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Molecular cloning and heterologous expression of β1,2-xylosyltransferase and core α1,3-fucosyltransferase from maize

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Abstract

Maize is considered a promising alternative production system for pharmaceutically relevant proteins. However, like in all other plant species asparagine-linked oligosaccharides of maize glycoproteins are modified with β1,2-xylose and core α1,3-fucose sugar residues, which are considered to be immunogenic in mammals. This altered *N*-glycosylation when compared to mammalian cells may reduce the potential of maize as a production system for heterologous glycoproteins. Here we report the cloning and characterization of the cDNA sequences coding for the maize enzymes β1,2-xylosyltransferase (XylT) and core α1,3-fucosyltransferase (FucT). The cloned XylT and FucT cDNAs were shown to encode enzymatically active proteins, which were independently able to convert a mammalian acceptor glycoprotein into an antigen binding anti-plant *N*-glycan antibodies. The complete sequence of the XylT gene was determined. Evidence for the presence of at least three XylT and FucT gene loci in the maize genome was obtained. The identification of the two enzymes and their genes will allow the targeted downregulation or even elimination of β1,2-xylose and core α1,3-fucose addition to recombinant glycoproteins produced in maize.

Keywords: *Zea mays*; Gramineae; Glycosyltransferase; *N*-Glycan; Fucose; Xylose; Cross-reactive carbohydrate determinant

1. Introduction

N-Glycosylation is considered to be one of the most important posttranslational protein modifications. In both animal and plant cells, a highly ordered biosynthetic pathway accounts for the addition of N-linked oligosaccharides to selected asparagine residues of newly synthesized proteins and their subsequent maturation (Kornfeld and Kornfeld, 1985). The basic steps of the *N*-glycosylation pathway are evolutionarily highly conserved, with both oligomannosidic and complex-type *N*-glycans being present in animal and plant glycoproteins. However, the structures of mature *N*-glycans differ between plants and mammals because of major differences in the final steps of the biosynthetic pathway. While mature mammalian *N*-glycans are primarily of the complex type, mature plant *N*-glycans are mainly truncated $Man_3GlcNAc_2$-structures containing β1,2-xylose and core α1,3-fucose residues. Being absent in mammals, these latter two residues are responsible for the fact that the glycan moiety of plant glycoproteins by itself constitutes an immunogenic determinant in mammals (Faye and Chrispeels, 1988; Faye et al., 1993; van Ree et al., 2000; Bencurova et al., 2004; Jin et al., 2006).

Maize (*Zea mays*) has received considerable attention during the last years as a production system for recombinant biopharmaceuticals, such as antibodies (Hood et al., 2002; Ma et al., 2005; Karnoup et al., 2005). Unless special

Abbreviations: A1AT, α1-antitrypsin; FucT, core α1,3-fucosyltransferase; XylT, β1,2-xylosyltransferase.

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measures are undertaken to prevent a plant-like glycosylation (Koprivova et al., 2004; Strasser et al., 2004) recombinant glycoproteins produced in maize will carry complex type *N*-glycans containing immunogenic β1,2-xylose and core α1,3-fucose epitopes (Samyn-Petit et al., 2001; Bardor et al., 2003). The presence of these immunogenic determinants on a therapeutic glycoprotein appears problematic since it can reduce the efficacy of a drug or lead to adverse side reactions (Gomord et al., 2005). This major drawback can be overcome by generating maize lines lacking the potentially immunogenic carbohydrate epitopes. However, for this purpose it is necessary to identify the maize genes responsible for the biosynthesis of the plant-specific complex-type *N*-glycans. Here we report the cloning, genomic characterization and functional expression of maize β1,2-xylosyltransferase (XylT; EC 2.4.2.38) and core α1,3-fucosyltransferase (FucT; EC 2.4.1.214).

2. Results and discussion

2.1. Cloning of maize XylT

XylT cDNAs have been cloned previously from *Arabidopsis thaliana* (Strasser et al., 2000) and rice (Leonard et al., 2004). Conserved regions from the corresponding amino acid sequences were selected and used to search the maize genome in the plant genome database (PlantGDB, Dong et al., 2004). These searches allowed us to retrieve several Genome Survey Sequences (GSS, e.g. ZmGSStuc04-27-04.120712.1) representing genomic DNA fragments, which displayed significant homology to *A. thaliana* and rice XylT sequences. Based on the sequence information a set of primers was designed for amplification of the full-length cDNA. A cDNA fragment of 1023 bp (GenBank Accession No. AY964642) was obtained from maize seedlings, which contained 949 bp of the open reading frame and 74 bp of 3′-untranslated region but lacked the 5′-end of the cDNA. Despite various attempts with different primer combinations and 5′-RACE using different reverse transcriptase enzymes for cDNA synthesis, it was not possible to amplify a cDNA fragment representing the missing putative 5′-end of the coding region. However, a region corresponding to the 5′-end could be amplified from genomic DNA. Since this missing part consists of a GC-rich region (>72%) it is likely that the reverse transcriptase reaction was inhibited due to the high GC-content and strong secondary structure at the 5′-end of the XylT mRNA. Similar difficulties to obtain a full-length cDNA were described for rice XylT (Leonard et al., 2004). The assembled open reading frame for the putative maize XylT consists of 1545 bp, encodes a protein with 514 amino acids and displays 98% identity with an unpublished maize XylT mRNA sequence recently deposited in GenBank (Accession No. AM179857). Primary sequence analysis predicted that maize XylT contains a single transmembrane domain at the N-terminus (comprising amino acids 20–39, TMHMM prediction http://www.cbs.dtu.dk/services/TMHMM/) and thus displays a type II membrane protein topology as typical for glycosyltransferases. In contrast to the *A. thaliana* enzyme (Bencur et al., 2005), maize XylT contains only one potential *N*-glycosylation site at position 294. A pairwise BLAST alignment (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) revealed that the protein sequence of maize XylT displays 80% identity (86% similarity) to the rice enzyme, 58% identity (70% similarity) to *A. thaliana* XylT, and 40% identity (54% similarity) to the enzyme from the moss *Physcomitrella patens* (Fig. 1). All these XylT amino acid sequences lack any significant similarity with the only other plant xylosyltransferase cloned so far, *A. thaliana* α1,6-xylosyltransferase. This is not surprising since the latter enzyme is not involved in glycoprotein processing, but fulfils a very different task in hemicellulose biosynthesis (Faik et al., 2002).

2.2. Cloning of maize FucT

cDNAs coding for FucT have been cloned previously from other plant species (mung beans: Leiter et al., 1999; *A. thaliana*: Wilson et al., 2001; rice: Leonard et al., 2004; *Medicago truncatula*: Castilho et al., 2005). The cloning strategy for maize FucT was similar to the one described for maize XylT, with the difference that the complete open reading frame (1497 bp) could be obtained directly from maize seedling cDNA (GenBank Accession No. AY964641). This sequence shares 91% identity with an unpublished maize FucT mRNA sequence recently deposited in GenBank (Accession No. AM162281). The deduced maize FucT protein sequence (498 amino acids) contains a single N-terminal transmembrane domain (amino acids 19–38) and displays a typical type II membrane protein topology similar to other plant fucosyltransferases. The amino acid sequence of maize FucT contains one potential *N*-glycosylation site at position 334, which is conserved in all plant core α1,3-fucosyltransferases sequenced so far (Fig. 2). The maize FucT displays 81% identity (88% similarity) to rice, 64% identity (77% similarity) to mung bean, 62% identity (76% similarity) to *M. truncatula*, 59% identity (73% similarity) to both *A. thaliana* enzymes and 54% identity (70% similarity) to the *P. patens* orthologue. All these FucT amino acid sequences lack any significant similarity with α1,4-fucosyltransferase, an enzyme involved in outer chain elongation of plant *N*-glycans (Wilson et al., 2001), and α1,2-fucosyltransferases involved in xyloglucan biosynthesis (Sarria et al., 2001).

2.3. Gene organization of XylT and FucT

To analyze the gene organization of XylT in maize, PCR was performed with genomic DNA and the amplified products were subcloned or directly sequenced. By comparison of the obtained genomic DNA with the cDNA sequence the gene organization was deduced (Fig. 3). The 2752 bp genomic sequence of XylT is organized as three exons of 675, 150 and 720 bp with two introns of 643 and 564 bp

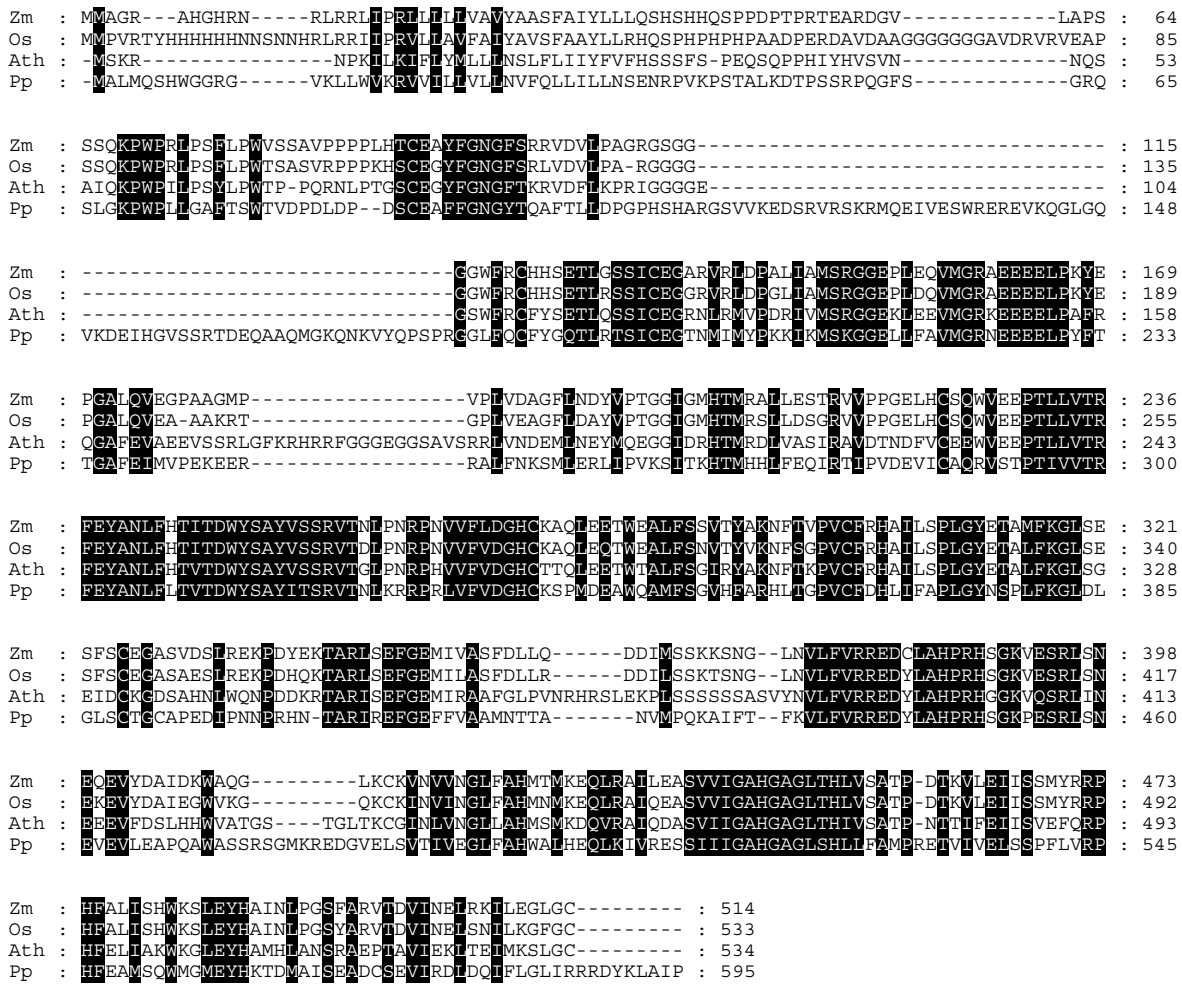

Fig. 1. Alignment of XylT amino acid sequences. The alignment contains only sequences for which activity was shown either by in vitro assays (Strasser et al., 2000; Leonard et al., 2004) or indirectly by analysis of knockouts (Koprivova et al., 2004). Residues conserved in all four sequences are shaded in black. The important KPWP motif, which marks the beginning of the catalytic domain, is highlighted. Maize (Zm), rice (Os), *A. thaliana* (Ath) and *P. patens* (Pp).

(Fig. 3A). The exon/intron junctions are conserved between the maize (GenBank Accession No. DQ026518), *A. thaliana* (NM124632) and rice genes (AP004190).

For maize FucT gene no genomic DNA fragment could be amplified, which covers the whole gene. To obtain information about the putative gene structure the cloned FucT cDNA was used to search the maize genome database (MaizeGDB, http://www.maizegdb.org/). Based on the sequence data obtained from the database and additional sequencing of exon/intron junctions from amplified FucT gene fragments we predict a gene structure with seven exons and six introns (Fig. 3B). Thus, the number of exons is the same as for *A. thaliana* (At1g49710 and At3g19280) and rice (AP004457) but is different from the *M. truncatula FUT2* gene (AY557602) organization, which displays five exons (Castilho et al., 2005) and from the mung bean FucT gene (Y18529), which harbors four exons (Leiter et al., 1999).

To estimate the number of XylT gene loci, maize genomic DNA was exhaustively digested with enzymes having either no (*Bam*HI and *Kpn*I), one (*Hin*dIII at 2521 bp in exon 3) or two (*Eco*RI at 1272 bp in intron 1 and at 2247 bp in exon 3) restriction nuclease cleavage sites within the gene sequence. Subsequently, Southern blotting with three different cDNA probes was carried out (Fig. 3A): (i) P1 covering exon 1 (partially) and exon 2, (ii) P2 corresponding to the 3′-region of exon 3, and (iii) P3 recognizing all three exons. Hybridization with the 5′-probe P1 showed one band for *Hin*dIII and two bands for *Eco*RI (Fig. 4A). The lower band in the *Eco*RI digest is the fragment generated by cleavage at the two *Eco*RI sites within the XylT gene (Fig. 3A). On the other hand, hybridization with the 3′-probe P2 yielded three bands for *Hin*dIII and three bands for *Eco*RI (Fig. 4B). These results indicate the existence of a minimum of three different gene loci which lack *Eco*RI and *Hin*dIII polymorphisms in their 5′-regions. As

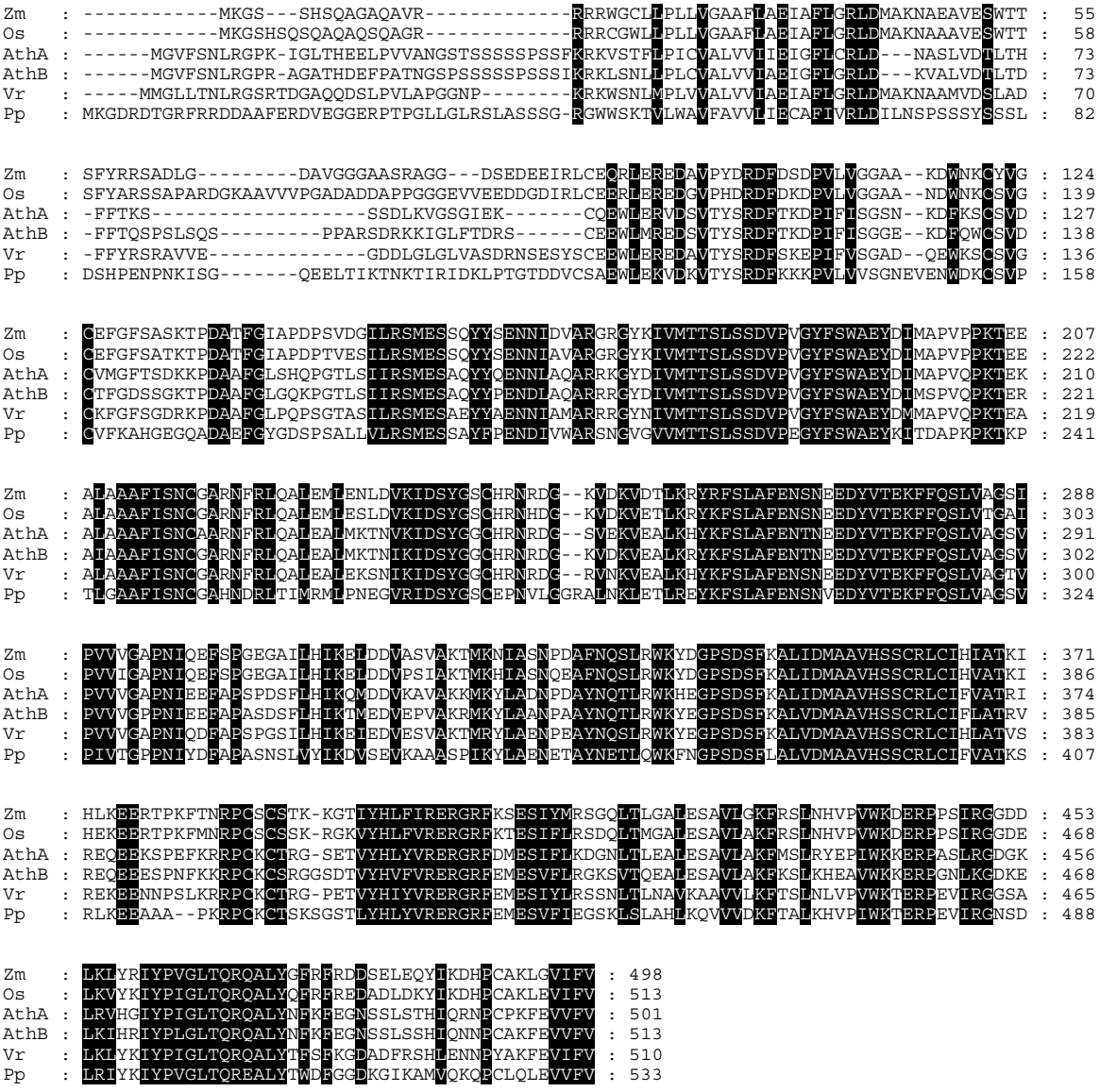

Fig. 2. Alignment of FucT sequences. The alignment contains only sequences, for which activity was shown either by in vitro assays (Leiter et al., 1999; Wilson et al., 2001; Bakker et al., 2001; Leonard et al., 2004) or indirectly by analysis of knockouts (Strasser et al., 2004; Koprivova et al., 2004). Residues conserved in all six sequences are shaded in black. Maize (Zm), rice (Os), *A. thaliana* FucTA (AthA) and FucTB (AthB), mung bean (Vr) and *P. patens* (Pp).

expected, all *Eco*RI and *Hin*dIII fragments obtained with P1 and P2 were also detected by hybridization with P3, a probe which covers the whole gene (Fig. 4C). Interestingly, four bands were detected by hybridization of *Bam*HI digested DNA with P3, indicating that more than three XylT gene loci may exist in the maize genome. The fragments obtained upon *Kpn*I digestion were too large to allow discrimination of individual bands (Fig. 4C).

The multiple copies of the XylT gene could be due to the segmental tetraploid origin of the maize genome and transposition events (Swigonova et al., 2004; Messing et al., 2004).

The estimation of the maize FucT gene loci by Southern blotting is not straightforward as the complete sequence of the gene is not yet available. We have therefore concentrated in our genomic analysis on the 3′-region of the gene. *Eco*RI, *Hin*dIII and *Kpn*I genomic DNA digests were hybridized to a 319 bp probe consisting of exons 5 and 6 (probe P4, Fig. 3B). The results presented in Fig. 4D show at least five well-discriminated bands for *Hin*dIII, three bands for *Kpn*I and three strong bands besides some fainter signals for *Eco*RI digested DNA. A minimum of 3 loci should be present in the maize genome to explain the observed *Hin*dIII, *Eco*RI and *Kpn*I fragment patterns. A high number of FucT gene loci were also described for *M. truncatula*, where five chromosomes carry a gene locus and in some cases more than one locus per chromosome was found (Castilho et al., 2005). It will be possible to

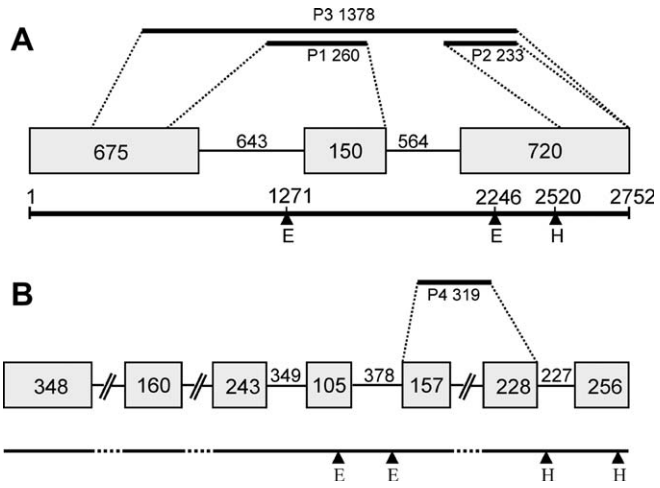

Fig. 3. Schematic presentation of the gene structure for (A) XylT and (B) FucT. Boxes represent exonic sequences connected by strait lines representing introns. Vertical lines in introns denote incomplete sequence information. Black bars on top represent the sequences used as probes (P1–P4) for Southern blot hybridizations. Genomic regions recognized by the different probes are shown by the connecting dashed lines. Restriction sites for *Eco*RI (E) and *Hin*dIII (H) are mapped in the bottom line. All numbers are in base pairs.

obtain further information about the locus number and the chromosome allocation of the XylT and FucT genes once the complete maize genome sequence is available.

2.4. Heterologous expression in insect cells

Heterologous expression of maize XylT and FucT was achieved in *Spodoptera frugiperda* Sf21 cells by baculovirus mediated infection. For this purpose the catalytic domain of XylT lacking 55 amino acids at the N-terminal end was cloned into the baculovirus transfer vector pVTBacHis1 (Sarkar et al., 1998). The engineered protein lacks the putative cytoplasmic tail and transmembrane domain of XylT but still contains the conserved tetrapeptide KPWP, which is required for XylT activity and indicates the beginning of the catalytic domain (Bencur et al., 2005). The FucT construct encodes a protein lacking the N-terminal 43 amino acids. Immunoblot analysis of cell extracts and culture supernatants revealed that both XylT and FucT deletion mutants were successfully expressed in Sf21 cells and in the culture medium (Fig. 5). The detected sizes of the recombinant polypeptides were in close agreement with their theoretical molecular masses. No specific signals were obtained with lysates and conditioned media of uninfected cells (data not shown).

2.5. Biochemical characterization

In order to verify that the expressed proteins are functional and display XylT and FucT activity, respectively, the culture supernatants from the infected insect cells were assayed for the corresponding activities. The supernatant

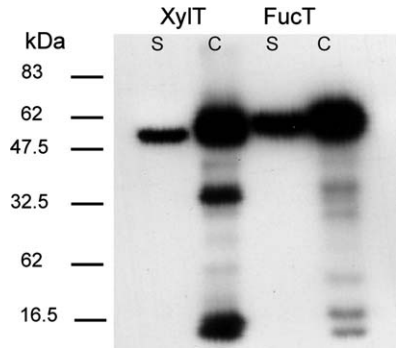

Fig. 5. Heterologous protein expression of soluble forms of maize XylT and FucT in insect cells. Equivalent amounts of culture supernatants (S) and protein extracts from Sf21 cells (C) infected with recombinant baculoviruses encoding either maize XylT or FucT were separated under reducing conditions by SDS–PAGE, blotted and probed with mouse anti-enterokinase recognition site antibodies binding to the N-terminal leader sequence of the fusion proteins. The observed sizes of the major bands (XylT: 56 kDa; FucT: 57 kDa) are in close agreement with the theoretical molecular masses of the recombinant polypeptides. The migration positions of molecular-mass standards are indicated.

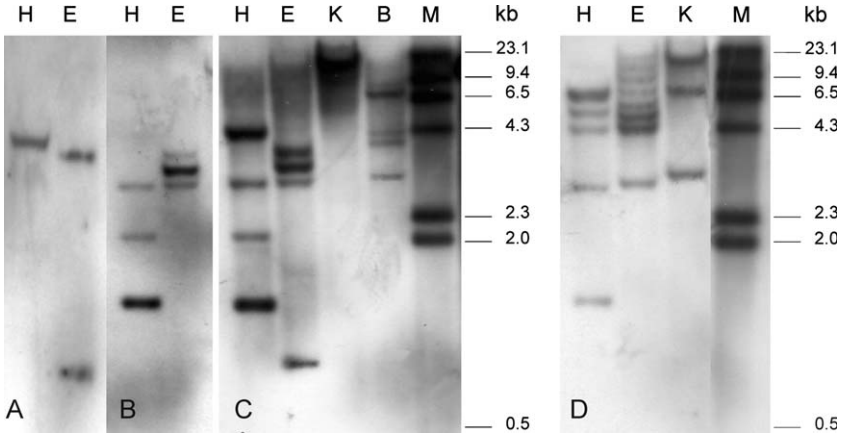

Fig. 4. Southern blot analyses of maize genomic DNA digested with *Hin*dIII (H), *Eco*RI (E), *Kpn*I (K) and *Bam*HI (B). Hybridization pattern of (A) XylT probe P1, (B) XylT probe P2, (C) XylT probe P3, and (D) FucT probe P4. M, marker fragments.

from the XylT expressing cells was analyzed by MALDI-TOF MS using dabsylated GnGn-glycopeptide as substrate (Fig. 6). The soluble recombinant maize XylT was expressed in an active form as judged by the increase in the mass of the acceptor substrate (132 Da for a single xylose residue). This increase was not detected when supernatants of uninfected cells or cells infected with a control baculovirus were analyzed.

To analyze the metal ion requirement of maize XylT, enzyme activity was monitored by reversed phase HPLC (Fig. 7). Assays were performed using pyridylaminated-GnGn as a substrate in the absence or presence of different Mn^{2+} concentrations or in the presence of EDTA. Maize XylT was active in the presence of 10 mM EDTA and did not require the addition of any metal ions for activity. 10 mM and higher amounts of $MnCl_2$ inhibited the enzyme activity. In this respect maize XylT resembles the *A. thaliana* enzyme (Bencur et al., 2005) and is different from rice XylT, which requires divalent cations for full activity (Leonard et al., 2004).

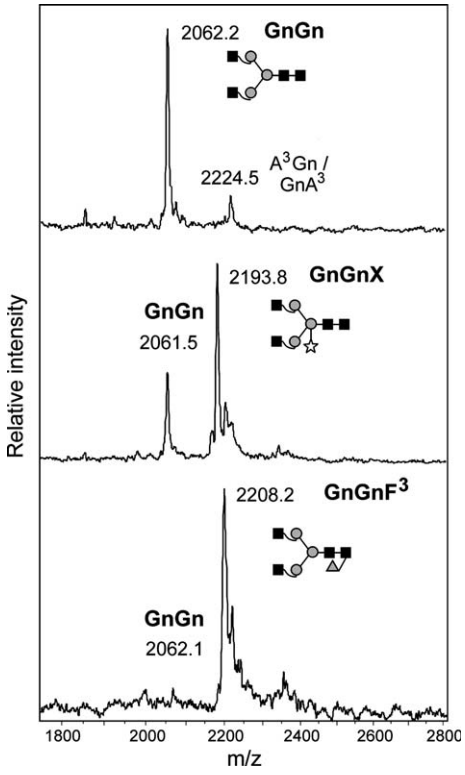

Fig. 6. Assays of recombinant maize XylT and FucT. Assays were performed using dabsylated GnGn-glycopeptide (*m*/*z*: 2062) as acceptor substrate and analyzed by MALDI-TOF MS. Upper panel: dabsylated GnGn-glycopeptide incubated with culture supernatant from insect cells infected with a control baculovirus. The peak at 2224 is derived from a minor fraction of dabsylated GnGn-glycopeptide carrying a single β1,3-linked galactose residue (A^3Gn or GnA^3, for a detailed explanation of the glycan structures and their nomenclature see www.proglycan.com). Middle panel: dabsylated GnGn-glycopeptide incubated with culture supernatant from insect cells producing maize XylT. Lower panel: dabsylated GnGn-glycopeptide incubated with culture supernatant from insect cells producing maize FucT.

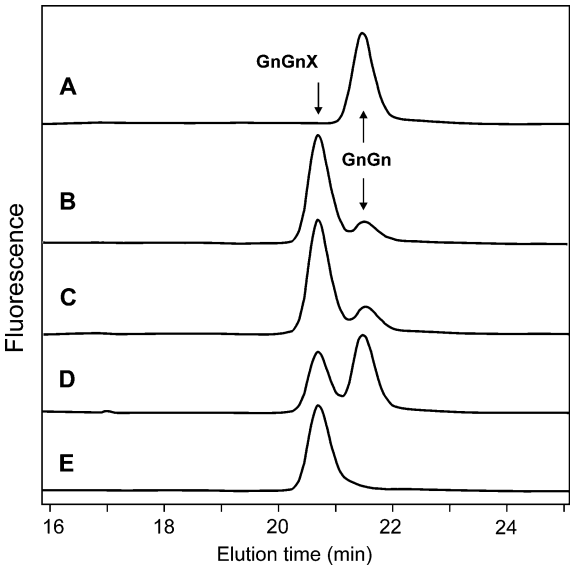

Fig. 7. Metal ion-dependence of maize XylT activity: the activity of XylT was determined by RP-HPLC using pyridylaminated GnGn as acceptor substrate in the absence (trace A) or presence (traces B–E) of UDP-xylose. Chromatograms B and C show the result of incubation in the presence and absence of 10 mM EDTA, respectively. Sample D contained 10 mM Mn^{2+}. Trace E is a GnGnX standard generated with recombinant *A. thaliana* XylT.

Maize FucT activity was assayed using dabsylated GnGn-glycopeptide as substrate and analyzed by MALDI-TOF MS. The detected increase in mass of the acceptor substrate corresponded to the molecular mass of a single fucose residue (146 Da) (Fig. 6), which demonstrated that the cloned maize cDNA codes for an active fucosyltransferase. No comparable activity was observed

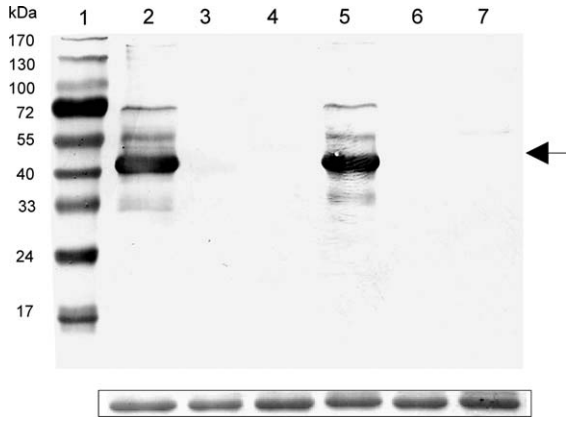

Fig. 8. Western blot with rabbit anti-glycan antibodies (anti-HRP). The migration positions of prestained molecular-mass standards are shown (lane 1). Equal amounts of human α1-antitrypsin were incubated with insect cell supernatant containing maize XylT in the presence (lane 2) or absence (lane 4) of UDP-xylose, or with insect cell supernatant containing maize FucT in the presence (lane 5) or absence (lane 6) of GDP-fucose. Samples incubated with supernatant of insect cells infected with a control baculovirus in the presence of UDP-xylose (lane 3) or GDP-fucose (lane 7) are also shown. Coomassie blue staining of the samples is shown in the lower panel.

in supernatants of insect cells infected with a control baculovirus.

We also determined the ability of the enzymes to produce cross-reactive *N*-glycan epitopes on a mammalian glycoprotein. To this end enzymatically modified human α1-antitrypsin (A1AT), carrying non-antigenic GnGn structures, was incubated with supernatants of maize XylT or FucT expressing insect cells, respectively. Subsequently the A1AT glycoprotein was analyzed by Western blot using antiserum raised against horseradish peroxidase (HRP), a typical plant glycoprotein, which was shown to react with *N*-glycans containing β1,2-xylose or core α1,3-fucose (Bencurova et al., 2004; Faye et al., 1993). As expected only A1AT incubated with either XylT or FucT reacted with HRP-antibody (Fig. 8). This demonstrates that both maize XylT and FucT are able to produce cross-reactive *N*-glycan epitopes on mammalian glycoproteins.

3. Conclusion

We have cloned and characterized maize cDNAs encoding active β1,2-xylosyltransferase and core α1,3-fucosyltransferase proteins. This finding is supported by the fact that the cloned enzymes share substantial sequence identity with previously characterized XylT and FucT enzymes from other plant species and display the same acceptor substrate specificity. For *A. thaliana* it has been clearly shown that XylT and FucT are the only enzymes capable of catalyzing the transfer of β1,2-xylose and core α1,3-fucose residues, respectively, to complex *N*-glycans *in planta* (Strasser et al., 2004).

The cloning of maize XylT and FucT cDNAs and gene segments enables the development of strategies for the targeted elimination of these enzymatic activities. This may increase the value of maize as potential host for the production of recombinant therapeutic glycoproteins. Since our results indicate the presence of at least three XylT and FucT gene loci in the maize genome, knockouts by T-DNA or transposon insertion, like it has been performed for *A. thaliana* (Strasser et al., 2004) will be very difficult to achieve. RNA interference strategies are therefore of higher promise to obtain a significant reduction or perhaps even complete elimination of the unwanted sugar residues.

4. Experimental

4.1. Plant material

Zea mays (cultivar Monalisa) seeds were germinated in water for 3–5 days at room temperature or directly transferred to soil. Seedlings were cultivated in a growth chamber at 22 °C, with a 16 h light/8 h dark light-cycle.

4.2. RNA/DNA extraction

The plant tissues were frozen in liquid nitrogen and ground to a fine powder using a bead mill (Retsch, Haan, Germany). Total RNA was isolated from seedlings using the TRIzol reagent (Invitrogen, Lofer, Austria) according to the supplier's instructions. First strand cDNA was synthesized from 0.5 μg of total RNA using AMV reverse transcriptase (Promega, Mannheim, Germany) and oligo-dT primers. Genomic DNA extraction was carried out from maize leaves with a GenElute™ Plant Genomic DNA Miniprep Kit (Sigma–Aldrich, Vienna, Austria).

4.3. Isolation of FucT and XylT coding sequences

The maize FucT open reading frame was amplified from maize cDNA with the primer pair M_FT2F (5′-GCGAGGAGGGCCAGCGATGAA-3′) and M_FT2R (5′-TACAGTACAACTGAAACTCGCACACAGA-3′) under standard PCR conditions with Expand High Fidelity Polymerase (Roche, Vienna, Austria). The primer sequences were derived from GSSs obtained by a BLAST search (tblastn) of the plant genome database (PlantGDB, http://www.plantgdb.org/PlantGDB-cgi/blast/PlantGDB-blast) with conserved amino acid regions from *A. thaliana*, rice and mung bean FucT.

PCR amplification of XylT gene was carried out in two steps since the full open reading frame could not be amplified from cDNA. To overcome this, a 3′-end fragment (1023 bp) containing most of the putative catalytic domain and 74 bp of 3′-untranslated region was amplified from cDNA with primers M_XT10F (5′-CGGGATCGGGATGCACACTATG-3′) and M_XT7R (5′-CCAAGTATGGGTGATGAAGTAGACACG-3′) as described above. Subsequently an overlapping 5′-end fragment of 2117 bp was PCR amplified from genomic DNA with primers M_XT6F (5′-CGACACCCCTCGATCTGCG-3′) and M_XT11R (5′-AAAGTGCTTCCCATGTTTCCTCTAAT-3′). To reconstruct the full length open reading frame, we used the unique *Xho*I site within the overlapping sequences to ligate the cDNA and gDNA PCR fragments.

4.4. Characterization of FucT and XylT gene organization

For the analysis of maize XylT gene organization, two overlapping PCR fragments were amplified from genomic DNA using primer combination M_XT6F/M_XT11R and M_XT10F/M_XT7R and subsequently sequenced. For the analysis of FucT gene organization, a BLAST search in the GSS maize genome database (http://www.maizegdb.org/blast.php) was performed using the cloned maize FucT cDNA. This allowed us to retrieve all exon/intron junctions except for exon 2. The following maize genome sequences were used to create the gene structure as shown in Fig. 3B: GenBank Accession Nos.: CC441955, CG196327, CC404690, CG196327, CG162290, BZ309235, BH781621, BZ623225, CG273090,

BZ306160, and CG151370. To confirm the presence of the missing intron 2 within the FucT gene, PCR was performed using primers M_FTE2F (5′-TTGGAATAAATGCTACGTAGGATGTGAA-3′) and M_FTE3R (5′-ACTGGTACGTCTGAAGAAAGGCTGGTT-3′) and the obtained PCR product was sequenced.

4.5. Subcloning and sequencing

XylT and FucT PCR fragments from cDNA or genomic DNA were cloned into pGEM®-T (Promega) and sequenced using the PRISM BigDye Terminator Cycle Sequencing Kit and the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

4.6. Southern blot hybridization

To estimate the minimum number of XylT and FucT gene loci, 5 μg of genomic DNA were exhaustively digested with *Eco*RI, *Hin*dIII, *Bam*HI and *Kpn*I restriction enzymes (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The digested DNA was separated on a 1% (w/v) agarose gel along with DIG labelled markers (Roche, Mannheim, Germany) and blotted to positively charged nylon membranes (Roche). Hybridization and post-hybridization washes were carried out under high stringency conditions according to the DIG Application Manual (Roche).

The probes used for Southern blot hybridization are represented in Fig. 3A and B. For the XylT gene three probes were used: (i) P1: a 260 bp fragment covering the 3′-region of exon 1 and exon 2 was amplified from XylT cDNA by PCR with primers M_XT10F (5′-CGGGATCGGGATGCACACTATG-3′) and M_XT11R (5′-AAAGGCTTCCCATGTTTCCTCTAAT-3′), purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Freiburg, Germany) and used as a template for DIG labelling with primer M_XT10F; (ii) P2: a 233 bp fragment derived from the 3′-region of exon 3 was labelled using the primer M_XT18R (5′-TATAGGTACCTCAGCATCCGAGACCTTCCAGTA-3′) and (iii) P3: a 1378 bp XylT cDNA fragment was amplified with primers M_XT19F (5′-TATAGCGGCCGCCCGTGACGGTGTCCTG-3′) and M_XT18R and used for DIG labelling with primer M_XT18R.

For the FucT probe P4, a 319 bp fragment covering exons 5 and 6 was amplified from FucT cDNA by PCR with the primer pair M_FTE5F (5′-CAGGTTCTATTCCGGTTGTTGTTGGT-3′)/M_FTE6R (5′-TGGTGGAACAGCTACAAGGACGATT-3′) and the purified fragment was used for DIG labelling with primer M_FTE5F. Labelling reactions contained 50 ng of purified PCR product, 24 pmol of primer, 1.5 μl DIG-dNTP mix (1 mM of each dATP, dCTP, dGTP; 0.75 mM dTTP and 0.25 mM DIG-11dUTP, Roche) and 5 U *Taq* polymerase in a final volume of 50 μl *Taq* polymerase buffer. DIG labelled probes were quantified by dot blot according to the protocol of the Dig High Prime DNA Labelling Kit (Roche).

4.7. Expression of XylT and FucT in insect cells

A truncated form of maize XylT comprising amino acids 56–514 was generated by PCR from the in vitro assembled open reading frame using primers M_XT19F (5′-TATAGCGGCCGCCCGTGACGGTGTCCTG-3′) and M_XT18R (5′-TATAGGTACCTCAGCATCCGAGACCTTCCAGTA-3′). The PCR product was cleaved with *Not*I and *Kpn*I restriction enzymes at the underlined sites and ligated into pVTBacHis1 baculovirus transfer vector (Sarkar et al., 1998) digested with the same enzymes. In this construct, the truncated XylT protein is placed downstream of the melittin signal peptide, a 6× His tag and an enterokinase cleavage site. For maize FucT, a N-terminal deletion construct containing amino acids 44–498 was amplified by PCR from the open reading frame with primers M_FT7F (5′-TATAGCGGCCGCGAAGAACGCCGAGGCGGTCG-3′) and M_FT4R (5′-TATAGGTACCTTACACAAAAATTACTTCAAGCTTTGC-3′) and cloned into pVTBacHis1 vector as described above for XylT.

Expression in *S. frugiperda* Sf21 cells was performed exactly as described previously (Bencur et al., 2005). Briefly, the recombinant transfer vector (1 μg) was co-transfected with 200 ng of BaculoGold viral DNA (BD Biosciences, Erembodegem, Belgium) into Sf9 cells using Lipofectin (Invitrogen) as recommended by the manufacturer. After 5 days at 27 °C, supernatants containing recombinant virus were used for infection of Sf21 cells. Cells and culture media were harvested after 4 days at 27 °C and subjected to immunoblotting and enzymatic analyses.

For Western blot analysis baculovirus-infected and non-infected Sf21 cells were lysed in SDS–PAGE sample buffer. Cell lysates and culture supernatants of infected Sf21 cells were subjected to 10% SDS–PAGE under reducing conditions. Fractionated proteins were electrophoretically transferred onto Hybond-C membranes (Amersham Biosciences) and subsequently incubated with a mouse monoclonal antibody recognizing the enterokinase sequence (Invitrogen). Detection of bound antibodies was achieved with goat anti-mouse immunoglobulin G antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch, Soham, Cambridgeshire, UK) using the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

4.8. Enzyme assay for XylT and FucT

For XylT and FucT enzyme activity assays, dabsylated GnGn-glycopeptide (see Fig. 6 for explanation of the abbreviation) derived from bovine fibrin with *N*-glycans carrying two non-reducing terminal *N*-acetylglucosamine residues was used as a substrate. The standard reaction

mixture consisted of 10 µl supernatant from infected cells, 1 nmol acceptor substrate, 0.5 mM activated nucleotide sugar donor substrate (UDP-xylose or GDP-fucose), 10 mM $MnCl_2$ and 0.1 M Mes buffer pH 7.0 in a total volume of 20 µl. After incubation for 16 h at 37 °C the glycans were purified from the assay mixture as described by Kolarich and Altmann (2000) and analyzed by MALDI-TOF MS (Thermo BioAnalysis, Hemel Hempstead, UK) with 1% α-cyano-4-hydroxycinnamic acid in 70% (v/v) acetonitrile as a matrix.

For the HPLC enzyme activity assay, the supernatant of Sf21 cells expressing maize XylT was incubated with 100 pmol of pyridylaminated-GnGn as acceptor substrate along with 0.5 mM UDP-xylose in 100 mM Mes buffer pH 7.0 overnight at 37 °C. Samples were analyzed with reversed phase HPLC as described previously (Altmann et al., 1993). Metal ion dependency of the enzyme was analyzed in the presence and absence of the indicated amounts (Fig. 7) of $MnCl_2$ or EDTA under the same conditions.

4.9. Western blot analysis

Human α1-antitrypsin (A1AT) (Sigma–Aldrich, St. Louis, MO, USA) was converted to the GnGn-glycoform by neuraminidase and β-galactosidase treatment as described (Bencurova et al., 2004). The GnGn-A1AT was modified with maize XylT and FucT to generate GnGnX-A1AT and GnGnF-A1AT. 4 µg of human GnGn-A1AT was first incubated overnight at 37 °C with 10 mM $MnCl_2$, 0.1 M Mes buffer pH 7.0, 10 µl of supernatant from infected insect cells (XylT, FucT or control) and 0.5 mM UDP-xylose and GDP-fucose, respectively. Subsequently, samples were incubated with β-*N*-acetylglucosaminidase (0.5 mU/µg A1AT) from *Streptococcus pneumoniae* (Sigma–Aldrich) in 50 mM sodium citrate buffer pH 4.5 overnight at 37 °C to create modified MM-A1AT, MMX-A1AT and MMF-A1AT. Modified α1-antitrypsin proteins (2 µg) were separated on 12% SDS–PAGE and either stained with Coomassie blue or blotted onto a nitrocellulose membrane. The membrane was blocked with 0.5% (w/v) bovine serum albumin in 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween and subsequently incubated for 1 h with rabbit anti-HRP-antibody (Wilson et al., 1998) diluted 1:2000 in blocking solution. Alkaline phosphatase conjugated anti-rabbit antibody (Sigma–Aldrich) was used as secondary antibody at 1:2000 dilution in blocking solution. Finally, the blot was stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma–Aldrich).

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