular endothelial growth factor
CaMV	cauliflower mosaic virus

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Introduction

Plants have a high potential to be used as bioreactors for the economic and safe production of biopharmaceuticals, i.e., recombinant proteins for therapeutic or diagnostic use (Ma et al., 2003; Fischer et al., 2004). They overcome the main limitations of the commonly used expression systems, i.e., bacteria and mammalian cell lines. The risk of product contamination by bacterial endotoxins, human pathogenic viruses, prions, or oncogenic DNA is minimised in transgenic plants (Hood et al., 2002; Raskin et al., 2002), overall costs are outstandingly low and the capacity for scale up of production volumes is high (Ma et al., 2003; Schillberg et al., 2003).

As opposed to prokaryotes, plants and mammals share similar post-translational protein modifications. Both perform eukaryotic folding, phosphorylation, complex glycosylation, and multimeric assembly (Raskin et al., 2002; Obermeyer et al., 2004). The major issue for plant-derived pharmaceuticals appears in protein N-glycosylation. Plant-derived proteins lack the terminal β(1,4)-galactose and acetylneuraminic acid (sialic acid) residues that are normally found in humans, but they have fucose α(1,3)-linked, instead of α(1,6)-linked, to the core glycan (Fig. **1**), and contain β(1,2)-xylose, a sugar which is absent in N-glycans of human proteins (van Ree et al., 2000; Bakker et al., 2001). The specific α(1,3)-fucose and β(1,2)-xylose epitopes are known to be immunogenic (Wilson et al., 2001; Bardor et al., 2003) in humans and mice and may cause allergic reactions (Garcia-Casado et al., 1996; van Ree et al., 2000; Foetisch et al., 2003; Westphal et al., 2003). It was also shown previously that variations in N-glycosylation pattern may influence pharmacokinetics and activity of biopharmaceuticals (Wright et al., 2000; Lisowska, 2002; Shields et al., 2002; Gomord and Faye, 2004). Thus, the use of plants for the production of pharmaceutical proteins inescapably necessitates the adaptation of the N-glycosylation from plants to humans, i.e., by knockout of plant-specific glycosyltransferase genes as well as the insertion of human glycosyltransferases. Attempts to engineer plant glycosylation by insertion of the human β(1,4)-galactosyltransferase *(GT)* gene have been per-

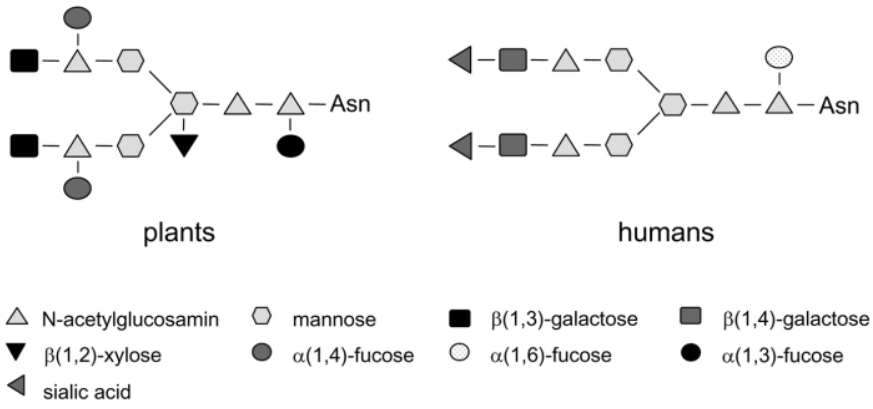


Fig. 1 Complex-type glycan structure in plants and humans. The core glycan, consisting of four N-acetylglucosamine and three mannose residues is identical in both carbohydrate structures. The different sugar moieties of which the complex-type glycans consist are indicated in the lower part of the figure. The most critical differences between plant and mammalian N-glycans are the plant-specific β(1,2)-xylose as well as the core-fucose moiety which is α(1,3)-linked in plants and α(1,6)-linked in mammals.

formed before (Palacpac et al., 1999; Bakker et al., 2001). Loss of immunogenic plant-specific glycosylation was achieved recently by screening for T-DNA insertions within the α(1,3)-fucosyltransferase *(FT)* and β(1,2)-xylosyltransferase *(XT)* genes in the model plant *Arabidopsis thaliana* (Strasser et al., 2004).

We established the moss *Physcomitrella patens* as a system for producing complex biopharmaceuticals (Decker and Reski, 2004). Photoautotrophic cultivation in bioreactors provides controlled conditions in strict biological containment (Hohe et al., 2002) where recombinant proteins can be produced under GMP (good manufacturing practices) conditions. The possibility to secrete heterologous proteins into the medium has major advantages for product purification, resulting in reduced downstream processing costs (Twyman et al., 2003; Decker and Reski, 2004; Gomord et al., 2004). Although separated from seed plants by approximately 450 million years of evolution, the *P. patens* protein N-glycosylation pattern has been shown to be organised in a similar way to that of higher plants (Koprivova et al., 2003; Vietor et al., 2003). Based on these results, we disrupted the moss-α(1,3)-*FT* and -β(1,2)-*XT* genes by knockouts via homologous recombination (Koprivova et al., 2004) which occurs in the moss at an exceptionally high rate (Girke et al., 1998; Holtorf et al., 2002; Schaefer, 2002), thus allowing targeted manipulation of the nuclear genome.

Here, we present for the first time a combined approach of targeting plant-specific glycosyltransferases and simultaneous insertion of a human terminal galactosyltransferase. Transgenic moss strains were created in which the α(1,3)-*FT* and β(1,2)-*XT* genes were disrupted by the β(1,4)-*GT* coding sequence (knockout/knockin). The transgenics displayed a humanised protein N-glycosylation pattern with a lack of immunogenic α(1,3)-fucose and β(1,2)-xylose residues and addition of terminal β(1,4)-galactose, but showed no changes in morphology and overall growth. Moreover, the plants were able to express and secrete a human protein, the vascular endothelial growth factor (VEGF), with the same efficiency as unmodified moss.

Materials and Methods

Construction of plant transformation vectors

The coding sequence of human β(1,4)-galactosyltransferase *(GT)* was amplified from human liver cDNA (Invitrogen) according to the manufacturer's instructions. *Xho*I and *Xba*I restriction sites introduced at the 5′- and 3′-end, respectively, were used for cloning the PCR product into pRT101 vector (Topfer et al., 1987) resulting in plasmid pRT101-GT with the *GT*-cDNA under control of the CaMV 35S promoter (35S-P) and 35S terminator (35S-T). For generating the knockout/knockin constructs, two fragments were PCR-amplified from *P. patens* genomic DNA. With the primers XTB-F (5′-TTG GAT CCT CAA TTA CGA AGC ACA CCA TGC-3′) and XTB-R (5′-TTG GAT CCT CCC AGA AAC ATC TGA TCC AG-3′), a 1.56-kb *XT* fragment was amplified. A 2.63-kb *FT* fragment was produced by use of primers FTB-F (5′-TAG GAT CCA GAT GAT GTC TGC TCG GCA GAA TGG-3′) and FTB-R (5′-CTG GAT CCT TGT AGA TCC GAA GGT CTG AGT TCC-3′). Additionally, a *Hin*dIII site was created in exon 3 of the *FT* fragment by insertion of a 60-bp double-stranded oligonucleotide (*SacHin*dins*Nde,* 5′-CAC AGC AAC GGA GTG GGT GTG GTG ATG ACT ACA AGC TTG TCA TCG GAT GTT CCA-3′) between restriction sites *Sac*I in intron 2 and *Nde*I in intron 5. Both fragments were cloned into pCR4-TOPO (Invitrogen), generating pCR4-FT and pCR4-XT plasmids. Ligation of *Hin*dIII-digested pRT101-GT with the accordingly digested pCR4-FT and pCR4-XT, respectively, created the gene-targeting constructs pCR4-FTko-GTki and pCR4-XTko-GTki.

Plant material and transformation

Physcomitrella patens (Hedw.) B.S.G. was cultivated according to Reski and Abel (Reski and Abel, 1985). Protoplasts were isolated from semi-continuous photoautotrophic stirred tank bioreactor cultures and transformed as described (Koprivova et al., 2004). Co-transfections with pCR4-FTko-GTki or pCR4-XTko-GTki were performed with pRT101neo vector (Girke et al., 1998) containing the neomycin phosphotransferase (nptII) cartridge. Selection was carried out on Knop medium supplemented with 25 µg/ml G418. For creation of double knockout/knockin plants, protoplasts of a XT knockout/GT knockin plant (*XT*koki) were co-transfected with the pCR4-FTko-GTki plasmid and vector pCAMBIA 1305 (Cambia, Australia), mediating hygromycin resistance. For selection, 30 µg/ml hygromycin were used.

Molecular analysis of transgenic plants

The screening for transformed plants after three weeks of selection pressure was performed by direct PCR (Schween et al., 2002) using the primers 1 (5′-CAT CGT GTG GGC GCG AAG GTG-3′), 2 (5′-GGG ACC ACT GTC GGT AGA GG-3′), 3 (5′-CCG

CCC ACG AAA AGT AGC CC-3′) for *FT*koki, and 8 (5′-GAA TTG CCG CTA TCT ACT TGT ATG C-3′), 9 (5′-AGC TGT AGT ATT CAT AGC TGC AAC G-3′), 10 (5′-GTA GTA AAC GAG GGT GAC GCC AAG G-3′), 11 (5′-GCT CTC TGA TGG TTT GAA CTC ACT CAC C-3′) for *XT*koki. Plant leaves were incubated for 30 min in PCR buffer with $(NH_4)_2SO_4$ at 45 °C, frozen for 3 h at – 20 °C and thawed at 45 °C. The achieved extract was then used for the multiplex primer PCR. Isolation of total RNA and DNA from frozen moss tissue was carried out as described previously (Bierfreund et al., 2003). For verification of *GT* transcript and the absence of corresponding wild-type *FT* and *XT* transcripts, reverse transcription (RT) PCR analyses were done according to standard protocols using the primers 4 (5′-CAG TCC CTA TTG TAA CGG GAC-3′), 5 (5′-CCT TTA ACC CCT TAT CAC CAC C-3′), 6 (5′-AGC CCA GTC GTG GAT TCT G-3′), and 7 (5′-ACA TAG ATG CCA TAG TCC AGC-3′) in the case of *FT*koki plants, and 12 (5′-TCG AAA TCA TGG TCC CAG AGA AGG AGG-3′), 13 (5′-AGC TGT AGT ATT CAT AGC TGC AAC G-3′), 6 (see above) and 14 (5′-AGG AAA TCC ATT GAT GGT TAG-3′) for *XT*koki plants. First strand synthesis was performed with Superscript II reverse transcriptase (Invitrogen).

Northern- and Southern-blot analysis

Northern blotting was accomplished as described previously (Bierfreund et al., 2003). Hybridisation was performed with [α^{32}P]-dCTP-labelled cDNA probes for *GT* for *FT*koki analysis and alkaline phosphatase (AP) labelled *GT* probes for detection in *XT*koki. Southern blotting was carried out as described previously (Lorenz et al., 2003). DNA of *FT*koki plants was digested with *Cfr*9I, which cut inside the *GT* gene. The *GT* gene targeting cartridge was cut out of *XT*koki plant DNA with *Hin*dIII. In both cases AP-labelled *GT* probes were used for hybridisation.

Western-blot analysis

For isolation of total protein from *FT*koki and *XT*koki plants, 200 mg frozen moss material were ground to fine powder in liquid nitrogen and mixed with 1 ml SDS buffer containing 10% (w/v) SDS, 50% (v/v) glycerol and 0.005% (w/v) Bromophenol blue in 62.5 mM Tris (pH 6.8). The suspension was incubated with agitation at 95 °C for 5 min. Cell remnants were removed by centrifugation at 15 000 × g for 10 min. The protein suspensions were run on 10% SDS-PAGE and blotted to Immobilon-P PVDF membranes (Millipore, Germany) or Optitran nitrocellulose membrane (Schleicher and Schuell, Germany) according to standard protocols. Equal amounts of loaded protein were determined by coomassie staining using PhastGel Blue R (Amersham, Germany). Immunodetections with anti-β(1,2)-xylose and anti-α(1,3)-fucose antibodies were carried out as described previously (Faye et al., 1993) using blocking and detection components of the DIG Glycan Differentiation Kit (Roche, Germany). For anti-HRP Western blots, 100 mg fresh weight material of wild type, *XT*koki and double knockout/knockin plant (*FT*/*XT*dokoki) were disrupted using a Mixer Mill (Retsch, Germany). After addition of 150 µl 1 × PBS and centrifugation (16 060 × g, 3 min), protein concentrations within the supernatants were determined by the Bradford assay. 20 µg of total protein from each sample were separated on 15% SDS-PAGE. Western blot was carried out using Optitran membranes. Equality of the protein transfer was controlled by Ponceau staining. Anti-HRP (Rockland, USA, 1 : 1000) and anti-rabbit (Amersham, 1 : 8000) antibodies diluted in 1 × HSTBST (0.5 M NaCl, 20 mM Tris (pH 7.5), 0.1% Tween 20) with 2.5% (w/v) skim milk powder were used for immunodetection. Visualisation was carried out using ECL Advance Western Blotting Detection Kit (Amersham).

Detection of β(1,4)-galactose residues on glycoproteins from FT*koki plants*

Glycoproteins from *FT*koki plants were concentrated via affinity chromatography using agarose-bound *Ricinus communis* agglutinin 120 (RCA_{120}) (Vector Laboratories, Inc.). Total protein was precipitated with acetone and re-suspended in one volume 0.1% (w/v) SDS in 5 mM DTT followed by dialysis in 5000 volumes of 0.1% (v/v) Tween 20 in 50 mM ammonium bicarbonate. The protein solution was lyophilised, resolved in 15 volumes 0.1% (v/v) Tween 20 in HEPES buffer (10 mM HEPES, 150 mM NaCl) and applied onto the RCA_{120} agarose. After incubation for 2 h at 4 °C and vertical rotation with 200 rpm, the RCA_{120} matrix was washed five times with nine volumes of 0.1% (v/v) Tween 20 in HEPES buffer. The chromatography material was supplemented with one volume SDS buffer containing 10% (w/v) SDS, 50% (v/v) glycerol and 0.005% (w/v) Bromophenol blue in 62.5 mM Tris (pH 6.8) before bound glycoproteins were denatured by heating the RCA_{120} agarose to 95 °C for 10 min. SDS-PAGE of glycoprotein samples and blotting onto Immobilon-P PVDF membrane was carried out as described previously. Terminal galactose-β(1,4)-N-acetylglucosamine on membrane-bound glycoproteins was detected with peroxidase-conjugated *Datura stramonium* agglutinin (DSA) (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Transient expression and secretion of recombinant human VEGF

Pre-cultivation and isolation of protoplasts were performed as described previously (Hohe et al., 2002; Hohe and Reski, 2002). Transformation of the protoplasts was carried out as described recently (Jost et al., 2005) using 30 µg plasmid DNA per transformation. Construct 5′PpAct5::Vegf (Weise et al., in preparation) uses the 5′ region of *P. patens* actin5 gene (acc. no. AY745190) to control VEGF expression.

ELISA analysis

Supernatants from transiently 5′PpAct5::Vegf-transformed protoplasts were analysed by sandwich ELISA according to standard protocols. Anti-VEGF antibodies (#AF-293-NA, R&D, Germany) coated onto immunosorb plates (Nunc, Denmark) were used for capturing the secreted VEGF, the detection occurred via biotinylated anti-VEGF (#BAF-293, R&D) and peroxidase-linked streptavidin (Roche Molecular Biochemicals). Purchased VEGF121 (#298-VS, R&D) served as standard. Dilutions were made in 1 × PBS with 0.1% (w/v) BSA (Serva, Germany). All samples were measured in duplicate.

Results

Generation of transgenic FT*ko*/GT*ki and* XT*ko*/GT*ki plants*

Attempting a humanisation of the plant glycosylation pattern, we cloned gene targeting constructs for knockout of plant-specific *FT* and *XT* genes by simultaneous introduction of human β(1,4)-*GT* (Fig. **2A**). *Physcomitrella* protoplasts were transfect-

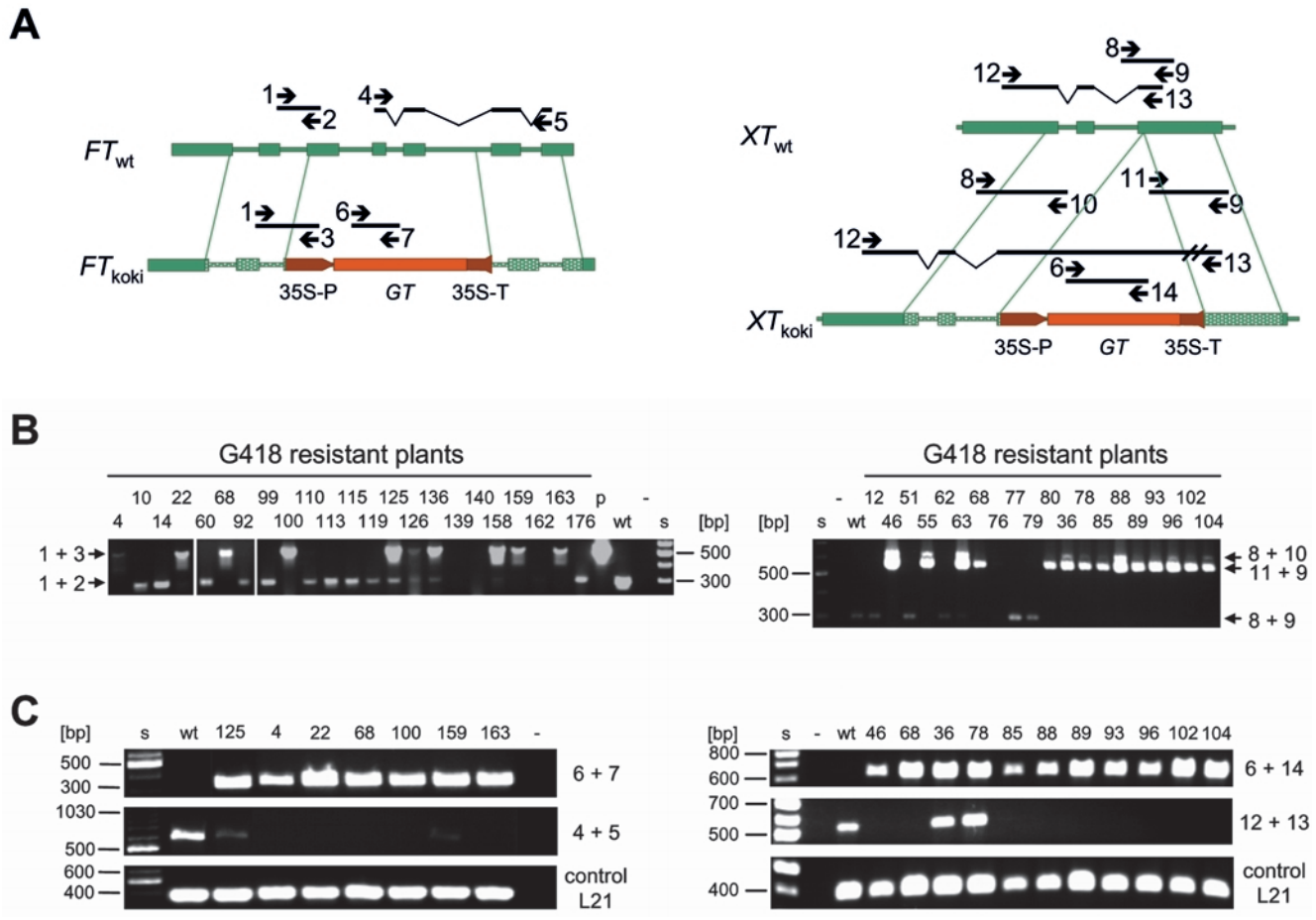


Fig. 2 Identification of transgenic *FT*koki and *XT*koki plants. (**A**) Schematic representation of the gene structure of *FT* and *XT* genes in wild type (wt) and knockout/knockin plants (koki). Lines represent introns and bars indicate exons. The diagonal lines enclose the homologous parts of wild type genes and *GT* gene-targeting cartridge necessary for the homologous recombination process. Arrows indicate PCR primer positions; possible PCR products are shown as horizontal lines. 35S-P: CaMV-35S promoter; 35S-T: CaMV-35S terminator. (**B**) PCR analysis for screening of G418-resistant plants. In case of *FT*koki the primers 1, 2, and 3 were utilised in parallel. Absence of a PCR product with primers 1 + 2 in combination with a PCR product obtained with primers 1 + 3 indicates a knockout of the *FT* locus. For *XT*koki the PCR was accomplished with primers 8, 9, 10, and 11. Absence of a PCR product of 300 bp, obtained with primer pair 8 + 9 indicates knockout of the *XT* gene, with primers 8 + 10 as well as 11 + 9 the presence of the gene-targeting cartridge was shown. The plasmid pCR4-FTko-GTki (p) served as positive control for insertion, *P. patens* wild type extract (wt) as negative control. DNA size marker (s); water control (–). (**C**) RT-PCR analysis. RNA from protonema tissue of *P. patens* wildtype (wt), seven plants transgenic for the *FT*koki and eleven plants transgenic for the *XT*koki construct were reversely transcribed into cDNA. *GT* expression was proved using primers 6 and 7 in case of *FT*koki as well as 6 and 14 for *XT*koki. Primer combinations 4 + 5 and 12 + 13 indicate transcripts derived from intact wild-type gene loci. The constitutively expressed mRNA of the ribosomal protein L21 served as an internal control. DNA size marker (s); water control (–).

ed via PEG-mediated gene transfer. About 100 plants each were screened for insertion of *GT* and disruption of the original *FT* and *XT* genes by PCR on genomic DNA (examples are shown in Fig. **2B**). Seven putative *FT*knockout/*GT*knockin (*FT*koki) and eleven putative *XT*knockout/*GT*knockin (*XT*koki) plants were identified (Fig. **2B**) and chosen for further analysis. Based on RT-PCR, the expression of *GT* as well as the absence of wild-type *FT* or *XT* transcripts verified five definitive *FT*koki and nine *XT*koki plants (Fig. **2C**).

Characterisation of FT*koki and* XT*koki plants*

Copy numbers, as well as transcript levels, of recombinant *GT* in the transgenic plants were estimated via Southern- and Northern-blot analysis (Fig. **3**). The accumulation of *GT* transcripts in both groups of transformants was analysed by Northern hybridisation, in which highly diverse mRNA amounts were detected (Figs. **3C,D**). With the exception of one *FT*koki plant (no. 22), transcript levels were in proportion to transgene copy numbers (Figs. **3A,B**). In addition to these molecular analyses, the *FT*koki and *XT*koki plants were grown in modified Knop medium (Reski and Abel, 1985), either in suspension cultures or on agar plates (data not shown). They did not show any phenotypical deviations when compared to growth, development, and morphology of wild-type moss plants.

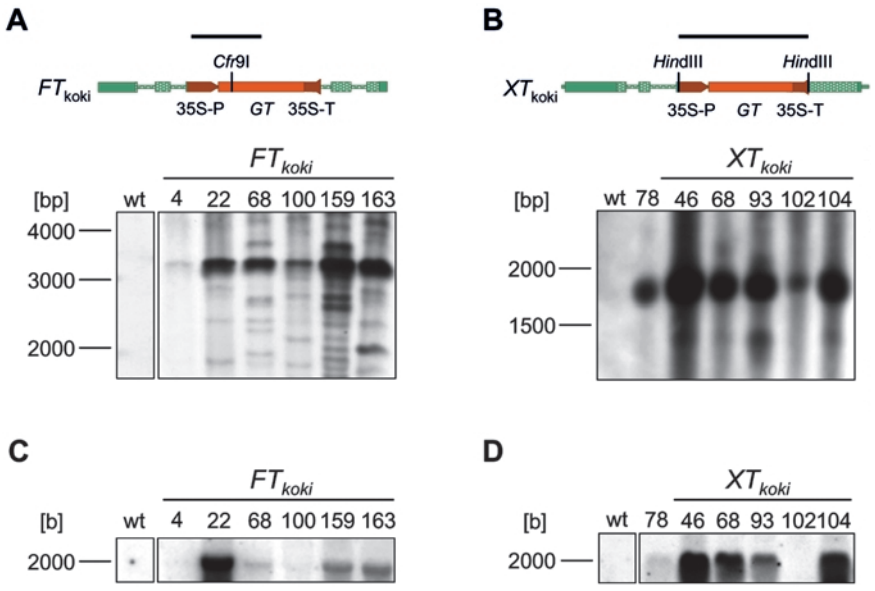


Fig. 3 Molecular analysis of *FT*koki and *XT*koki plants. Genomic DNA and RNA were extracted from protonema material of *P. patens* wild type (wt) and six transgenic *FT*koki (4, 22, 68, 100, 159, 163) plants as well as five *XT*koki (46, 68, 93, 102, 104) and one plant in which the gene-targeting construct integrated by illegitimate recombination (78). (**A**) Southern-blot analysis of *FT*koki plants. Digestion of the DNA was carried out using *Cfr*9I, which cut inside the *GT*. (**B**) Analysis of *XT*koki plants by Southern blot. *Hin*dIII digestion of DNA revealed in isolation of the GT gene-targeting cartridge. Restriction sites are indicated in the scheme above the Southern blots and the probe used for hybridization is shown as black horizontal line. (**C, D**) Northern-blot analysis for *GT* transcript accumulation in *FT*koki and *XT*koki plants, respectively.

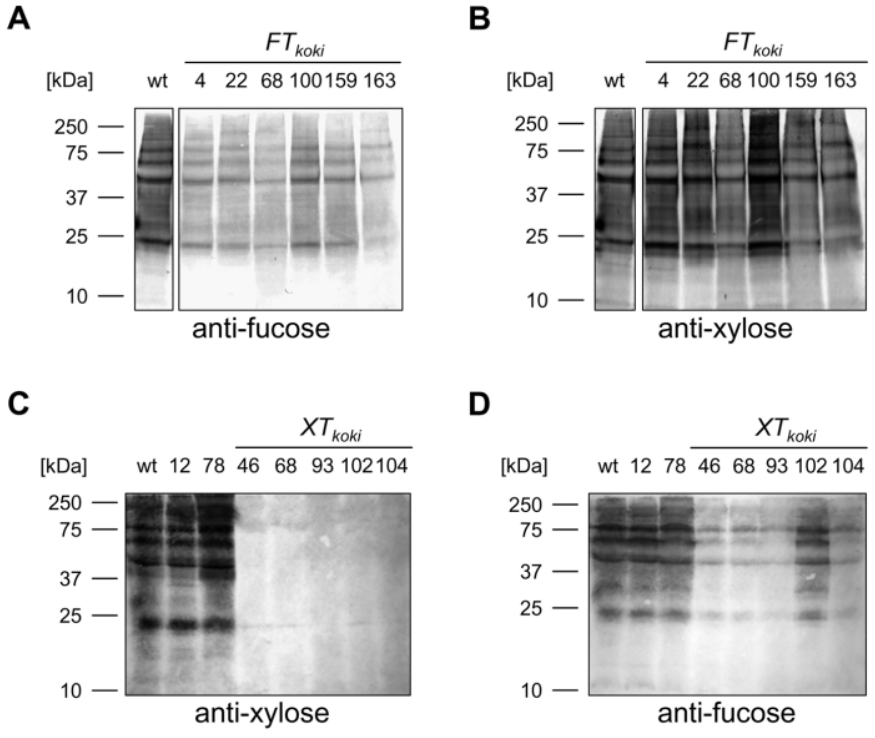


Fig. 4 Western-blot analysis to prove the absence of plant-specific carbohydrate residues in glycoproteins of *FT*koki (**A, B**) or *XT*koki (**C, D**) plants. Six *FT*koki plants (4, 22, 68, 100, 159, 163) and five *XT*koki plants (46, 68, 93, 102, 104) were tested using α(1,3)-fucose (**A, D**)- and β(1,2)-xylose (**B, C**)-specific antibodies. Controls: *P. patens* wild type (wt); for *XT*koki investigation plant 78 with illegitimate recombination of the gene-targeting construct and one transformed plant that has not inserted the *GT* gene-targeting construct (12).

Analysis of the protein N-glycosylation within transgenic moss strains

After molecular confirmation of *FT* and *XT* gene knockout, along with human *GT* transcription, we analysed the transgenic strains for absence of plant-specific α(1,3)-fucose and β(1,2)-xylose carbohydrate residues within glycosylated proteins. Immuno-blot analyses were performed with anti-α(1,3)-fucose and anti-β(1,2)-xylose antibodies purified from anti-HRP serum (Fig. **4**). Six *FT*koki plants (4, 22, 68, 100, 159, 163) and five *XT*koki plants (46, 68, 93, 102, 104) were tested using α(1,3)-fucose (**A, D**)- and β(1,2)-xylose (**B, C**)-specific antibodies. As negative controls, *P. patens* wild type (wt) was used, and, for *XT*koki investigation, control plants were those that have integrated the construct in a heterologous locus (78) or have not integrated the construct into its genome (12). Detection of N-glycans by the anti-fucose antibody was severely reduced in *FT*koki plants when compared to wild-type moss (Fig. **4 A**). In contrast, *FT*koki plants were recognised by the anti-xylose antibody in the same manner as the wild type (Fig. **4 B**). *P. patens*

*FT*ko plants, which were proven to no longer contain any 1,3-linked core fucose residues (Koprivova et al., 2004), were suitable as a positive control in *FT*koki analysis and reacted in the same way as the *FT*koki plants (data not shown). The weak residual bands detected by the antibody may be due to weak recognition of terminal 1,4-linked fucose residues. The four *XT*koki plants no longer reacted with the anti-xylose antibody (Fig. **4C**) whereas the anti-fucose antibody still bound to *XT*koki plants although somewhat less than to the control plants (Fig. **4D**). This reduction of endogenous sugar residues might be caused by the activity of the human-type galactosyltransferase (Palacpac et al., 1999), which probably interfered with protein fucosylation. As a loading control, Coomassie blue staining was performed (data not shown). *Datura stramonium* agglutinin (DSA), which binds specifically to galactose-β(1,4)-N-acetylglucosamine, was used in lectin affinity blotting (Fig. **5**). Protein recognition by peroxidase coupled DSA, exemplarily performed for *FT*koki plants, proved that the protein N-glycans within the *FT*koki transgenics contained human-type terminal β(1,4)-galactose residues.

Creation and analysis of transgenic FT/XT *double knockout/*GT *knockin plants*

Subsequent to the analysis of the single knockout plants, *XT*koki plant 104 was chosen as a platform for the generation of *FT/XT* double knockout/*GT* knockin (*FT/XT*dokoki) plants. Protoplasts were transfected with the *FT* gene targeting construct pCR4-FTko-GTki, which was used previously for the creation of *FT*koki plants. After one round of selection, twelve surviving plants were chosen for further analysis via PCR (Fig. **6A** and data not shown). Four of these did not show integration of the *FT* knockout/knockin construct at all, in five plants illegitimate integration was observed, and three plants were proven as *FT/XT*dokoki. One of these plants was selected for additional immunoblot and secretion experiments. Western-blot analysis with anti-HRP serum (Fig. **6B**), which shows cross-reactivity to both sugars, α(1,3)-fucose and β(1,2)-xylose, revealed that the *FT/XT*dokoki plant synthesised proteins without either of the two plant-specific carbohydrate moieties. Protein extract from *XT*koki 104, which was the base for *FT/XT*dokoki generation, was still recognised by the anti-α(1,3)-fucose antibody fraction of the anti-HRP serum.

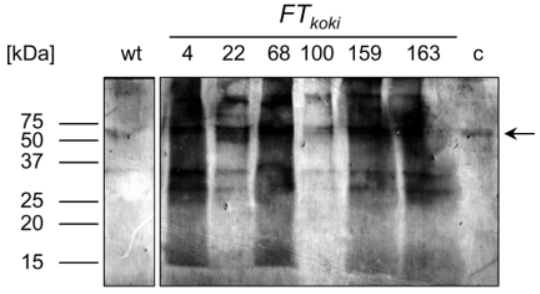


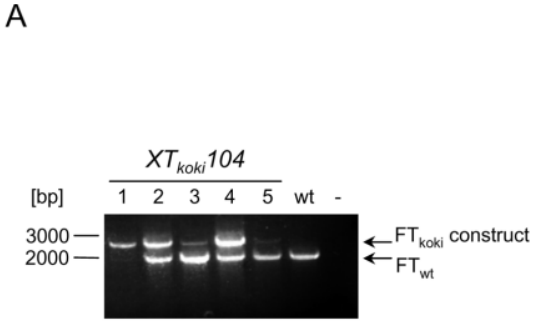
Fig. 5 Affinity-blot analysis for verification of terminal β(1,4)-galactose residues. Glycoprotein fractions of *FT*koki plants (4, 22, 68, 100, 159, 163) and *P. patens* wild type (wt), were separated on 10% SDS-PAGE and blotted onto PVDF membrane, respectively. The β(1,4)-galactosylated 68 kDa protein Fetuin (100 ng/lane) (Roche Molecular Biochemicals) is indicated by an arrow and served as positive control (c). Detection was carried out with *Datura stramonium* agglutinin (DSA).

Transient expression and secretion of recombinant human VEGF by wild type and FT/XT*dokoki protoplasts*

Since alteration of post-translational modification may have an impact on protein secretion, we checked secretion ability of the *FT/XT*dokoki strain. Protoplasts of wild type and the transgenic *FT/XT*dokoki plant were transiently transfected with a VEGF expression vector. Recombinant human VEGF in supernatants of all transfection experiments was quantified by VEGF ELISA (Fig. **7**). The amount of secreted VEGF from transfected wild-type protoplasts was set to 100%. Concentration of VEGF from *FT/XT*dokoki protoplasts of three independent transformations was between 85.3 and 116.4%, on average reaching 99.6%. Our data proved that the secretion capacity remains unaltered by the performed humanisation of the plant glycosylation pattern.

Discussion

Although post-translational protein modifications show high degrees of conservation among higher eukaryotes, the synthesis of complex N-glycans differs across the biological kingdoms. Plant-specific glycan structures have been shown to cause immune responses in mammals, as indicated by the presence of anti-sugar antibodies (Bardor et al., 2003) or even



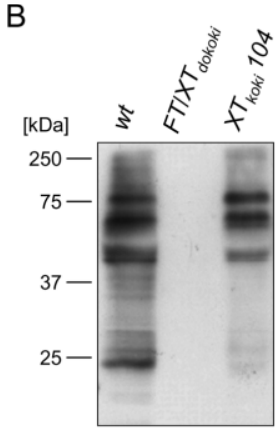


Fig. 6 Analysis of transgenic *FT/XT* double knockout/*GT* knockin plants. (**A**) PCR analysis of five plants transgenic for the *FT/XT*dokoki construct. Plant 1 was considered as *FT/XT*dokoki as the lower band indicating the wild-type *FT* locus disappeared while in plants 2–5 the construct integrated via illegitimate recombination. *P. patens* wild type (wt); water control (–). (**B**) Western-blot analysis to verify the lack of plant specific xylose and fucose residues on protein extract from *FT/XT* double knockout/*GT* knockin plant. 20 µg of total protein from *P. patens* wild type (wt), *FT/XT*dokoki and *XT*koki plant 104 on which the subsequent transformation with *FT* gene-targeting construct was based, were separated on 15% SDS-PAGE. After protein transfer onto nitrocellulose membrane the carbohydrate epitopes were detected via anti-HRP antibodies.

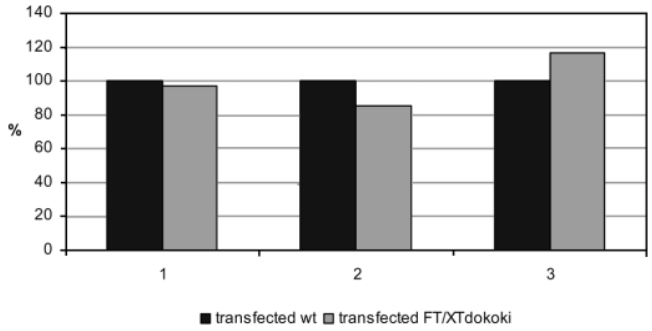


Fig. 7 Quantification of secreted recombinant human VEGF (VEGF) from 5′PpAct5::Vegf transformed *P. patens* wild type (wt) and *FT/XT*dokoki suspension by sandwich ELISA. Samples of three independent transformations were measured (1, 2, 3). The amount of VEGF from wild type plants were set to 100% proportionally for it the amount secreted by *FT/XT*dokoki.

by allergic reactions of some patients (Garcia-Casado et al., 1996; Zeng et al., 1997; van Ree et al., 2000; Westphal et al., 2003). Therefore, differences in glycosylation are the major limitation for flexible use of plant-made glycosylated pharmaceuticals, especially when applied intravenously. Currently several strategies are followed for adaptation of protein N-glycans to the human pattern. Using fusion to retention signals for the endoplasmic reticulum, antibodies were produced that no longer carry plant-specific Golgi modifications and therefore lacked the immunogenic α(1,3)-fucose and β(1,2)-xylose sugar residues (Sriraman et al., 2004; Tekoah et al., 2004). However, carrying exclusively high mannose-type but no complex N-glycans, these proteins displayed only poor stability after injection into mice (Ko et al., 2003). A way to achieve improved N-glycans without such a decrease in function would be the inactivation of plant-specific glycosyltransferases and the introduction of human glycosyltransferases. Whereas post-transcriptional gene silencing of plant glycosyltransferase genes has not resulted in lack of specific carbohydrate structures (Strasser et al., 2004), the targeted disruption of gene loci is an alternative that guarantees stable inherited modifications. The characterisation of *Arabidopsis thaliana* T-DNA insertions for α(1,3)-*FT* and β(1,2)-*XT* was published recently (Strasser et al., 2004), as were the targeted gene knockout of these plant-specific glycosyltransferases in *Physcomitrella* (Koprivova et al., 2004). These approaches lead to plants that synthesised proteins lacking α(1,3)-fucose and β(1,2)-xylose residues without obvious alterations in phenotype and development. Whereas the functional expression of human terminal β(1,4)-*GT* in tobacco cells (Palacpac et al., 1999; Bakker et al., 2001) caused synthesis of recombinant antibodies with β(1,4)-galactosylated N-glycans (Bakker et al., 2001).

Here, we combined both approaches to generate moss plants with a humanised N-glycosylation pattern by targeted insertion of the human β(1,4)-*GT* into the moss α(1,3)-*FT* and β(1,2)-*XT* gene loci via homologous recombination. Regarding protein N-glycosylation, we expected the disappearance of immunogenic α(1,3)-fucose and β(1,2)-xylose residues and the appearance of sugar structures carrying the terminal β(1,4)-linked galactose. Western-blot analysis revealed the lack of the plant-specific carbohydrate structures due to the loss of function in corresponding glycosyltransferases. The synthesis of human β(1,4)-galactose was shown by lectin affinity blots for *FT*koki plants. As the single knockout/*GT*knockin plants did not show any obvious changes in morphology compared to wild-type moss, these protein modifications do not seem to be of essential importance when the plants are cultivated under standard growth conditions. Based on these results, we aimed to achieve *P. patens* strains that no longer express either of the two objectionable plant enzymes but only the human galactosyltransferase, i.e., to generate double *FT/XT*knockout/*GT*knockin plants (*FT/XT*dokoki). One of the resulting plants was analysed by Western blotting which revealed complete lack of α(1,3)-fucose and β(1,2)-xylose sugar moieties. Lectin binding assays with a pharmaceutical protein produced in the *FT/XT*dokoki line confirmed the presence of terminal galactose residues not present in moss wild type (Jost et al., in preparation). As with the single knockout/knockin plants, growth, phenotype, and morphological characteristics were not affected by these modifications. Furthermore, the *FT/XT*dokoki moss strain showed no significant differences when compared to unmodified moss regarding secretion efficiency for the glycosylated recombinant human VEGF protein. This indicates that the alteration of plant sugar structures does not interfere with efficient secretion of extracellular proteins.

To our knowledge, this is the first targeted approach that has resulted in the complete lack of the immunogenic α(1,3)-fucose and β(1,2)-xylose moieties, combined with the introduction of terminal human β(1,4)-galactosyltransferase in a plant genome.

With the targeted disruption of plant-specific glycosyltransferases, we recently resolved the major drawback for the production of non-immunogenic plant-based biopharmaceuticals (Koprivova et al., 2004). By knockin of the human β(1,4)-galactosyltransferase into the plant-specific glycosyltransferase loci, we now present the next generation of engineered *Physcomitrella* that will lead towards a safe and flexible system for the production of fully processed, stable, and functional biopharmaceuticals with humanised N-glycosylation profiles.

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