Plant members of the α1→3/4-fucosyltransferase gene family encode an α1→4-fucosyltransferase, potentially involved in Lewis[a] biosynthesis, and two core α1→3-fucosyltransferases[1]

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Abstract Three putative α1→3/4-fucosyltransferase (α1→3/4-FucT) genes have been detected in the *Arabidopsis thaliana* genome. The products of two of these genes have been identified in vivo as core α1→3-FucTs involved in *N*-glycosylation. An orthologue of the third gene was isolated from a *Beta vulgaris* cDNA library. The encoded enzyme efficiently fucosylates Galβ1→3GlcNAcβ1→3Galβ1→4Glc. Analysis of the product by 400 MHz ^{1}H-nuclear magnetic resonance spectroscopy showed that the product is α1→4-fucosylated at the *N*-acetylglucosamine residue. In vitro, the recombinant *B. vulgaris* α1→4-FucT acts efficiently only on neutral type 1 chain-based glycan structures. In plants the enzyme is expected to be involved in Lewis[a] formation on *N*-linked glycans. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Fucose; Fucosyltransferase; Glycosyltransferase; Lewis[a]

1. Introduction

Plants have great potential for the safe production of human proteins for research purposes or pharmaceutical applications. Many of such proteins are glycoproteins, and it is known that cell and species specific glycosylation modulates numerous protein characteristics [1,2]. Obviously, detailed knowledge of the glycosylation potential of plants is important to devise strategies for genetic modification of plants to enable the production of proteins with a specific mammalian-like glycosylation. In many plants, Lewis[a] containing glycans are found [3–5] whereas typical mammalian Galβ1→4GlcNAc and Galβ1→4[Fucα1→3]GlcNAc (Lewis[x]) structures are lacking. The availability of the complete sequence of the *Arabidopsis thaliana* genome facilitates the identification of plant orthologues of human glycosyltransferase genes. Our aim in this study was to learn the potential of plants to fucosylate complex-type glycans by characterizing enzymes encoded by the putative members of the α1→3/4-fucosyltransferase (α1→3/4-FucT) gene family that were identified in the *A. thaliana* genome.

Members of the α1→3/4-FucT gene family are found in eukaryotes as well as prokaryotes [6]. In general, they catalyze the final and sometimes crucial step in the synthesis of a range of biologically active glycoconjugates. Six genes encoding α1→3/4-FucTs have been identified in humans, encoding FucT-III to FucT-VII and FucT-IX [7–16]. These FucTs have distinct acceptor specificities but in general catalyze the transfer of a fucose (Fuc) residue in α-linkage to the C3 of *N*-acetylglucosamine (GlcNAc) in Galβ1→4GlcNAc (type 2) structures occurring on glycolipids and glycoproteins. FucT-III and FucT-V have a dual α1→3 and α1→4 specificity for type 1 or type 2 chains, respectively, and a higher affinity for type 1 (Galβ1→3GlcNAc) containing structures than for type 2 structures [10,17]. In humans, the α1→3/4-FucTs are involved in the synthesis of the Lewis blood group determinants: Lewis[a], sialyl-Lewis[a], Lewis[b], Lewis[x], sialyl-Lewis[x] and Lewis[y].

In plants, as well as in some invertebrates, a FucT activity (FucT-C3) has been described that does not occur in mammals and catalyzes the transfer of a Fuc in α1→3-linkage to the innermost asparagine-linked GlcNAc of *N*-glycans [18–20]. The immunogenic core α1→3-fucosylated *N*-glycan synthesized by this enzyme is an abundant carbohydrate epitope in plants and invertebrates [21–25], and contributes to the carbohydrate-based cross-reactivity that has long been observed in immunoassays of glycoproteins of plants, insects and mollusks [26,27]. Recently the cDNAs for two FucT-C3s have been cloned, from mung bean and *Drosophila me-*

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[1] Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AJ315848 (Bv-α1→4FucT), AJ345084 (At-FucT-C3-1) and AJ345085 (At-FucT-C3-2).

Abbreviations: GlcNAc, *N*-acetylglucosamine; Fuc, fucose; FucT-(-C3), (core α1→3-)fucosyltransferase; EST, expressed sequence tag; LNT, lacto-*N*-tetraose; LNFP II, lacto-*N*-fucopentaose II

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lanogaster, and identified as members of the same gene family as the mammalian α1→3/4-FucTs [28,29]. In the genome of *A. thaliana*, three putative FucT genes of the α1→3/4-FucT family can be detected. While this manuscript was in preparation, Wilson et al. [30] reported that one of these genes (FucTA, At-FucT-C3-1 in this manuscript) encodes a FucT-C3 activity in vitro, whereas expression of FucTB (At-FucT-C3-2 in this manuscript) did not result in any FucT activity in vitro and was suggested to be a redundant non-active FucT-C3. Here, we demonstrate that both *A. thaliana* enzymes show FucT-C3 activity in vivo when expressed in CHO cells. In addition, we report the isolation of an orthologue of the third *A. thaliana* FucT gene from a *Beta vulgaris* (beet) cDNA library. This cDNA encodes an α1→4-FucT, catalyzing the transfer of a Fuc residue to the C4 of GlcNAc, with a strict substrate specificity for neutral type 1 chain-based glycan structures.

2. Materials and methods

2.1. Materials

Unlabeled GDP-Fuc was purchased from Boehringer Mannheim. GDP-[^{14}C]Fuc (250 Ci/mol) was purchased from New England Nuclear Corp. (Boston, MA, USA) and diluted with the unlabeled nucleotide sugar to obtain a specific radioactivity of 5 Ci/mol. Dr. Monica Palcic (University of Alberta, Edmonton, AB, Canada) kindly provided the 8-methoxycarbonyloctyl glycoside acceptors.

2.2. Cloning of plant cDNAs

A cDNA clone encoding At-FucT-C3-1 (accession no. AJ345084) was isolated from an *A. thaliana* selique cDNA library in the mammalian expression vector pABE [31] by a PCR screening method and sibling selection as described previously for the isolation of a GlcNAcT-I cDNA [32]. The cDNA clone was completely sequenced and contained the complete open reading frame as was predicted from the genomic sequence (protein accession no. BAB02969). As the cDNA insert was in the wrong orientation for expression, the plasmid was digested with *Bst*XI and the insert religated in the same vector. An At-FucT-C3-2 cDNA clone (accession no. AJ345085) was isolated by PCR using primers covering the putative start and stop (ttgattcatcctctaggttaatg and tccagaggaatgaaactagacg) codons from selique cDNA. *Bst*XI linkers (Invitrogen) were ligated to the PCR fragment and inserted into *Bst*XI-digested pABE. Sequencing confirmed that the sequence was identical to the prediction from the *A. thaliana* genome (protein accession no. AAG13053). Sequences of At-FucT-C3-1 and At-FucT-C3-2 are also identical to those cloned by Wilson et al. [30] (DNA accession no. AJ404860 (FucTA) and AJ404861 (FucTB), respectively) except that At-FucT-C3-2 is not the alternative spliced form cloned by Wilson et al. [30], but is identical to the described correctly spliced form. Bv-α1→4FucT cDNA (accession no. AJ315848) was recognized as an orthologue of At-FucT-3 (protein accession no. AAG52222) in a *B. vulgaris* root expressed sequence tag (EST) library in SK$^+$ made within Plant Research International (accession no. AJ315848). The cDNA was sequenced and appeared to contain the complete putative coding region. The cDNA insert was isolated from SK$^+$ and introduced into pABE by *Hin*dIII/*Xba*I digestion and ligation (pABE-Bv-α1→4FucT).

2.3. Expression of the plant cDNAs in CHOP8 cells and immunohistochemistry

The putative FucT genes were transiently expressed in CHOP8 cells [33]. Cells were grown and transfected as described before [32,34]. Two days after transfection cells were washed with phosphate-buffered saline (PBS), fixed in 1% glutaraldehyde in PBS, washed with Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris–HCl pH 7.5), blocked with 2% milkpowder/TBS and immunostained by a core α1→3-Fuc specific antibody in 2% milkpowder/0.1% Tween 20/TBS. This antibody was purified from commercially available rabbit anti-horseradish peroxidase (HRP) antiserum (Rockland, Gilbertsville, PA, USA), that has a dual specificity for the β1→2-xylose and core α1→3-Fuc epitope of HRP *N*-glycans. By affinity chromatography with honeybee venom phospholipase A2 (Sigma), that carries *N*-linked glycans with core α1→3-Fuc, but differs from HRP by lacking the β1→2-xylose epitope, a core α1→3-Fuc specific antibody was obtained, as described previously by Faye et al. [35]. The secondary antibody was an alkaline phosphatase-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories). For color development, Fast-red (Sigma) was used.

2.4. Expression of Bv-α1→4-FucT in COS-7M6 cells and FucT activity

COS-7 cells (ATCC 1651) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1/50 penicillin/streptomycin solution (all from Life Technologies). The cells were transiently transfected with pABE-Bv-α1→4-FucT using the calcium phosphate precipitation method as described previously [36]. After 2 days, cells were washed with PBS and collected in 50 mM sodium-cacodylate buffer pH 7.2 at a concentration of 2.5 mg/ml, and assayed for FucT activity. The standard reaction mixture (50 μl) contained 5 nmol GDP-[^{14}C]Fuc (4–5 Ci/mol), 2.5 μmol sodium-cacodylate buffer pH 7.2, 1 μmol $MnCl_2$, 0.2 μmol ATP, 20 nmol acceptor substrate, 0.1% (v/v) Triton X-100 and 20 μl homogenate. Reactions were incubated for 2 h at 37°C, in which period no more than 10% of the limiting substrate was converted. Incorporation of Fuc was determined as described [37]. Values were corrected for incorporation into endogenous acceptors. Three replicates were used per data point, and the assays were performed twice.

2.5. Product characterization

Lacto-*N*-tetraose (LNT) (250 nmol) was incubated with 500 nmol GDP-[^{14}C]Fuc (0.2 Ci/mol), and 250 μl cell extract under otherwise standard incubation conditions and incubated for 18 h at 37°C. The product was separated from unincorporated nucleotide sugar by ion exchange on a 5 ml column Dowex 1-X8 (Cl^- form), isolated on a Spherisorb S5 NH2 column (Waters, 4.6×250 mm) run in acetonitrile/15 mM KH_2PO_4 (80:20) using a linear gradient (0.3%/min, flow 2 ml/min), and desalted on a column (0.7×45 cm) of Bio-gel P-2 (200–400 mesh) run in water. The fucosylated product was exchanged in D_2O (99.75 atom%, Merck) three times with intermediate lyophilization. Finally, the sample was redissolved in 400 μl D_2O (99.96 atom% D, Aldrich, Milwaukee, WI, USA). ^{1}H-nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker MSL 400 spectrometer operating at 400 MHz at a probe temperature of 300 K. Resolution enhancement was achieved by Lorentzian to Gaussian transformation. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ=2.225 ppm in D_2O).

3. Results

3.1. Cloning of plant FucT cDNA clones

Screening of the *A. thaliana* genomic database with mammalian and putative bacterial sequences of the α1→3/4-FucT gene family resulted in the identification of three putative α1→3/4-FucT genes. All three showed a low overall homology with the known α1→3/4-FucTs, but did show a conserved sequence motif (α3 motif II) found in the mammalian and bacterial genes (Fig. 1, [6]). A cDNA clone encoding At-FucT-C3-1 was isolated from an *A. thaliana* selique cDNA library by a PCR screening method and sibling selection as described previously for the isolation of a GlcNAcT-I cDNA [32]. Another cDNA, At-FucT-C3-2, was isolated by PCR using primers covering the putative start and stop codons from selique cDNA. The third putative gene, At-FucT-3, could not be isolated from the same cDNA source using this approach. ESTs containing part of the At-FucT-3 sequence, however, can be found in GenBank. An orthologue of At-FucT-3 was found in an EST library from *B. vulgaris*. The EST clone contained a complete open reading frame with 64% identity to At-FucT-3 and was named Bv-α1→4-FucT.

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At1 M-----------------GVFSNLRGPKIGLTHEELPVVANGSTSSSSSPSSFKRKVSTFLPICVALVVIIEIGFLCRLDNASLVD  69
At2 M-----------------GVFSNLRGPRAGATHDEFPATNGSPSSSSSPSSSIKRKLSNLLPLCVALVVIAEIGFLGRLDKVALVD  69
At3 MPM-----------RYL-NAMAALLMM----FFTLLILSFTGILEFPSASTSMEHSIDPEPKLSDSTSDPFS-DVLVAYKKWDFEV  69
Bv  MRS-----------KPLINPISITIMLSISIFFILFFSGFFQVPSFASPSI-IDQTIKSN------KPDPFT-DLLGAFRKWDSQM  67
H3  MDPLGAAKPQWPWRRCLAARLFQLLVAVC--FFSYLRVSRDDATGSPRAPSGSSRQDTT----------PTRPTLLILLWTWPFHI  74

                                                  *
At1 TLTHFFTKSSS----------DLKVGSGIEK-CQEWLERVDSVTYSRDFTKDPIFISGS-NKDFK-SCSVDCVMGFTSDKKPDAAF 142
At2 TLTDFFTQSPSLSQSPPARSDRKKIGLFTDRSCEEWLMREDSVTYSRDFTKDPIFISGG-EKDFQ-WCSVDCTFGDSSGKTPDAAF 153
At3 GCARFRENHKDAILGNVSSG------SLQEFGCG----K-LKMKHVKVLVKGWTWIPDNLENLYSCRCGMTCLWTKSSV-LADSPD 143
Bv  GCLKFREKYQNWGFSNASVS--LQFSRIDEVGCD----KELKLKHVNVLVKGWTWIPDNLENLYECKCGLSCFWTKSSV-LSDPPD 146
H3  PVA--LSRCSEMVPGTA--------------DCHITADRKVYPQADTVIVHHWDIMSNPKSRL------------PPSPRPQGQR 131

                                    α-3 motif I
                                          S     G
At1 GLSHQPGTLSIIRSM-ESAQYYQENNLAQARRKGYDIVMTTSLSSDVPVGY----F---------SWAEYDIM-----APVQPKTE 209
At2 GLGQKPGTLSIIRSM-ESAQYYPENDLAQARRRGYDIVMTTSLSSDVPVGY----F---------SWAEYDIM-----SPVQPKTE 220
At3 ALLFETTTPPLQRRVGDPLRVYMELEAGRKRSGREDIFISYHAKDDVQTTYAGSLFHNNRNYHISPHKNNDVLVYWSSSRCLPHRD 229
Bv  ALLFETTTPPLQRRVGDPLRIYMDLEAGRKRSGREDIFVSYHAKDDVQATYAGALFHNNRNYYVSSWKKNDTLVYWSSSRCLPERN 232
H3  WIWFNLEPPPNCQHLEALDRYF-------------NLTMSYRSDSDIFTPYG--------------WLEP-----WSGQPAHPPLN 185

                                                                  α-3 motif II
                                                              Y F I  EN     YITEK
At1 KALAAAFISNCAARNFR-----LQALEALMKTNVKIDSYGGCHRNRDGSV---EKVE-ALKHYKFSLAFENTNEEDYVTEKFF-QS 285
At2 RAIAAAFISNCGARNFR-----LQALEALMKTNIKIDSYGGCHRNRDGKV---DKVE-ALKRYKFSLAFENTNEEDYVTEKFF-QS 296
At3 R-LAKSLLDLIPHHSFGKC---LNNVGGLDSA---LSMYPECVAEHNAEAKWYDHLHCAMSHYKFVLAIENTAVESYVTEKLF-YA 307
Bv  D-IAKRLLSLLSHHSFGKC---LNNVNGLDKA---LYMYPECAKDPNSAPKWWDHLHCAMSHYKFVLAIENTMTESYVTEKLF-YA 310
H3  L-SAKTELVAWAVSNWKPDSARVRYYQSLQ-AHLKVDVYG---RSHKPLPK--GTMMETLSRYKFYLAFENSLHPDYITEKLWRNA 264

      IPV
At1 LVAGSVPVVVGAP--NIEEFAPSPDSFLHIKQMDDVKAVAKKMKYLADNPDAYNQTLRWKHEGPSDSFKALIDMAAVHSSCRLCIF 369
At2 LVAGSVPVVVGPP--NIEEFAPASDSFLHIKTMEDVEPVAKRMKYLAANPAAYNQTLRWKYEGPSDSFKALVDMAAVHSSCRLCIF 380
At3 LDSGSVPIYFGAS--NVQDFVP-PHSVIDGSKFGSMQELAAYVKRLGDDPVAYSEYHAWRRCGLMGNYGKTRAVSLDTLPCRLC-- 388
Bv  LDSGAVPIYFGAP--NVWDFVP-PHSIIDGSKFSSLEELASYIKALANDPVAYAEYHAWRRCGVLGNYGKTRATSIDTLPCRLC-- 391
H3  LEAWAVPVVLGPSRSNYERFLP-PDAFIHVDDFQSPKDLARYLQELDKDHARYLSYFRWRETLRPRSFSWALDF------CKAC-- 341


At1 VATRIREQEEKSPEFKRRPCKCTRG-SETVYHLYVRERGRFDMESIFLKDGNLTLEALESAVLAKFMSLRYEPIWKKERPASLRGD 454
At2 LATRVREQEEESPNFKKRPCKCSRGGSDTVYHVFVRERGRFEMESVFLRGKSVTQEALESAVLAKFKSLKHEAVWKKERPGNLKGD 466
At3 ------------EEISRRGGKNA-GV 401
Bv  ------------EAVSRRGGRSARAL 405
H3  -----WKLQQESRYQTVRSIAAWFT 361

At1 GKLRVHGIYPIGLTQRQALYNFKFEGNSSLSTHIQRNPCPKFEVVFV 501
At2 KELKIHRIYPLGLTQRQALYNFKFEGNSSLSSHIQNNPCAKFEVVFV 513
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Fig. 1. Amino acid sequence alignment of three *A. thaliana* members of the α1→3/4-FucT gene family (At1 and At2 are FucT-C3-1 and -2, At3 is the *A. thaliana* orthologue of the *B. vulgaris* α1→4-FucT), the *B. vulgaris* α1→4-FucT (Bv) and the human FucT-III (H3, protein accession no. BAA96390). Amino acids that are conserved in all five members are shaded. Above the alignment, two conserved boxes (α3 motifs) are indicated that were previously identified based on sequence alignments of animal and bacterial FucTs [6]. Within the motifs, the previously indicated most conserved amino acid residues are shown. An * indicates the position of the tryptophan (W) residue, that determines the α1→4-FucT specificity of mammalian FucT-III [42]. The presented *A. thaliana* sequences are identical to the predictions from the genome sequence, except that At-FucT-3 was extended with eight amino acids at the N-terminus, as these aligned well with Bv-α1→4-FucT.

At-FucT-C3-1 and At-FucT-C3-2 each show 77% mutual similarity and are considered orthologues of the mung bean core α1→3-FucT. At-FucT-3 and Bv-α1→-4FucT are about 17% identical to At-FucT-C3-1 and At-FucT-C3-2. The similarity was mainly found around a conserved FucT motif (α3 motif II) that was previously identified in animal and bacterial α1→3/4-FucTs (Fig. 1, [6]). These data indicate that At-FucT-3 and Bv-α1→-4FucT form a distinct subfamily of plant FucTs within the α1→3/4-FucT family.

3.2. Expression of the plant cDNAs in CHOP8 cells

The three cDNAs have been inserted in the mammalian expression vector pABE [31] and transiently expressed in CHOP8 cells [33]. CHOP8 is a Chinese hamster ovary (CHO) cell line of the Lec8 complementation group. Lec8 cells are deficient in UDP-galactose transport and were chosen since their cell surface glycans, which lack galactose, are expected to be efficient acceptor substrates for core α1→3-FucTs [38]. Transfected cells were stained with an affinity-purified antibody specific for the core α1→3-Fuc structure. Expression of either At-FucT-C3-1 or At-FucT-C3-2 cDNA in CHOP8 cells resulted in cells that were recognized by the anti-core Fuc antibody (Fig. 2), indicating that both cDNAs encode core α1→3-FucTs. In vitro FucT assays of the transfected CHOP8 cells, using GDP-Fuc as donor substrate and glycopeptides of asialo-agalacto IgG as acceptor as described previously [20], demonstrated that At-FucT-C3-1 indeed showed core α1→3-FucT activity, whereas no enzymatic activity could be detected using cells transfected with At-FucT-C3-2 cDNA (results not shown).

Expression of the Bv-α1→4-FucT cDNA in CHOP8 cells did not result in positive staining of transfected cells, indicating that the Bv-α1→4-FucT cDNA does not encode a functionally active core α1→3-FucT.

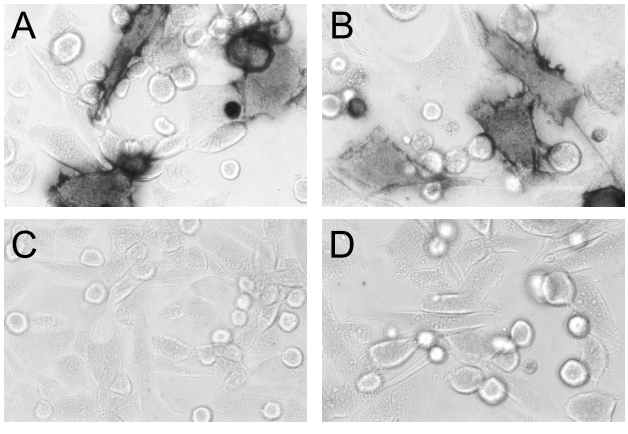

Fig. 2. CHOP8 cells transiently expressed with At-FucT-C3-1 (A), At-FucT-C3-2 (B) and Bv-α1→4-FucT (C) and an empty vector control (D). Cells were stained with an antibody specific for core α1→3-linked Fuc.

Table 1
Acceptor specificity of recombinant Bv-α1→4-FucT towards reducing oligosaccharides and 8-methoxycarbonyloctyl glycoside acceptors

Substrate	FucT activity (%)
Type 1 chain-based oligosaccharides	
1. Galβ1→3GlcNAc	42
2. Galβ1→3GlcNAcβ1→3Galβ1→4Glc (LNT)	59
3. Galβ1→3GlcNAcβ-R[a]	59
4. Fucα1→2Galβ1→3GlcNAcβ-R	100
5. NeuAcα2→3Galβ1→3GlcNAcβ-R	<0.1
Type 2 chain-based oligosaccharides	
6. Galβ1→4GlcNAc	1
7. Galβ1→4GlcNAcβ1→3Galβ1→4Glc	0.7
8. Galβ1→4GlcNAc-R	2
9. Fucα1→2Galβ1→4GlcNAc-R	4
10. NeuAcα2→3Galβ1→4GlcNAc-R	3

Relative rates for each acceptor (final concentration reducing oligosaccharides: 1 mM and 8-methoxycarbonyloctyl glycoside acceptors 0.4 mM) are expressed as percentage of the incorporation with compound #4. 100% activity: 90 pmol/min/mg.
[a]R, O-$(CH_2)_8$-$COOCH_3$.

3.3. Characterization of Bv-α1→4-FucT

To investigate the enzymatic properties of Bv-α1→4-FucT, homogenates of COS-7 cells transfected with pABE-Bv-α1→4-FucT were incubated with radiolabeled GDP-Fuc and a panel of oligosaccharide acceptor substrates. COS-7, a monkey kidney cell line, was chosen for these experiments as they facilitate plasmid replication, usually resulting in a higher expression level of recombinant proteins than CHO cells. The results (Table 1) show that the enzyme efficiently acts on acceptor substrates containing neutral type 1 chain-based structures (Galβ1→3GlcNAc), presumably producing Lewis[a] and Lewis[b] determinants, respectively. Bv-α1→4-FucT failed to act on sialylated type 1 substrates and all type 2 chain-based acceptor substrates were used with poor efficiency.

To establish the structure of the product synthesized with the recombinant Bv-α1→4-FucT, 200 nmol of fucosylated LNT was prepared by incubation of 250 mmol LNT, the transfected COS cells and GDP-Fuc. The product from this large-scale enzyme reaction appeared to be a pentasaccharide, as was observed by high-performance liquid chromatography. The structure of this pentasaccharide (#F-LNT) was analyzed by 400 MHz ^{1}H-NMR spectroscopy. Relevant chemical shifts are listed in Table 2, together with the chemical shifts of reference compounds LNT and lacto-*N*-fucopentaose II (LNFP II). Chemical shifts of LNT and LNFP II were essentially as reported before [39,40]. The chemical shifts of the fucosylated product (#F-LNT) appeared to be similar to those of LNFP II. Thus, the Fuc residue appears to be attached to the GlcNAc residue in an α1→4-linkage. In the spectrum of #F-LNT no signals were found typical for a Fuc in α3-linkage to Glc (two doublets of a H-1 of a residue in α-linkage at δ= 5.427 and at δ= 5.371 ppm; [17]). The H-1 residue of the Gal of the Galβ1→4GlcNAc element is shifted downfield in the spectrum of #F-LNT (δ= 4.506 ppm), due to the presence of a Fuc residue at the GlcNAc. Furthermore, the H-1 of the α-anomeric Glc residue (δ= 5.221 ppm) is not shifted upfield, compared to the H-1 of a Glc which is fucosylated (compounds #F3 and #F10 in [17]). Similarly, in the spectrum of #F-LNT there are no signals indicative for a Fuc in α2-linkage to Gal, indicating that the Bv-α1→4-FucT lacks any α1→2-FucT activity.

Thus, the Bv-α1→4-FucT can be identified as a GDP-Fuc:GlcNAc α1→4-FucT capable of synthesizing Lewis[a] and Lewis[b] epitopes.

4. Discussion

Three plant members of the α1→3/4-FucT gene family have been cloned and, based on immunohistochemical and in vitro enzymatic studies, their function in plant glycosylation is proposed. Two cDNAs with high sequence similarity to the mung bean core α1→3-FucT gene were isolated from an *A. thaliana* cDNA library and designated At-FucT-C3-1 and At-FucT-C3-2. We were not successful in isolating the third cDNA from *A. thaliana*, but found and cloned instead an orthologous EST of this gene from the sugar beet, *B. vulgaris*.

Transient transfection of the plant cDNAs in CHOP8 cells resulted in binding of an antiserum specific for core α1→3-Fuc to the At-FucT-C3-1 and At-FucT-C3-2, but not to the Bv-α1→4-FucT-transfected cells. These data indicate expression of core α1→3-fucosylated *N*-glycans on the cell surface

Table 2
400 MHz ^{1}H-NMR chemical shift values and *J* constants of structural reporter group protons for the constituent monosaccharides of the product synthesized by enzymatic transfer of Fuc by Bv-α1→4-FucT to LNT (#F-LNT)

Residue	Reporter group	Anomer	Chemical shifts (*J* constants) (ppm) (Hz)		
			#F-LNT	LNT	LNFP II
Fuc(1-4)	H-1	α,β	5.026 (3.6)	–	5.026 (4.0)
	H-5	α,β	4.872	–	4.873
	H-6	α,β	1.180 (6.5)	–	1.180 (6.6)
Gal(1-4)	H-1	α	4.438 (7.8)	4.441 (7.7)	4.441 (7.8)
	H-1	β	4.438 (7.8)	4.441 (7.7)	4.436 (7.9)
	H-4	α,β	4.155 (3.0)	4.153 (3.4)	4.154 (3.3)
Gal(1-3)	H-1	α,β	4.506 (7.7)	4.480 (7.8)	4.505 (7.7)
GlcNAc	H-1	α	4.704 (8.3)	4.737 (8.3)	4.707 (8.5)
	H-1	β	4.704 (8.3)	4.732 (8.4)	4.703 (8.5)
	H-3	α,β	4.082 (10.1)	N.D.	4.082 (9.1)
	NAc	α,β	2.033	2.028	2.030
Glc	H-1	α	5.221 (3.7)	5.220 (3.9)	5.220 (3.8)
	H-1	β	4.663 (7.9)	4.662 (7.9)	4.662 (8.0)
	H-2	β	3.280	3.278	3.279

Reference compounds are LNT and LNFP II (LNFP II was purified from human milk according to Donald and Feeney, 1988). Chemical shifts are given in ppm downfield from DSS in D_2O, at 300 K, acquired at 400 MHz (but were actually measured relative to internal acetone, δ= 2.225 ppm). N.D., not determined; –, not applicable.

of the antiserum positive cells, suggesting that both cDNAs encode functional core α1→3-FucTs. The identity of At-FucT-C3-1 could be established by in vitro FucT assays using a homogenate of the transfected cells. In contrast and for reasons unknown we were not able to demonstrate in vitro core α1→3-FucT activity using the At-FucT-C3-2-transfected cells as enzyme source, although staining intensity of transfected cells appeared to be similar. Similarly, Wilson et al. [30] reported the in vitro core α1→3-FucT activity of FucTA (=At-FucT-C3-1) and the in vitro inactivity of the correctly spliced form of FucTB (=At-FucT-C3-2) and concluded that FucTB could possibly be a redundant product of duplication of an original FucT-C3 gene. Our immunohistochemistry results, however, strongly favor the possibility that At-FucT-C3-2 is a functionally active core α1→3-FucT. The inability to show in vitro activity might be caused by a variety of reasons, i.e. the enzyme may have a low affinity towards the substrates used in vitro, or may be unstable in the assay conditions used. Furthermore, it should be considered that in the CHOP8 cells overexpression of both enzymes is achieved. Possibly, the activity of At-FucT-C3-2 is low compared to that of At-FucT-C3-1, and under the detection limit of the in vitro assay. This activity, however, might be sufficient to fucosylate in vivo. To get insight in the contribution of At-FucT-C3-1 and At-FucT-C3-2 to the fucosylation of *N*-glycans in *A. thaliana*, however, the fucosylation capacity of selective knock-outs of both the respective genes in *A. thaliana* should be investigated.

To define the enzymatic properties of the putative FucT encoded by the Bv-α1→4-FucT cDNA, we used COS-7 cells transiently transfected with this cDNA as enzyme source. Our data clearly show that Bv-α1→4-FucT has similar properties as the human Lewis-type FucT (FucT-III). Out of a panel of type 1 and type 2 chain containing structures, Bv-α1→4-FucT has a strict preference for neutral type 1-based acceptor substrate. Still the substrate specificity of the plant enzyme is distinct from the specificity of the human Lewis enzyme FucT-III [37], since the sialylated type 1 acceptor #5 was not a substrate for the plant enzyme and type 2 acceptors were very poor substrates. The enzyme also lacked the ability to transfer Fuc to the C3 of Glc in LNT [17], as appeared from the structural identification of the product by ^{1}H-NMR spectroscopy. The enzymatic properties of Bv-α1→4-FucT resemble those of a FucT activity purified from mung bean seedling [41], indicating that in mung bean a similar gene is expressed. Interestingly, in human FucT-III, a tryptophan residue (W^{111}) has been identified that is responsible for α1→4-FucT specificity [42]. The same residue can be found in FucT-V, the second mammalian enzyme with α1→4-FucT activity, but an arginine residue (R) is found at the corresponding position in the mammalian α1→3-FucTs. A single amino acid substitution W^{111}→R in FucT-III was sufficient to change the specificity of Fuc transfer from H-type 1 to H-type 2 acceptors [42]. In agreement with the proposed function of this region in acceptor binding, W^{114} in Bv-α1→4-FucT and W^{111} in its *A. thaliana* orthologue align with W^{111} of FucT-III, whereas the two *A. thaliana* core α1→3-FucTs did not have either W or R in the corresponding position. Comparison of the Bv-α1→4-FucT area around W^{114} showed several more conserved amino acids (see Fig. 1) with FucT-III, that may contribute to the α1→4 structural motif, although they cannot be found in FucT-V. We were not successful in isolating a full length cDNA of the putative *A. thaliana* α1→4-FucT, although EST fragments have been detected in the databases. Wilson et al. [30], however, have isolated the full coding region of the putative *A. thaliana* α1→4-FucT, but were unable to assign a function to the gene, which may be in accordance with previous findings that Lewis^a has not been detected in *Arabidopsis* [3–5].

Lewis^a epitopes, however, are found on many other plants [3–5], and we could show that this structure is readily synthesized in vitro by Bv-α1→4-FucT, indicating that the Bv-α1→4-FucT gene described here may have a function in the formation of Lewis^a glycan epitopes on *N*-linked glycans in beet. It is not clear yet if the capacity of Bv-α1→4-FucT to catalyze the synthesis of the Lewis^b epitope has a functional relevance. So far, the Lewis^b epitope has not been described in plants. A prerequisite for the formation of Lewis^b structures is the coordinate expression of an α1→2-FucT that catalyzes the transfer of Fuc in α1→2-linkage to Gal in Galβ1→3GlcNAc-R, i.e. an orthologue of the human FucTs I and II, belonging to the gene family of α1→2-FucTs. So far, such an enzyme has not been described in plants, but several sequences that putatively encode plant members of this gene family can be detected in the databases. Recently, plant α1→2-FucTs belonging to this family have been cloned [43,44]. These enzymes, however, seem to catalyze specifically the synthesis of α1→2-fucosylated xyloglucan structures.

In conclusion, we have identified two core α1→3-FucT genes and one α1→4-FucT gene in plants. So far, no other α1→3/4-FucT homologues have been found in plants, suggesting that none of the plant FucTs identified so far encodes an enzyme that is able to α1→3-fucosylate type 2 chain-based (Galβ1→4GlcNAc, LacNAc) structures, thus forming Lewis^x or Lewis^y determinants. So far, no LacNAc structures have been found on plant glycoproteins. We have shown previously that the glycosylation potential of plants can be modified, by introducing the human β1→4-galactosyltransferase gene in tobacco. This resulted in the formation of *N*-glycans containing typical mammalian LacNAc structures [45], an achievement that is promising for future genetic glycan engineering of plants. Knowing the specificities of the endogenous plant FucTs is important as it facilitates the prediction of the fucosylation of recombinant glycoproteins produced in these plants, as well as the design of additional genetic modifications that will be required to produce for example Lewis^x, Lewis^y and Lewis^b structures in plants.

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