Distantly related plant and nematode core α1,3-fucosyltransferases display similar trends in structure–function relationships

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Here, we present a comparative structure–function study of a nematode and a plant core α1,3-fucosyltransferase based on deletion and point mutations of the coding regions of Caenorhabditis elegans FUT-1 and Arabidopsis thaliana FucTA (FUT11). In particular, our results reveal a novel “first cluster motif” shared by both core and Lewis-type α1,3-fucosyltransferases of the GT10 family. To evaluate the role of the conserved serine within this motif, this residue was replaced with alanine in FucTA (S218) and FUT-1 (S243). The S218A replacement completely abolished the enzyme activity of FucTA, while the S243A mutant of FUT-1 retained 20% of the “wild-type” activity. Based on the results of homology modeling of FucTA, other residues potentially involved in the donor substrate binding were examined, and mutations of N219 and R226 dramatically affected enzymatic activity. Finally, as both FucTA and FUT-1 were shown to be N-glycosylated, we examined the putative N-glycosylation sites. While alanine replacements at single potential N-glycosylation sites of FucTA resulted in a loss of up to 80% of the activity, a triple glycosylation site mutant still retained 5%, as compared to the control. In summary, our data indicate similar trends in structure–function relationships of distantly related enzymes which perform similar biochemical reactions and form the basis for future work aimed at understanding the structure of α1,3-fucosyltransferases in general.

Keywords: core α1,3-fucosyltransferase / GDP-fucose binding domain / homology modeling / N-glycosylation / site-directed mutagenesis / structure–function

Introduction

Fucosyltransferases are, in general, divided into two superfamilies according to their sequence similarities and type of linkage formed. The members of the first superfamily are α1,2-, α1,6- and protein-O-fucosyltransferases which belong to the GT11, GT37, GT23, GT65 and GT68 families according to the Carbohydrate Active enZymes database (CAZy: http://www.cazy.org/; Cantarel et al. 2009). All α1,3- and α1,4-fucosyltransferases belong to the second superfamily, whether they are core α1,3 or Lewis-type; they are defined as members of the GT10 family. In contrast to Lewis-type enzymes that modify glycan antennae, core α1,3-fucosyltransferases (EC 2.4.1.214) catalyze addition of 1-fucose from GDP-Fuc to the reducing terminal N-acetylgalactosamine (GlcNAc) residue of N-glycans, thereby these enzymes form the core α1,3-fucose epitope which is a conserved feature of plants and invertebrates (Paschinger et al. 2009). As mammals lack this type of fucosylation, this epitope is recognized by mammalian immune systems as a foreign element; indeed, core α1,3-fucose is an epitope for IgE from the sera of many patients with insect, pollen or food allergy and for IgG directed against immunized plant and insect glycoproteins (Wilson et al. 1998; Hemmer et al. 2001).

During the last two decades, several studies dealt with bioinformatic analysis of protein sequences of enzymes belonging to the GT10 family. The story started with the discovery of the so-called α1,3-FucT motif (Martin et al. 1997) which was also described by Breton et al. (1998) as motif II, in addition to another motif called I (see also Figure 1). These motifs were observed during the analysis of the sequences of the human Lewis-type FucT III–VII and FucT-IX by hydrophobic cluster analysis (HCA). Later, a third conserved peptide motif (I/V/F)HH(R/W)[D/E][I/V/L] was revealed and called the acceptor motif, because replacement of a single residue Trp111 to Arg within this motif of FucT-III led to the formation of the Leβ epitope (α1,3-fucosylation, which requires β1,4 bound galactose to the GlcNAc) instead of the Leα epitope (α1,4-fucosylation,
which requires β1,3 bound galactose to the GlcNAc; Dupuy et al. 1999, 2002). A fourth conserved motif, located toward the N-terminus, prior to the acceptor motif, was later called motif III (Dupuy et al. 2002, 2004). Dupuy et al. (2002) claimed that motif III may correspond to the beginning of the catalytic domain of vertebrate α1,3/4-fucosyltransferases. As the result of a recent study in which a fifth conserved motif was found (Mollicone et al. 2009), the five motifs were renamed according to their order in the protein sequence (Figure 1A).

Recently, the 3D structure of a highly C-terminally truncated form of a prokaryotic Lewis-type α1,3-fucosyltransferase (FucT) from Helicobacter pylori, a member of the GT10 family, was solved by X-ray crystallography. FucT crystals soaked with GDP-fucose revealed the donor substrate binding site (Sun et al. 2007). It is evident from the structure that the C-terminal portion of the catalytic domain is responsible for the donor substrate binding; while, as predicted for other GT10 members, the N-terminal subdomain interacts with the acceptor substrate. FucT and FucU (another Lewis-type α1,3-fucosyltransferase homolog) from H. pylori J99 share some conserved features with other GT10 members such as the α1,3-FucT motif. The main difference is that, unlike the eukaryotic GT10 members, they are not type II transmembrane proteins. Furthermore, they have a repetitive sequence on their C-terminus which is responsible for their dimerization and membrane anchoring (Sun et al. 2007).

Eukaryotic GT10 members, as type II transmembrane proteins, have a topology typical of Golgi glycosyltransferases with a so-called cytoplasmic/transmembrane/stem (CTS) region followed by a globular C-terminal catalytic domain (Grabenhorst and Conradt 1999). In addition, plant core α1,3-fucosyltransferases are extended by a C-terminal region of ≏100 residues when compared with other GT10 members. This C-terminal tail does not show similarity to any other known sequence. Core α1,3-fucosyltransferases from the invertebrates Caenorhabditis elegans and Drosophila melanogaster do not possess such a region, even though they catalyze the same or similar reaction (Fabini et al. 2001; Paschinger et al. 2004). Furthermore, consistent with their localization within the secretory pathway, plant and invertebrate core α1,3-fucosyltransferases possess potential N-glycosylation sites, as do other eukaryotic GT10 members. It is interesting to note that GT10 members, as well as some other glycosyltransferases with this proposed topology, are sensitive to deletions at the C-terminus, while most N-terminal CTS regions are not essential for the enzymatic activity (Xu et al. 1996; Sherwood and Holmes, 1999).
In the past, the structure–function relationships of especially human Lewis-type fucosyltransferases were examined; here, however, we focus on two core α,1,3-fucosyltransferases from different organisms, which have subtly divergent substrate specificities (see Scheme 1). Whereas the nematode enzyme (C. elegans FUT-1) accepts paucimannosidic glycans lacking nonreducing terminal N-acetylglucosamine residues, the plant enzyme (Arabidopsis thaliana FucTA) requires at least one such residue on its acceptors. Previously, both enzymes were expressed in Pichia pastoris as recombinant soluble forms lacking the putative cytoplasmic and transmembrane domains (Wilson et al. 2001; Paschinger et al. 2004). In order to assess minimal active variants of both enzymes, the importance for catalytic activity of selected conserved residues and the role of predicted N-glycosylation sites, we extended the previous studies by preparing a series of truncation and site-directed mutants. Furthermore, our data allow us to define for the first time a putative donor-binding motif also present in Lewis-type fucosyltransferases.

**Scheme 1** Reactions catalyzed by nematode and plant core α,1,3-fucosyltransferases. Both possible in vivo and in vitro reactions of C. elegans and A. thaliana core α,1,3-fucosyltransferases are shown; the C. elegans FUT-1 catalyzes the transfer of fucose to MMF6 in vivo, but the transfer to Man5 has also been observed (see this study). On the other hand, the A. thaliana FucTA probably accepts xylosylated and nonxylosylated forms of MGn or GnGn in vivo, but can transfer to α,1,6-fucosylated glycans in vitro, even though these glycans are not naturally present in plants (hence “reaction possible in vitro” as opposed to “possible reaction in vivo”). N-glycan abbreviations are based on the complex N-glycan nomenclature of Schachter (Fabini et al. 2001) and the schematic representations for N-acetylglucosamine, fucose and mannose are those proposed by the Consortium for Functional Glycomics.

### Results

**Expression of recombinant variants of A. thaliana FucTA**

As previously shown, employment of the yeast α-mating factor signal sequence at the N-terminus of the untagged truncated FucTA mutant Δ1-88 (which lacks the CTS domain) resulted in secretion of the fusion protein into the P. pastoris conditioned medium (Bencúrová et al. 2003), whereas the Δ1-95 form was apparently inactive. By recloning a reading frame encoding residues 89–501 of FucTA into a redesigned version of the pPICZαC vector, a new recombinant form of FucTA with both His- and FLAG tags at its mature N-terminus was prepared. Successful expression in P. pastoris was shown by activity tests and Western blotting with anti-FLAG; the enzyme could also be purified by nickel chelation chromatography using either Ni-NTA or HisTrap columns. The calculated $M_r$ of this form of FucTA is ~49 kDa, whereas Western blotting indicates an $M_r$ of 60–70 kDa (Figure 2A, lane REF and 2C, lane a); the difference is probably due to glycosylation as shown by its sensitivity to PNGase F (Figure 2C, lanes a and b). Further experiments regarding truncation as well as examination of putative catalytically important residues and predicted N-glycosylation sites of FucTA were performed using this HisFLAG-tagged form.

An initial experiment was aimed at defining whether the last 113 residues, delineating the C-terminal subdomain unique to plant core α,1,3-fucosyltransferases, are necessary for the enzyme activity. Deletion of the C-terminal subdomain of the Δ1-88 form resulted in expression of a double truncated mutant of FucTA (ΔΔ1-88;388–501: Figure 2A lane ΔΔ); Based on use of anti-FLAG enzyme-linked immunosorbent assay (ELISA) to determine the relative expression levels, we observed that this deletion results in a dramatic decrease of the specific activity of the purified protein, which retains a mere 1% of the original activity of Δ1-88 (Figure 2B).

Considering also the aforementioned previous results, the minimum catalytically active region of FucTA commences between residues 89 and 94, whereas the extended C-terminal region is still important for activity or stability.

**Expression of recombinant variants of C. elegans FUT-1**

In addition to analysing a FLAG-tagged form of FUT-1 lacking the N-terminal 32 residues, a set of mutants of this enzyme, engineered to lack either N-terminal and/or C-terminal residues, were expressed in P. pastoris. Truncations were engineered based on the protein sequence homology of the five different α,1,3-fucosyltransferases previously cloned from C. elegans (Paschinger et al. 2004) in order to estimate the boundary between the CTS and the catalytic domain. It was attempted to generate the following mutants possessing N-terminal FLAG-tags: Δ1-78, ΔΔ1-78;424–433, Δ1-95, ΔΔ1-95;424–433. However, only two of the truncated mutants Δ1-78 and ΔΔ1-78;424–433 were successfully expressed in P. pastoris and further characterized (Figure 2D and E).

As the C-terminal region of some fucosyltransferases is essential for catalytic activity (Xu et al. 1996; Tanaka et al. 2001), the impact of C-terminal truncation on the catalytic activity of FUT-1 was also studied. In accordance with expectations, deletion of 10 amino acids from the C-terminus, to yield the double truncated mutant ΔΔ1-78;424–433, resulted in an
almost complete loss of activity (0.2% of the specific activity of the control Δ1-78) as well as reduced protein expression (Figure 2D and E). Considering all our data, it appears that residues 79–433 approximately delineate the minimal region of FUT-1 that can be expressed efficiently in an active form.

Therefore, point mutations in the fut-1 open reading frame were prepared based on the truncated form Δ1-78. Two of these mutants (N194Q and T361A) affect two putative N-glycosylation sites; a proof that FUT-1 is indeed glycosylated is offered by the effect of PNGase F digestion on this enzyme (Figure 2C, lanes c and d), a result also observed when examining FUT-1 expressed in insect cells (data not shown).

Enzymatic characterization of soluble forms of FucTA and FUT-1

For an exact enzymological characterization of recombinant FucTA (Δ1-88), a dansylated NST-GnGnF6 glycopeptide was used as a substrate in an reversed-phase high-performance liquid chromatography (RP-HPLC)-based assay; the product eluting at 9 min was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS/MS to have a composition consistent with a GnGnF3F6 structure, whereas this peak was absent in the control (Figure 3A). Recombinant FUT-1 (Δ1-33) also catalyzes the synthesis of anti-horseradish peroxidase (HRP) epitope in vitro, but converts MM and MMF6 glycopeptides to MMF3 and MMF3F6, respectively; therefore, it uniquely accepts paucimannosidic, rather than complex, N-glycans as substrates (Paschinger et al. 2004). Considering data suggesting that C. elegans mutants with increased levels of Man5GlcNAc2 also possess anti-HRP epitope (specifically, the GlcNAcT-I triple mutant; Zhu et al. 2004), we reappraised whether dansylated NS-Man5 glycopeptide is a FUT-1 substrate; previously, we stated that we had not detected activity (Paschinger et al. 2004). However, when using more enzyme and longer incubation times, we could observe that Man5 was indeed fucosylated as suggested by the appearance of a peak of earlier retention time (9.5 min); the rate of transfer was around an order of magnitude less than that to MMF6. The actual transfer of fucose to the reducing-terminal GlcNAc of Man5 was demonstrated by LC-MS/MS (Figure 3B).
Using the HPLC-based assay, the optimal pH reaction for *Arabidopsis* FucTA (Δ1-88) was found to be between pH 6 and 7. Maximal activity at the optimal temperature (30°C) was obtained at pH 6.5 (data not shown). Similarly, the optimal pH for *Caenorhabditis* FUT-1 (Δ1-33) was previously found to be between pH 6.5 and 8, with a maximal activity at pH 7.5 and a temperature optimum between 25 and 30°C (Paschinger et al. 2004); these data were also confirmed when directly comparing FUT-1 activity in culture supernatants of yeast expressing the Δ1-33 and Δ1-78 forms (data not shown).

The divalent metal ion dependency of FucTA (Δ1-88) was examined with an enzyme preparation (HisTrap-purified) deprived of divalent metal ions by exhaustive dialysis against a dilute Tris buffer; activity assays were then performed in the absence and presence of 10 mM divalent metal ions. The highest relative enzyme activity was obtained in the presence of Mn(II) ions; the enzyme was also active in the presence of Mg(II), Co(II), Ni(II) and Ca(II) and exhibited ~5% activity even in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) or in the absence of divalent metal ions. Zn(II) and Cu(II) ions did not activate the enzyme; on the contrary, they had an inhibitory effect with activities in the range of 3% when compared with that in the presence of Mn(II) ions (Figure 3C).

In the case of FUT-1, the divalent metal ion dependency was examined with an Affigel Blue purified preparation of the Δ1-33 form. Similar to previous results on either *C. elegans* extracts or crude *Pichia* supernatants (Paschinger et al. 2004),


(A) *Arabidopsis* FucTA was characterized using an RP-HPLC-based assay; the dansyl-GnGnF₆ substrate was converted to a product displaying an earlier elution time consistent with formation of GnGnF₃F₆. This product was collected and analyzed by MALDI-TOF MS/MS. The loss of m/z 794.1 from the m/z 2183.1 parent ion is consistent with a concomitant loss of both one terminal GlcNAc residue and the dansylated Asn-Ser-Thr peptide moiety (see putative fragmentation pattern); the two fucose residues are apparently linked to the same reducing-terminal GlcNAc residue as shown by consecutive fragments of m/z 1096.0, 1242.0 and 1389.0. The differences in m/z are annotated with F, H or N, indicating either a difference corresponding to a fucose (strictly, a deoxyhexose), hexose or N-acetylhexosamine residue. (B) *Caenorhabditis* FUT-1 was characterized also in an RP-HPLC-based assay and the transfer of fucose was accompanied by a reduction in retention time as shown by the appearance of a new peak at 9.5 min in the chromatogram after incubating a dansylated Man5 glycopeptide in the presence of the enzyme, GDP-Fuc and Mg(II) ions. The product, absent when Cu(II) was included in the incubation, was analyzed by LC-MS/MS with the doubly charged species of m/z 909.0 being fragmented; the daughter ion with m/z 802.4 is consistent with the attachment of fucose to the asparagine-linked GlcNAc residue (see putative fragmentation pattern). (C) Enzyme activity of FucTA (Δ1-88) and FUT-1 (Δ1-33) in the presence of divalent metal ions. FucTA was purified by HisTrap affinity chromatography and incubated with dansyl-NST-GnGnF₆ as substrate, whereas Affigel Blue-purified FUT-1 was tested with dansyl-NS-MMF₆; GDP-Fuc was used as the donor substrate either in the presence or absence of different divalent metal ions or EDTA. For both enzymes, the highest enzyme activity was referred to be 100%.
the highest activity when using MMF as a substrate was obtained in the presence of Mg(II) ions. Although Zn(II) and Cu(II) ions completely inhibited the enzyme activity, the enzyme still showed activity in the presence of EDTA or without exogenous divalent metal ions (Figure 3C). When Man5 was used as a substrate (data not shown), the $K_m$ value of FucTA towards Man5-GnGnF6 was estimated to be 0.1 mM for both enzymes; therefore, the concentration of donor substrate used in our other experiments was in excess of the $K_m$ value. Regarding the acceptor substrate, the $K_m$ of FucTA towards NST-GnGnF6 was estimated to be 15 µM; in the case of FUT-1, the $K_m$ with NS-Man5 was determined to be ~400 µM, consistent with this being a relatively poor acceptor substrate.

Identification of the GT10 first cluster motif and site-directed mutagenesis

In a comprehensive survey, we analyzed protein sequences of 47 members of the GT10 family by Clustal W and HCA (Gaboriaud et al. 1987) and found the following peptide region significantly similar for all examined sequences: S-(N/H/D)-X$_9$-R-X$_7$-(L/I)-X$_5$-X$_3$-(V/L/I)-X$_3$-G (Figure 1B; see also Supplementary data, Figure S1). This motif, designated here as the first cluster motif, is located at a variable distance of between 20 and 30 residues N-terminal to the classical α1,3-FucT motif previously defined by Martin et al. (1997). The first cluster motif, which is in addition to the five conserved motifs defined by Mollicone et al. (2009), spans residues 218–244 of the FucTA and 243–269 of the FUT-1 protein (Figures 1 and 4); it comprises three completely conserved residues (Ser, Arg, Gly) and was confirmed to be present in all GT10 family members from H. sapiens, A. thaliana and C. elegans (Supplementary data, Figure S2) regardless of whether they are core or Lewis-type enzymes. Using the crystal structure of H. pylori FucT (Sun et al. 2007) as a template, possible binding modes of the donor substrate GDP-Fuc with the first cluster motif were retrieved demonstrating the formation of hydrogen bonds between the conserved serine and arginine of this motif and the donor substrate (Figure 5).

As part of our modeling approach, a good protein alignment of H. pylori FucT and A. thaliana FucTa was needed to model the donor-binding pocket of the latter. All of the important amino acid residues, which form hydrogen bonds with the donor substrate in the template, are present in the model and are almost in the same positions as in the crystal structure. Moreover, in our model one can see how an additional hydrogen bond is formed between the hydroxyl group of Ser253 and the oxygen of the guanine moiety of the donor substrate as well as a π–π interaction between the lone pair of the guanine’s N$^7$ and the aromatic π system of Y243 (Figure 5).

In order to evaluate the role of the conserved serine within the first cluster motif, which may form a hydrogen bond with the oxygen from the α-phosphate of GDP-Fuc, alanine mutants of FucTA (S218A) and FUT-1 (S243A) were prepared. Upon expression in P. pastoris, it was observed that the Ser$\rightarrow$Ala mutation of FucTA completely abolished enzyme activity, while such a mutation of FUT-1 decreased activity by about 81% (Figure 2B and E). Since site-directed mutagenesis was predicted computationally, not to affect the local protein structure (Jpred 3 secondary structure prediction server; Cole et al. 2008), we propose that this residue plays a significant role in donor substrate binding.

Furthermore, alanine mutation of residue R226 was performed. This residue, which is conserved among all catalytically active α1,3/4-fucosyltransferases, forms a hydrogen bond with the β-phosphate of the GDP ligand. This mutation led to a complete loss of activity (Figure 2B), whereas alanine replacement of N219, which forms a hydrogen bond with the β-phosphate of the GDP ligand in our model, led to a residual activity of ~1% (Figure 2B). Mutation of residue Y243, which may be involved in the aforementioned π–π interaction with the N$^7$ atom of the donor, decreased the enzyme activity by ~90% (Figure 2B).

In the region lying between the two conserved motifs (i.e., first cluster and α1,3 FucT motifs), which seems to be a loop, alanine replacement of residue S253, which might form a hydrogen bond with the guanine of the donor substrate (Figure 5),
was also performed. This mutant retained around 35% of activity (Figure 2B). 

$K_m$ values for GDP-Fuc of those first cluster mutants (N219A, Y243A and S253A) which retained some activity were, respectively, estimated to be 0.33, 0.24 and 0.1 mM, as compared to 0.1 mM for the wild-type enzyme.

The $\alpha_{1,3}$-FucT motif of FUT-1

As mentioned above, the $\alpha_{1,3}$-FucT motif (Martin et al. 1997) is found in $\alpha_{1,3}$/4-fucosyltransferases from different species in different degrees of conservation and has been defined as motif V by Mollicone et al. (2009). It is a 19-residue region, of which 10 residues are conserved in all members of the GT10 family (Oriol et al. 1999; Jost et al. 2005). Some of these residues are directly involved in the donor substrate binding as their functional groups form hydrogen bonds with GDP-Fuc. In the case of H. pylori FucT, the residues Y246, E249 and K250 form hydrogen bonds with GDP-Fuc (Sun et al. 2007).

In this context, it is interesting to consider the three $\alpha_{1,3}$-fucosyltransferase homologs (FucTA, -B, -C) from the honeybee (Apis mellifera), which were cloned and expressed in P. pastoris by Rendić et al. (2007). Despite various attempts, no activity for FucTB was detected. FucTB shows a high similarity with the other FucTs, but here glutamate is in the position that is normally occupied by aspartate in eukaryotic fucosyltransferases, including the FucTA and FucTC from the honeybee. To test whether a single change of Asp $\rightarrow$ Glu could lead to a loss of activity in a distantly related enzyme, a point mutation of C. elegans FUT-1 was prepared, in which D303 was switched to glutamate; this form was expressed in Pichia and possessed 20% of the activity of the wild-type form (Figure 2E). This result indicates sensitivity to changes within the $\alpha_{1,3}$-FucT motif, but the significant level of residual activity suggests that this sequence deviation may not be the only reason for an absolute lack of detectable activity of honeybee FucTB. Interestingly, in H. pylori FucT, this position is occupied by glycine (Figure 1B).

Putative N-glycosylation sites of FucTA and FUT-1

The Δ1-88 FucTA mutant contains three putative N-glycosylation sites at N337, N420 and N481 and, when expressed in P. pastoris, it appears as a major band of

Fig. 5. View of the donor-binding regions of H. pylori FucT and A. thaliana FucTA. (A) One view of the conserved residues of the first cluster motif of H. pylori FucT in interaction with GDP-Fuc. (B) GDP-Fuc accommodated in the enzyme donor substrate binding pocket (coloured part of the protein surface represents conserved residues of the first cluster motif). The Protein Data Bank data of H. pylori FucT soaked with GDP-Fucose (PDB code: 2NZY) were used as source and visualized by Chimera (Sanner et al. 1996; Pettersen et al. 2004; Goddard et al. 2005). (C) View of residues either of the first cluster motif or the loop connecting the first cluster and $\alpha_{1,3}$ FucT motifs (the latter also known as motif V) which may interact with the donor substrate via their side chains based on our model of FucTA’s donor-binding pocket (the regions around residues S128, N219, R226, Y243 and S253 are shown).
70 kDa, whose size is reduced upon digestion with PNGase F (Figure 2C). Analysis of N-glycans, released from the reference enzyme by PNGase F, was also performed and indicates a series of oligomannosidic structures with compositions ranging from Hex\(_{5-14}\)HexNAc\(_2\) (Figure 6A); such structures are in keeping with previous data on Pichia glycans (Montesino et al. 1999). Thereafter, the three potential N-glycosylation sites of FucTA were examined by alanine screening of the N-X-T/S sites of His FLAG-tagged FucTA. Four FucTA mutant forms (N337A, T339A, T422A and S483A) were expressed in \(P\). pastoris, purified and assayed for activity. N337 is the one glycosylation site conserved in probably all plant core \(\alpha_1,3\)-fucosyltransferases, including that from the moss \(Physcomitrella patens\).

The relative activities for the N337A, T339A, T422A and S483A mutants, when compared with the reference wild-type (Δ1-88) form, were, respectively, 18.6% (±0.8%), 12.1% (±1.5%), 36.5% (±2.5%) and 32.2% (±1.3%) (Figure 2B). Thereby, abolishing of the potential for glycosylation at the conserved N337 residue showed the greatest reduction in activity; when compared with the wild-type \(K_m\) of 0.1 mM, the T339A mutant displayed a \(K_m\) value for GDP-Fuc of 0.36 mM. Moreover, the active triple N-glycosylation site mutant (Δ1-88 FucTA T339A_T422A_S483A) gives a single
well-defined band of ~50 kDa, which corresponds to the predicted size of the unglycosylated protein (Figure 2A). The relative specific activity of this mutant is 5.5% (±0.5%) when compared with the reference enzyme. In the case of the nematode FUT-1, mutagenesis affecting two glycosylation sites (N194 and N359) was also performed; although the T361A mutant affecting the latter site was expressed at normal levels and displayed activity, the N194Q mutant was only detected at a low level in supernatants of recombinant yeast (Figure 2E). Perhaps, it is significant that N194 is the site conserved in all C. elegans GT10 family members.

In order to investigate the glycosylation of the recombinant FucTA mutants in more detail, mass spectrometric tryptic peptide mapping was employed. We analyzed the major ~70 kDa band of the ∆1-88 mutant, a fainter lower band of ~55 kDa representing the deglycosylated form (which appeared in some preparations) and the PNGase F treated form of ∆1-88 (~50 kDa; see Figure 2C) as well as the T339A and S483A mutants. Tryptic digestion of the lower band of ∆1-88 revealed an intense peak of m/z 1654.79 which may represent the fragment YLADNPDA YDQTLR, while the spectrum of the upper band lacks this peak completely (Figure 6B). Due to the Δm/z of +1 when compared with the expected peptide, the lower band may result from deglycosylation, by an endogenous yeast PNGase, of the higher molecular weight form at N337 and conversion of this residue to Asp; however, possibly due to the heterogeneity and size of yeast glycans (Figure 6A), the corresponding glycopeptide from the upper band was itself not detected. Consistent with actual glycosylation of N337 in the upper band form, an m/z 1654.72 peak was present after treatment of FucTA with PNGase F (Figure 6B).

In the case of the N420-containing peptide, a peak of m/z ~1643.9, potentially representing the nonglycosylated fragment DGNLITLEALESAVLAK (expected m/z 1643.88) was also identified in both bands. A potential shift in the isotopic distribution towards an m/z 1644.84 peak, which could correspond to DGDNLITLEALESVLAK, can be observed in the deglycosylated sample (Figure 6B) and is concluded to be compatible with partial glycosylation at this site. The third glycosylation site, N481, is within the sequence FEGNSSLTHIQR; the presence of species of m/z 1475.7 and 1476.7 (when compared with the expected nonglycosylated m/z of 1475.7) may also indicate the presence of forms of the enzyme which either were never glycosylated at this site or became deglycosylated during or after secretion. Finally, peptide maps of the T339A and S483A mutants indicate the success of site-directed mutagenesis as judged by the respective lack of the wild-type peptides (YLADNPDA YDQTLR and FEGNSSLTHIQR) and the appearance of the peptides predicted to correspond to the mutated sequences (data not shown).

Discussion

Delineation of the minimal catalytic region for the core fucosyltransferase FucTA and FUT-1

N- and C-terminal truncation studies were performed on plant and worm core fucosyltransferases FucTA and FUT-1 in order to determine the extent of their minimal catalytic domains. Due to the lack of expression of the ∆1-95 form, it can only be inferred that the border between the stem and catalytic domains of C. elegans FUT-1 is located between residues 79–95; previous studies on the other enzyme in this study, A. thaliana FucTA, suggested that this border is between residues 88 and 95 with C90 being the first generally conserved residue in plant core fucosyltransferases (Bencúrová et al. 2003). Furthermore, we also examined whether the extra C-terminal subdomain unique to plant core α1,3-fucosyltransferases is essential for the enzyme activity. From bioinformatic analyses, we proposed that the beginning of this subdomain should begin after a “hinge” sequence comprising more than one proline residue, located next to the region which shows a sequence similarity to other higher eukaryotic members of the GT10 family; in comparison, the nematode FUT-1 lacks such a C-terminal domain. However, the deletion of the final 113 residues of FucTA and the final 10 residues of FUT-1 led to dramatic (>99%) decreases in activity. Thereby, our results confirmed that GT10 members of higher eukaryotes are sensitive to manipulation at their C-termini (Augé et al. 2000), while the N-terminal CTS domain is not essential for the enzyme activity and can be replaced by other sequences such as His- or FLAG-tags. Interestingly, a contrast to this “rule” is the bacterial FucT from H. pylori, which is not a type II transmembrane protein and which retains activity despite deletion of its C-terminal domain consisting of 115 residues (Sun et al. 2007).

Biochemical characterization

Many other eukaryotic members of the GT10 family have been biochemically characterized over the years. The pH optimum for Golgi-type members of the GT10 family such as FucT from Vaccinium myrtillus (bilberry) is pH 7.0, while that of human FucT-VII encoded by the FUT7 gene is pH 7.5 and that of human FucT-V encoded by the FUT5 gene is between pH 5.0 and 7.0 (Holmes et al. 1995; Shimoda et al. 1997; Palma et al. 2001). The pH optimum of recombinant FucTA was found to be between pH 6.0 and 7.0 with maximal activity at pH 6.5, while recombinant FUT-1 displays maximal activity at pH 7.5. Therefore, the pH optima of our enzymes are within the usual range for Golgi-type α1,3-/1,4-fucosyltransferases. Since the Golgi complex displays pH around 6.5 in different organisms (Seksek et al. 1995; Kim et al. 1996; Llopis et al. 1998), such pH optima are consistent with the intracellular milieu of these enzymes.

Some glycosyltransferases including fucosyltransferases with a GT-B fold require divalent metal ions for their optimal activity, even if there is no evidence for a bound metal ion being involved in catalysis in such enzymes (Breton et al. 2006). On the other hand, glycosyltransferases sharing the GT-A fold or its variant very often possess a DXD motif (Breton et al. 2006) or a similar sequence such as EXD, DXE or EXE involved in coordination of divalent metal ions required for their activity. Consistent with the possession of a GT-B fold, divalent metal ions are not essential for the activity of human FucT-III encoded by the FUT3 gene, but addition of Mn(II) boosted the enzyme activity and caused an
increase in the affinity for the acceptor (Palma et al. 2004). The same feature was described in the case of human FucT-V and also alternative divalent metal cofactors were identified, such as Ca(II), Co(II) and Mg(II) (Murray et al. 1997). The Le^3-forming FucT from Silene alba requires Mg(II) or Mn(II) for activity and is inhibited by Zn(II) (Léonard et al. 2005). In the case of recombinant FucTA, assaying dialyzed enzyme either in the presence of EDTA or the absence of exogenous divalent cations did not cause a complete lack of activity, but resulted in ∼95% less activity when compared with the control assay in which Mn(II) was present. Divalent metal ions such as Mg(II), Ca(II), Ni(II) and Co(II) ions also significantly stimulated the enzyme, while Cu(II) and Zn(II) ions were inhibitory. Comparable results were obtained in the case of FUT-1 except that this enzyme is not so dependent on divalent metal ions; in the presence of EDTA the enzyme retained some 20% of its control activity.

In the case of the GDP-Fuc donor substrate, the \( K_m \) value was determined to be 100 \( \mu \)M for soluble forms of both FucTA and FUT-1, when compared with 70 \( \mu \)M for \( H. pylori \) FucT (Ma et al. 2006), 20 \( \mu \)M for human FucT-VI (Jost et al. 2005) and 16 \( \mu \)M for human FucT-VII (Shinoda et al. 1997). Thus, our \( K_m \) values are within a similar range to those of other GT10 family members.

First cluster motif mutants

Based on our bioinformatic analyses, we define a new motif (first cluster) present in GT10 fucosyltransferases, whether core or Lewis-type, located between the donor-binding motifs IV and V as defined by Mollicone et al. (2009). As the conserved serine of this motif forms a hydrogen bond with the donor substrate, we compared the impact of mutating this residue in our two enzymes. The FUT-1 (S243) mutation did not lead to complete abolition of enzyme activity (20% residual activity) as observed in the case of FucTA (S218); this result was unexpected as all the previously described mutations of the conserved residues of the donor-binding pocket led to a dramatic drop of activity (Sherwood et al. 1998; Jost et al. 2005). Since FUT-1 uses also a different acceptor substrate compared to other core \( \alpha_1,3 \)-fucosyltransferases which are dependent on the presence of the GlcNAc residue, transferred by GlcNAcT-I, the binding pocket might be changed in a way that a modification of this conserved residue which otherwise leads to a loss of activity has less dramatic effects on the activity of the FUT-1. Evidently, this conserved serine residue is not the only determinant of the donor substrate binding and its importance may vary among the GT10 members.

Other alanine replacements were based on modeling of FucTA using the \( H. pylori \) FucT as a template. For instance, N219 and R226 of FucTA, which are predicted to form hydrogen bonds with the \( \beta \) phosphate of the GDP ligand were examined. Whereas the R226A mutant displayed no activity, the N219A mutant showed a residual activity of ∼1% and an apparent increase in \( K_m \) value for GDP-Fuc. Alanine replacement of Y243, which may interact with the lone pair of guanine’s N7, decreased the enzyme activity by ∼90% and also resulted in an increase in the \( K_m \) value for GDP-Fuc; these data indicate roles for these three residues in donor binding. Interestingly, alanine replacement of S253, which might form a hydrogen bond with the guanine of the donor substrate, resulted in retention of around 35% of activity and no change in the \( K_m \) value for GDP-Fuc. Considering the distance between this residue and the assumed active site, the contribution of S253 to the reaction mechanism remains unclear.

N-glycosylation site mutants

As with many Golgi proteins, eukaryotic fucosyltransferases are N-glycosylated. For instance, human \( \alpha_1,3/4 \)-fucosyltransferases III, -V, and -VI (FucT-III, -V, and -VI) contain two conserved C-terminal N-glycosylation sites (FucT-III: N154 and N185; FucT-V: N167 and N198; and FucT-VI: N153 and N184). Compared to wild types, FucT-V and -VI mutants of the first conserved glycosylation site as well as double mutations led to a loss of activity whereas the N198Q and N185Q mutants decreased the activity more than three-fold. The FucT-III glycosylation site mutant N154Q exhibited ∼15%, the N153Q/N185Q double mutant ∼5%, and N184Q ∼38% of the control activity. Moreover, tunicamycin-mediated inhibition of N-glycosylation in cells expressing FucT-III resulted in a complete absence of activity of the recombinant enzyme, while castanospermine treatment, which affects processing of N-linked oligosaccharides, diminished activity by ∼60% when compared with the native enzyme (Christensen, Bross, et al. 2000; Christensen, Jensen, et al. 2000).

The first of the sites conserved in human FucT-III, -V and -VI is also conserved in \( C. elegans \) fucosyltransferases (Paschinger et al. 2004). In the case of \( C. elegans \) FUT-1, the corresponding glycosylation site mutant (N194Q) was only expressed at a low level, which may be due to improper folding. On the other hand, the analysis of N-glycosylation site mutants of \( A. thaliana \) FucTA indicates that N-glycosylation is not absolutely essential for activity. Certainly, we observed that \( P. pastoris \) produces incompletely glycosylated isoforms of FucTA; in part, as judged by the presence of Asp at predicted Asn sites in samples not treated with PNGase F, this may be due to an endogenous PNGase activity. Indeed, the results of mass spectral fingerprinting, glycan analysis, Western blotting and mutagenesis suggest that all the three N-glycosylation sites may be glycosylated by a range of oligomannosidic \( N \)-glycans, but that occupation of these sites is incomplete; it is unknown whether partial occupancy of glycosylation sites is also a phenomenon in planta.

Conclusion

To summarize, our data support the premise that N-glycosylation is necessary for the expression of full enzymatic activity of GT10 family members and in some cases may also be important for the stability and proper folding of the enzyme. Thereby, at least one or more potential sites have to be occupied for optimal activity; however, results with the triple mutant of FucTA reveal that an unglycosylated core \( \alpha_1,3 \)-fucosyltransferase is still catalytically active. Furthermore, the definition, modeling and mutagenesis of the first cluster motif extends our knowledge regarding the mode of donor substrate binding in the GT10 family; nevertheless, clear conclusions can only be made using a crystal structure...
and preparation of the unglycosylated form of FucTA may represent a way forward in terms of obtaining more detailed data on eukaryotic α1,3-fucosyltransferases.

Materials and methods

Homology modeling

The structure of FucT from H. pylori (Sun et al. 2007; PDB code: 2NZY) served as a template for homology modeling. The most conserved regions of H. pylori FucT and A. thaliana FucTA were aligned to model the donor-binding pocket of the A. thaliana FucTA. Several alignments of these sequences were used to build models by Modeller9v1 (Eswar et al. 2007). The obtained model was then visualized and prototyped using the UCSF Chimera program (Sanner et al. 1996; Pettersen et al. 2004; Goddard et al. 2005) in the presence of the donor substrate. Data for GDP-Fucose were retrieved from the PDB: 2NZY structure. Charge -2 was assigned to the donor substrate and a subsequent energy minimization was performed using the AMBER’s (v. 10) molecular mechanical force field 99SB, where charges for the donor substrate were assigned by Gasteiger charges (Ponder and Case 2003; Case et al. 2008).

Preparation of pPICZαFLAGC3 and pPICZαHisFLAG

The pPICZαC vector (Invitrogen, Carlsbad, CA, USA) was modified by inverse PCR to insert a region encoding a FLAG tag or hexahistidinyl-FLAG tag just upstream of the multiple cloning site but downstream of the region encoding the Ste13 signal cleavage site. These derivatives are designated pPICZαFLAGC3 and pPICZαHisFLAG.

Cloning of A. thaliana FucTA mutants

For the study of core α1,3-fucosyltransferase A (FucTA) from A. thaliana, a set of gene constructs was ligated into forms of the pPICZαC vector. Truncation mutants of FucTA were prepared by PCR amplification from the construct pPICZαC/Δ1-66 FucTA (Bencuvrová et al. 2003) using relevant pairs of primers (Table I), the DynAzyme EXT polymerase (Finzymes, Espoo, Finland) and the buffer supplied by the manufacturer containing 15 mM MgCl2 under the following conditions: one cycle of 95°C for 5 min, 35 cycles of 1 min at 95°C, 45 s at 55°C, 1–2 min at 72°C and with a final extension step at 72°C for 7 min. PCR fragments were gel-purified (Wizard® SV Gel and PCR Clean-Up System; Promega, Madison, WI, USA) and incubated for 1 h with KpnI at 37°C prior to addition of EcoRI, after which time the digestion was continued for one more hour. The pPICZαFLAGC3 and pPICZαHisFLAG plasmids were cut under the same conditions as for the PCR products and were generally treated with calf intestine alkaline phosphatase (Fermentas, St. Leon-Rot, Germany) during the final half-hour of restriction digestion. PCR fragments were then ligated into either pPICZαFLAGC3 or pPICZαHisFLAG vector for 3 h at room temperature using T4 DNA ligase (Fermentas) and a standard ligase buffer.

Point mutants of FucTA were prepared by inverse PCR amplification using pPICZαFLAGC3/Δ1-88 FucTA or pPICZαHisFLAG/Δ1-88 FucTA as templates and pairs of phosphorylated mutation primers (listed in Table I). To perform the inverse PCR, Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) together with its buffer was used under the following conditions: one cycle of 95°C for 2 min, 16 cycles of 45 s at 95°C, 1 min at 55°C, 13 min 40 s at 68°C. Inverse PCR products were gel-purified and ligated as above. After transformation into E. coli DH5α cells and plating the cells on LB/low-salt agar containing zeocin (25 µg/mL; Invitrogen), positive clones were selected after PCR screening and by sequencing to confirm the reading frame.

Cloning of C. elegans FUT-1 mutants

To facilitate expression in P. pastoris of FUT-1 variants, the pPICZαFLAGC3 shuttle vector was used. Fragments encoding truncated forms of FUT-1 were amplified using PCR and primers specific for the K08F8.3 reading frame (Table I). The Expand PCR High fidelity system (Roche Applied Science, Mannheim, Germany) was used with C. elegans cDNA as template (Paschinger et al. 2004). PCR conditions were as follows: one cycle of 95°C for 3 min, 30 s at 50°C, 2 min 30 s at 72°C, one cycle of 95°C for 30 s, 30 s at 51°C, 5 min at 72°C, 10 cycles of 95°C for 30 s, 30 s at 58°C, 3 min at 72°C, 25 cycles of 95°C for 30 s, 30 s at 58°C, 3 min +5 s per cycle at 72°C, and with a final extension step at 72°C for 7 min. PCR fragments and the vector were gel-purified (with the GFX kit); these were incubated at 37°C for 3 h with PstI, XbaI and BSA in Buffer 3 (all four components from New England Biolabs, Ipswich, MA, USA) and then heated for 15 min at 80°C to inactivate the enzymes. In case of the vector, alkaline phosphatase was added after 2.5 h. The restricted DNA was then purified by ethanol precipitation. PCR fragments were ligated into the pPICZαFLAGC3 vector.

Four different point mutations (N194Q, S243A, D303E, T361A) were generated by inverse PCR. The pPICZαFLAGC3/Δ1-78 FUT-1 construct was used as the basis for these mutations. For inverse PCR, the KOD hot start kit (Merek Biosciences, Darmstadt, Germany) and specific primers (Table I) were used under the following conditions: one step of 95°C for 3 min, 30 cycles of 95°C for 1 min, 1 min at 60°C, 5 min at 72°C, and a final extension step of 4 min at 72°C. Purified inverse PCR products were then ligated and selected positive clones were sequenced to confirm the reading frame and the mutation.

Electroporation of P. pastoris GS115 and heterologous expression of mutants

Two to ten µg of the expression construct plasmid DNA was linearized by PmeI (MssI) and used to electroporate P. pastoris competent cells (GS115), which were then plated onYPD agar containing zeocin (100 µg/mL). Screening by PCR amplification of genomic DNA, using plasmid or insert-specific primers, confirmed the integration of the relevant expression cassette. Selected colonies of recombinant P. pastoris were inoculated into 10 mL of preculture glycerol-containing MGYP medium supplemented with 100 µg/mL of zeocin. After overnight incubation at generally 30°C with continuous shaking (200 rpm) in baffled flasks, cells were

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collected by centrifugation at 1500 × g. The cells were washed once in 1.34% (w/v) yeast nitrogen base solution and resuspended in methanol-containing MMYC medium to a starting OD600 = 1.5. Every 24 h samples of the cultures cultivated at 18°C were removed and extra methanol was added to maintain a concentration of 1% (v/v).

Recombinant protein purification

Purification of the reference and mutant forms of FucTA (Δ1-88) was done by HisTrap affinity chromatography. After expression, phenylmethylsulfonyl fluoride (final concentration 0.1 mg/mL) was added to the culture medium (150 mL), which was then centrifuged at 1500 × g for 5 min. The resulting supernatant was centrifuged again at 45,000 × g for 15 min to remove insoluble material. The supernatant was then concentrated using an Amicon device (cut off 15 kDa) to ≏25 mL and dialyzed against equilibration buffer (25 mM Tris-HCl, 300 mM NaCl, 7 mM imidazole, pH 8.5) and loaded on the equilibrated HisTrap column. The column was then washed with 25 mM Tris-HCl, 300 mM NaCl, 7 mM imidazole, pH 8.5. Proteins were eluted with 25 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, pH 8.5 prior to extensive dialysis against 25 mM Tris buffer, pH 7, containing 150 mM NaCl to remove any Ni(II) leaching from the column.

The supernatant from the FUT-1 expression culture (30 mL) was concentrated on Amicon (cut off 10 kDa) and at the same time buffered to Affigel Blue running conditions (25 mM Tris-HCl, pH 7). The enzyme concentration was then incubated with ca. 6 mL of Affigel Blue for 1 h on a rotating mixer in the cool room. All chromatography steps were carried out at 4°C. After filling of the column, the gel was washed with two column volumes of starting buffer, while 2 mL of fractions were collected. Eluted protein was then concentrated (~50 times, same device as above) and rebuffered with 25 mM Tris, pH 7.

### Table I. PCR primers employed in this study

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primers</th>
<th>Sequence (5′ → 3′)</th>
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<tr>
<td>Arabidopsis FucTA</td>
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</tr>
<tr>
<td>Δ1-88</td>
<td>Dei1/EcoRI</td>
<td>CCGGAATTCCGAGAATGCCAGAGTTG</td>
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<tr>
<td>2Δ1-88;388–501</td>
<td>Dei1/EcoRI</td>
<td>CCGGAATTCCGAGAATGCCAGAGTTG</td>
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<td>388stopFucTA</td>
<td>S218A</td>
<td>GCAATTCGCGCCCTCGAGAATTTCCGC</td>
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<tr>
<td>S218A</td>
<td>S218Aev</td>
<td>GAAATAAGCGACAGCAAGCAGCTTTTCGTG</td>
</tr>
<tr>
<td>N219A</td>
<td>N219Afw</td>
<td>CCGAAATTAAGACAGCAAGCAGCTTTTCGTG</td>
</tr>
<tr>
<td>N219A</td>
<td>N219Arev</td>
<td>CCGAAATTAAGACAGCAAGCAGCTTTTCGTG</td>
</tr>
<tr>
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<td>R226Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
</tr>
<tr>
<td>Y243A</td>
<td>Y243Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
</tr>
<tr>
<td>S253A</td>
<td>S253Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
</tr>
<tr>
<td>N337A</td>
<td>N337Afw</td>
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</tr>
<tr>
<td>T339A</td>
<td>T339Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
</tr>
<tr>
<td>T422A</td>
<td>T422Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
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<tr>
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<td>Triple</td>
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<tr>
<td>S243A</td>
<td>S243Afw</td>
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</tr>
<tr>
<td>D303E</td>
<td>D303Efw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
</tr>
<tr>
<td>T361A</td>
<td>T361Afw</td>
<td>CCGTCGCAAGTTCTGAAGCC</td>
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<tr>
<td>Caenorhabditis FUT-1</td>
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<td></td>
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<tr>
<td>Δ1-78</td>
<td>K08F8.3/1 trunc1/PsrI</td>
<td>ATTCGCAGAAGACCTTTTTAATTTAACG</td>
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<tr>
<td>Δ1-78;424–433</td>
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<td>ATTCGCAGAAGACCTTTTTAATTTAACG</td>
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<tr>
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<td>K08F8.3/trunc2/PsrI</td>
<td>ATTCGCAGAAGACCTTTTTAATTTAACG</td>
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<tr>
<td>N194Q</td>
<td>K08F8.3_194Gln5’</td>
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<td>D303E</td>
<td>K08F8.3_308Glu5’</td>
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<td>T361A</td>
<td>K08F8.3_308Glu3’</td>
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<tr>
<td></td>
<td></td>
<td>ATTCGCAGAAGACCTTTTTAATTTAACG</td>
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</table>
**Anti-FLAG ELISA**

Up to 10 μL of *Pichia* supernatant or diluted purified enzyme preparations were pipetted into the wells of MaxiSorb ELISA plates (Nunc, Roskilde, Denmark), then 40 μL of 50 mM Na₂CO₃, pH 9, were added. The plate was then incubated at 37°C for 2 h (or overnight at 4°C). The wells were emptied, washed once with TTBS (200 μL), and then blocked at 37°C with TTBS containing 0.5% BSA (100 μL) for 1 h. The wells were then emptied and murine anti-FLAG (M2; Sigma-Aldrich, St. Louis, MO, USA), 1:5000 in TBS containing 0.5% BSA (50 μL), was applied. After 1 h incubation at 37°C, the wells were washed three times, then the secondary antibody, anti Mouse IgG alkaline phosphatase conjugate (1:5000 in TBS containing 0.5% BSA; 50 μL), was added. After incubation and washing as above, 1 mg/mL p-nitrophenyl-phosphate (pNP-phosphate) in 0.1 M Tris pH 8.5 was added (150 μL), the plate was incubated at 37°C for 1 h or measured each 10 min, then the reaction was stopped by the addition of 5 M NaOH (50 μL). The plate was then analyzed with an SLT Spectra plate reader at 405 nm. Calibration was done using a FLAG-BaP positive control (Sigma-Aldrich).

**A. thaliana FucTA enzyme assay**

Several forms of *A. thaliana* FucTA were assayed using GnGnF₆-NST-dansylated glycopeptide as an acceptor substrate. Reactions containing 0.05 mM acceptor, 1 mM GDP-Fuc, 10 mM MnCl₂, 50 mM 2-morpholinoethanesulphonic acid (MES), pH 6.5 and 4 μl of enzyme preparation at a suitable dilution, to avoid substrate conversion higher than 20% (thus staying in the linear range), were performed in a final volume of 10 μl. The amount of enzyme added to each reaction was calculated on the basis of anti-FLAG reactivity in ELISA as compared to a FLAG-BaP control which has a similar *M*<sub>f</sub> to FUT-1. For the *K*<sub>m</sub> value for GDP-Fuc as well as the comparison of FUT-1 mutants, assays (2 h at 23°C) were performed in the presence of HEPEs buffer (pH 7.5) with 0.02, 0.06, 0.1, 0.2 or 2 mM GDP-Fuc and dansyl-MMF<sup>6</sup> as an acceptor. The enzyme incubations were analyzed by reversed-phase HPLC employing an MZ Analysetechnik Hypersil column as above, but with 7% of solvent B for isocratic elution when using Man5 as the acceptor substrate or 8.5% when using MMF<sup>6</sup>.

**Western blotting, tryptic peptide mapping, PNGase F treatment and mass spectrometry**

SDS-PAGE was performed using 10% polyacrylamide gels; staining was performed using Coomassie blue. For Western blotting, a semi-dry transfer procedure was employed and after blocking, the membranes were incubated with a mouse monoclonal antibody raised against the FLAG-epitope (1:5000); proteins were visualized with alkaline phosphatase-conjugated antimouse IgG raised in goat (1:5000). As required, protein samples were precipitated using a five-fold excess of methanol prior to electrophoresis.

Mass spectral fingerprinting of SDS-PAGE separated proteins was performed after excision of bands of interest from the gel; after a series of washing steps, proteins were reduced and alkylated in situ, prior to further washing. After drying of the gel pieces, one gel volume of 12.5 μg/mL modified trypsin (sequencing grade, Roche Applied Science) in 20 mM NH₄HCO₃ was added. After an overnight incubation at 37°C, the gel pieces were washed with 50% acetonitrile/0.1% TFA to extract the peptides prior to MALDI TOF/MS.

A portion of the partially purified enzymes was deglycosylated using N-glycosidase F from Roche Applied Science, using the manufacturer’s standard protocol for complete removal of *N*-glycans, prior to SDS-PAGE. For analysis of released *N*-glycans, a portion of a tryptic digest of FucTA was incubated using glycerol free PNGase F from New England Biolabs, prior to purification of glycans with a mini-column (containing Dowex AG 50 and LiChroprep<sup>®</sup> resins) and analysis by MALDI TOF/MS.

MALDI-TOF MS and MS/MS was performed using a Bruker Ultraflex TOF-TOF (Bremen, Germany) instrument with 2,5-dihydroxybenzoic acid as matrix for dansylated glycopeptides and glycans or with α-cyano-4-hydroxycinnamic acid for dabsylated glycopeptides and tryptic peptides. MS/MS of the fucosylated Man₅GlcNAc₂ dansyl-peptide was performed on an ion trap LC-MS (Agilent XTC ultra).

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Abbreviations

CAzy, Carbohydrate Active enZymes database; CTS, N-terminal region of type II transmembrane proteins comprising cytoplasmic tail, transmembrane domain, and stem region; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FLAG-tag, the peptide sequence of the FLAG-tag is as follows: DYKDDDKD; GDP-Fuc (GDP-Fucose or GDP-1-fucose), guanosine diphosphate-1-fucose; GnGn, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; GnGnF3, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; GnGnF5, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; GnGnF6, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; GnGnF8, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; GnGnF8, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; GnGnF10, GlcNAcβ1-2Manβ1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; MM, Man5, Manα1-6(Manα1-3)Manβ1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc; MMGn, Manα1-6(GlcNAcβ1-2Manβ1-3)Manβ1-4GlcNAcβ1-4GlcNAc; MMF5, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; MMF5, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; MMF5, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc; MS, mass spectrometry; OD, optical density; RP, reversed phase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; YNB, yeast nitrogen base; YPD, yeast extract peptone dextrose medium; YNB–silica silica gel; YPD–silica silica gel.


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