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Towards abolition of immunogenic structures in insect cells: characterisation of a honeybee multi-gene family reveals both an allergy-related core α 1,3-fucosyltransferase and the first insect Lewis histo-blood group-related antigen synthesising enzyme

Dubravko Rendić*, Jaroslav Klaudiny[†], Ute Stemmer*, Julia Schmidt*, Katharina Paschinger* and Iain B. H. Wilson*[‡]

*Department für Chemie, Universität für Bodenkultur, Muthgasse 18, A-1190 Wien, Austria

[†] Department of Glycobiology, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia

[‡]To whom correspondence should be addressed: iain.wilson@boku.ac.at

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Abstract

Glycoproteins from *Apis mellifera*, such as phospholipase A₂ and hyaluronidase are well known major bee venom allergens. They carry N-linked oligosaccharide structures with two types of α 1,3-fucosylation: the modification by α 1,3-fucose of the innermost core GlcNAc, which constitutes an epitope recognised by IgE from some bee venom allergic patients, and a antennal Lewis-like GalNAc β 1,4(Fuc α 1,3)GlcNAc moiety. We now report the cloning and expression of two cDNAs encoding the relevant active α 1,3-fucosyltransferases. The first sequence, closest to *Drosophila melanogaster* FucTA, was found to be a core α 1,3-fucosyltransferase (EC 2.4.1.214), as judged by several enzyme and biochemical assays. The second cDNA encoded an enzyme, most related to *Drosophila* FucTC, was shown to be capable of generating the Le^x epitope *in vitro* and is the first Lewis-type α 1,3-fucosyltransferase (EC 2.4.1.152) to be described in insects. The transcription levels these two genes in various tissues were examined: FucTA was found to be predominantly expressed in the brain tissue and venom glands, whereas FucTC transcripts were detected at highest levels in venom and hypopharyngeal glands. Very low expression of a third homologue of unknown function, FucTB, was also observed in various tissues. The characterisation of these honeybee gene products not only accounts for the observed α 1,3-fucosylation of bee venom glycoproteins, but is expected to aid the identification and subsequent down-regulation of the fucosyltransferases in insect cell lines of biotechnological importance.

Introduction

The glycosylation potential of insect species is increasingly being investigated not only to understand the wide range of glycotypes in nature, but in order to determine the origin of immunogenic and allergenic cross-reactive carbohydrate determinants and to refine the use of insect-based expression systems in the production of pharmaceutically-relevant glycoproteins. In both these cases, the non-mammalian features of insect N-glycans are relevant: whereas many mammalian glycoproteins carry sialic acids on so-called 'complex' N-glycans, insect glycoproteins have distinct features which are 'foreign' to mammalian immune systems (Figures 1A and 1B). However, the 'humanisation' of glycans of insect cell expression systems may aid the manufacture of glycoprotein drugs, such as erythropoietin or clotting factors, which have otherwise to be produced at considerable cost from mammalian cell lines or with the risk of using natural sources contaminated with viruses or prions. From the biotechnology perspective, the complete lack (or very low levels) of terminal sialylation [1-6] and the processing of the oligosaccharides to 'paucimannosidic' forms (such as structures in Figure 1B) are a particular problem when using insect cell lines [7-10], since such glycoforms may be cleared quickly from the mammalian circulation by lectin-dependent mechanisms. Moreover, the presence of α 1,3-fucose bound to the proximal *N*-acetylglucosamine of an N-glycan (*i.e.*, core α 1,3-fucose; see Figures 1B and 1C), as also found in plants including on pollen and food allergens, is of concern for both allergologists and biotechnologists because of its immunogenicity [11, 12]. Indeed, core α 1,3-fucose constitutes a major epitope recognised by antisera raised in rabbits against insect and plant glycoproteins, such as bee venom or horseradish peroxidase [13, 14]. Furthermore, in one study, the sera of about 25% of patients with bee venom allergy were found to contain IgE cross-reacting with plant N-glycans, an observation explained by the presence of core α 1,3-fucose in both plants and insects [11]. Similar findings have been reported for patients allergic to fire ant [15] and wasp venoms [16].

Detailed analysis of the N-glycans of two components of bee venom, phospholipase A₂ and hyaluronidase (designated respectively as allergens Api m 1 and Api m 2), showed that these glycoproteins possess a number of non-mammalian features (see Figure 1D for the most complex structure observed). Besides the core α 1,3-fucosylation mentioned above, some glycoforms of both these proteins carry a Lewis-type fucosylated LacdiNAc structure [17, 18], which resembles the Lewis X structure (Le^x; Figure 1E), except that a GalNAc is present rather than a galactose. Fucosylated LacdiNAc is also present in parasitic trematode

and nematode worms (particularly *Schistosoma* spp. and *Haemonchus contortus*) and is a major epitope for antibodies from infected individuals or animals [19, 20]. In humans, Lewis-type epitopes containing galactose constitute a set of carbohydrate histo-blood group antigens. These moieties are often modified by sialic acids and sulphate groups, and as such play numerous roles including ones in inflammation and cancer [21-23].

Based on the aforementioned data, we hypothesised that at least two α 1,3-fucosyltransferases would be encoded by the honeybee genome. To date, all enzymes required for addition of α 1,3-linked fucose residues to glycans have been found to be members of one enzyme family, CAZy glycosyltransferase family 10 [24], which includes both core α 1,3-fucosyltransferases from plants, nematodes and insects as well as enzymes from bacteria, plants and enzymes of mammals capable of generating Lewis blood group antigens. Previously, in insects, the native enzyme activity responsible for the acquisition of the core α 1,3-fucose epitope has been described in bee venom glands [25] and in a cell line [26]. However, the only data previously obtained on recombinant insect fucosyltransferases pertains to *Drosophila melanogaster*: its genome encodes four α 1,3-fucosyltransferase homologues (DmFucTA, DmFucTB, DmFucTC and DmFucTD). Of these only DmFucTA, a core α 1,3-fucosyltransferase with orthologues in all other insect genomes examined, has a defined enzymatic activity [1, 27]. On the other hand, no Lewis-type enzyme is expected to be encoded by the fruitfly genome since no relevant epitopes have been described in *Drosophila* to date; thus, it is to be assumed that any honeybee enzyme with a Lewis-type fucosyltransferase may not be so closely related to the three 'orphan' fucosyltransferases (FucTB, FucTC and FucTD) from the fruitfly.

To test the hypothesis that a honeybee fucosyltransferase homologue least akin to DmFucTA may indeed be a Lewis-type enzyme able to fucosylate LacdiNAc, whereas putative orthologues of DmFucTA are solely core-type enzymes, we searched the draft honeybee genome and EST databases for α 1,3-fucosyltransferase homologues. Of three fucosyltransferase homologues identified, two were indeed expressed as active proteins in both insect cells and yeast and were subject to a variety of biochemical assays in order to define their enzymatic function.

Experimental Procedures

Cloning of fucosyltransferase cDNAs from the honeybee

Male honeybee larvae were collected from a hive in Schiltern near Krems in Lower Austria and cDNA was prepared by Trizol (Invitrogen) extraction and subsequent reverse transcription (Superscript III, Invitrogen) with oligo(dT)₁₈. A fragment with a partial reading frame of the FucTA homologue was amplified using the primers BBFT3 (GAGATATGGCACCGTATGC) and BBFT2/XbaI (GCTCTAGACCTAGGCAGAAAGTTTTTC) with Expand polymerase mix (Roche) and then cloned into the pGEM-T vector (Promega). The full length form was reconstructed by ligating a BstAPI/KpnI fragment from the pGEM-T/FucTA plasmid into the corresponding sites of the EST clone (GenBank BI503295, clone ID BB170010A20D04) in pT7T3-Pac encoding only the 5'- and the 3'-ends of honeybee FucTA. This reconstructed form was used as a template for PCR using KOD polymerase (Novagen) with the primers BBFT1/NotI (ATAAGAATGCGGCCGCATGGGTCTGCCGCG) and BBFT2/XbaI; the resulting fragment was then digested with NotI and XbaI and ligated into the pIZT-V5/His vector (Invitrogen).

The complete FucTB and FucTC open reading frames were prepared by PCR using, respectively, the primers BFTB1/KpnI (GGGGTACCGATGCTCGGAATTTTCACAA) with BFTB2/XbaI (GCTCTAGAGTCAGAAGGTACAATCTCGT) and BFTC1/KpnI (CGGGGTACCATGAGATTATGGATACTGGAAA) with BFTC2/XbaI (GCTCTAGATCAGTCTTTTAAAACGGAACC) in combination with Expand polymerase mix and the fragments were ligated also into the pIZT-V5/His vector. Partial reading frames corresponding to the catalytic and stem regions of FucTA, FucTB and FucTC were isolated using the primers BBFT3/PstI (AACTGCAGGAAATCTAGTGCAGGACGAG) with BBFT2/XbaI, BFTB3/PstI (AACTGCAGTTTATAAAGATGTATTTAT) with BFTB2/XbaI or BFTC3/PstI (AACTGCAGGTACCCCTCGACATATAATAC) with BFTC2/XbaI and ligated into a form of the pPICZ α B vector (Invitrogen) modified to encode a FLAG-tag.

Expression of honeybee putative fucosyltransferases

Constructs in the pIZT-V5/His and pPICZ α -type vectors were transformed respectively into insect Sf9 (*Spodoptera frugiperda*; fall armyworm) and *Pichia pastoris* GS115 cells as previously described [27, 28]. In *Pichia pastoris* the recombinant enzymes

were expressed at 16°C. Various strategies, as described below, were used to verify enzyme activities. Briefly, core α 1,3-fucosyltransferase activity was tested either by Western blotting of transfected Sf9 cells using rabbit anti-horseradish peroxidase (anti-HRP) or *in vitro* using various dansyl- and dabsyl-glycopeptides as substrates as used in previous studies [28, 29]. The Lewis-type fucosyltransferase was also assayed with dabsyl-glycopeptides as well as with pyridylaminated lacto-*N*-neo-tetraose (Gal β 1,4GlcNAc β 1,3Gal β 1,4Glc-PA) and lacto-*N*-tetraose (Gal β 1,3GlcNAc β 1,3Gal β 1,4Glc-PA). Where stated, *Pichia* supernatants were concentrated 10-fold using UltraFree centrifugal concentration devices (Millipore, MWCO 30000).

MALDI-TOF-MS fucosyltransferase assay

For analysis by MALDI-TOF-MS, the activity of recombinant α 1,3-fucosyltransferases from concentrated *Pichia* supernatants was measured using 40 mM MES buffer, pH 6.5, 10 mM MnCl₂, 1 mM GDP-fucose and 80 μ M of each of dabsyl-MM, -GnGn, -GalGal and - β GN β GN glycopeptides derived from bovine fibrin, and possessing the common peptide sequence GENR (see Figure 2 for oligosaccharide structures and Ref. 28). Reaction tubes were incubated at 30°C and analyzed after 12 h. Following incubation, 0.5 μ l of a 1:10 dilution of the reaction mixture was mixed with 0.5 μ l of 1% α -cyano-4-hydroxycinnamic acid (Fluka; in 70% acetonitrile) on a MALDI plate. An increase in glycopeptide m/z by 146.1 Da indicated the transfer of one fucose residue.

RP-HPLC based fucosyltransferase assays

For analysis by HPLC, the activity of concentrated supernatants of *Pichia* expressing honeybee FucTA was measured using 40 mM AMPD and MES buffer, pH 6.5-9.5 (MES buffer, pH 7.0 for temperature and cation dependency), 10 mM MnCl₂, 1 mM GDP-fucose, and 25 μ M dansylated GnGnF⁶-glycopeptide derived from human IgG, with NS as the peptide sequence (see Figure 2 for oligosaccharide structure). Reaction tubes were incubated at 23 °C and analyzed after 2 h. Following incubation an aliquot of the reaction was analysed on a reverse-phase HPLC system (Hypersil ODS) under isocratic conditions (9.5% acetonitrile, 0.045% trifluoroacetic acid).

The activity of unconcentrated supernatants of *Pichia* expressing honeybee FucTC was measured using 50 mM AMPD and MES buffer pH 6.5-9.5 (MES buffer, pH 7.5 for temperature and cation dependency), 100 μ g/ μ l bovine serum albumin, 12.5 mM MnCl₂, 1.25 mM GDP-fucose and 125 μ M pyridylaminated lacto-*N*-neo-tetraose. Reaction tubes were

incubated at 23 °C and analyzed after 2 h. Following incubation, an aliquot of the reaction was analysed on a reverse-phase HPLC system (Nucleosil C18) under isocratic conditions (0.094 M ammonium-formate, pH 4.0, 1.8% methanol).

Western blotting

GnGn, GalGal and β GN β GN isoforms of human apo-transferrin were prepared by enzymatic remodelling using *Clostridium perfringens* neuraminidase (yielding GalGal-transferrin), *Aspergillus oryzae* β -galactosidase (yielding GnGn- from GalGal-transferrin) and bovine β 1,4-galactosyltransferase I (yielding β GN β GN- from GnGn-transferrin since the enzyme also utilises UDP-GalNAc as a sugar donor [28, 30]). Recombinant honeybee FucTA (concentrated) and FucTC (unconcentrated) were incubated with 20 μ g of the transferrin neoglycoforms in 40 μ M MES, pH 7.0, 10 mM MnCl₂, 1 mM GDP-Fuc and 1 mM PMSF in a total volume of 5 μ l. The reactions were performed at 23°C for 48h. One-fifth of each incubation was mixed with an equal volume of 2 \times Laemmli buffer and analysed by Western blotting using 1:10000 dilutions of either rabbit anti-horseradish peroxidase (anti-HRP) or rat anti-Le^x (L5) as primary antibodies and 1:2000 dilutions of the relevant secondary alkaline phosphatase-conjugated antibodies followed by development using SigmaFAST BCIP/NBT solution.

Sf9 cells were maintained, transfected and lysed as previously described [27]. Briefly, pIZT/V5-His vectors carrying complete ORFs of all three *Apis mellifera* α 1,3-fucosyltransferase homologues with the native stop codon were used to transfect Sf9 cells (maintained in IPL41 medium supplemented with 3% FCS) using Cellfectin reagent (Invitrogen) following the manufacturer's protocol for insect cells. A small aliquot of the cells was analysed by confocal laser scanning microscopy with a UV-light source to confirm the presence of GFP fluorescence within the cells as indication of a successful transfection. Cells were collected 48h post-transfection, washed once with PBS and stored at -80°C. Cells were lysed using 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 supplemented with Complete-mini protease inhibitor cocktail without EDTA (Roche) or His-tag protease inhibitor cocktail (Sigma) for 10 minutes at 23°C and 20 minutes on ice followed by removal of insoluble material by centrifugation (14100g, 4°C, 30 min). Different volumes of the buffer were used to normalise the number of cells per volume (to 1.25 \times 10⁴ cells/ μ l). Cell lysates (2.5 μ l per lane) were mixed with 2 \times Laemmli loading buffer and analysed by Western blotting using a 1:20000 dilution of rabbit anti-HRP as primary antibody and a

1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) as secondary antibody followed by development using SigmaFAST BCIP/NBT solution. Prior to blocking, the nitrocellulose sheets were reversibly stained with 0.5% Ponceau S (in 1% acetic acid) to verify that equal amounts of proteins were present in every lane.

RT-PCR analyses

Honeybees (*Apis mellifera carnica*) were obtained from one colony at a private apiary located near Bratislava (Slovak Republic). Nurses were collected on a comb taken from the hive in the region containing cells with 1-3 day-old larvae. Foragers were caught in an insect net while trying to fly into or out of the hive. After collection, tissues and glands were dissected from distinct numbers of honeybees [thorax muscle (2 individuals), brains (10 individuals), hypopharyngeal glands (12 individuals) and venom glands (17 individuals)] into RNAlater solution and stored at -20° C. Total RNA from honeybee tissues and glands was prepared with SV Total RNA isolation System (Promega) after Ultra-Turrax homogenization in a denaturing solution. The quality and the spectrophotometrically-estimated concentration of the preparations were tested by agarose gel electrophoresis. RT-PCR assays were performed with the one-tube, two-enzyme Access RT-PCR System (Promega) using 40-160 ng (*FucTA*, *FucTC*, *actin*) or 100-400 ng (*FucTB*) of RNAs in a volume of 25 μ l. The primers used in the RT-PCRs had the same T_m for the specific sequences (58° C) and also worked with the same efficiencies in PCR using plasmid DNAs containing the corresponding *FucT* cDNAs. Primers were designed so that products would span at least one intron/exon boundary to ensure that cDNA amplicons could be distinguished from genomic DNA amplicons. The following primers were used: *FucTA*: BBFT3/PstI and BBFT2; *FucTB*: BFTB/5 (ATAGAACATCGAAAGCAAACG) and BFTB2/XbaI; *FucTC*: BFTC3/PstI and BFTC2/XbaI; *Actin*: Actinfor (MTCCGGIATGTGCAARGCCG) and Actinrev (CCTGCTCRAAGTCMAGIGC).

The primer concentration in the reaction was 0.8 μ M. The concentration of Mg^{2+} was 1.5 mM except for the amplification of *FucTB*, where the optimal concentration was 1.2 mM. Reverse transcription was performed at 48° C for 1 h. PCR after the initial denaturation for 2 min at 94° C was performed for 30, 34 or 50 cycles (30s at 94° C, 60s at 54° C and 150s at 68° C) followed by 7 min at 68° C. PCR products were analysed on 1% agarose gels.

Results

Cloning of fucosyltransferase cDNAs from the honeybee

The first target was to identify which gene is responsible in the honeybee for the occurrence of core α 1,3-fucose on bee venom glycoproteins; it seemed reasonable that the encoded protein would display homology to other core α 1,3-fucosyltransferases, including the known *Drosophila melanogaster* FucTA. During initial searches of the EST databanks in 2001, two partial bee brain cDNA sequences from the honeybee with homology to *Drosophila* FucTA were noted. The two relevant clones were ordered, but both were found to contain DNA encoding protein sequences with homology to only the N- and C-terminal regions of the fruitfly FucTA. These EST DNA sequences were then used to design specific primers in order to clone the full ORF as well as an ORF fragment encoding a form lacking the putative cytosolic and transmembrane regions. Only the latter fragment was actually isolated from honeybee cDNA, but due to the presence of a BstAPI site in the cDNA sequence and a KpnI site in both the cloning and EST vectors, it was possible to reconstruct the full length honeybee FucTA sequence encoding a protein of 452 amino acids (M_r 53 kDa), with 52% identity over 439 residues to *Drosophila* FucTA with a shorter putative stem region than the fly protein. The significance of the homology at the DNA level was also shown by the finding that a *Drosophila* FucTA probe could recognise the honeybee FucTA PCR product, but not that of fruitfly FucTB, at low stringency ($2\times$ SSC, 0.1 % SDS, 1 hr, 55 °C; data not shown). The determined honeybee FucTA cDNA sequence was identical to that later released for the predicted mRNA XM_392653 (based on honeybee Contig1747); a comparison of the cDNA and genomic sequences also showed that the honeybee FucTA is encoded by three exons.

Considering the presence of fucosylated LacdiNAc on bee venom glycoproteins, we also sought a second fucosyltransferase gene. Indeed, when using the fruitfly FucTC sequence to search the now available honeybee genome, another predicted fucosyltransferase was found (XM_394138; honeybee Contig655); the confirmed cDNA sequence encodes a protein of 399 residues (M_r 47 kDa), which we designate as FucTC. Furthermore, using the amino acid sequence of a *Glossina morsitans* putative fucosyltransferase (Genbank entry AJ582622), another potential homologous sequence was found on honeybee Contig4553; although this was not a predicted mRNA, a corresponding cDNA designated FucTB was then successfully cloned. This sequence encodes a protein of 431 amino acids (M_r 51 kDa), which instead of the DYI/VTEK motif typical of animal α 1,3-fucosyltransferases has an EYVTEK

sequence. The three honeybee fucosyltransferases display between 28 and 34% identity to each other over approximately 300 residues (Figure 3A).

Phylogenetic analysis (Figure 3B) groups the honeybee and fruitfly FucTAs together, whereas those designated as FucTB homologues appear to form two distinct groups: one group is more related to the human Lewis-type fucosyltransferases III-VII and IX, while the other is closer to the mammalian fucosyltransferase homologues X and XI. The honeybee FucTC appears to be closely related to a number of insect sequences; however, a branching into two distinct FucTC groups cannot be excluded. Indeed, the occurrence of two FucTC groups would be in agreement with the apparent inability of fruitfly FucTC to utilise various N-glycan substrates [1, 27], whereas, as shown below, honeybee FucTC does accept such glycans.

Expression of honeybee fucosyltransferases in Pichia

Constructs encoding FLAG-tagged soluble forms of the three honeybee fucosyltransferase homologues were prepared for expression in the yeast *Pichia pastoris*. Culture supernatants were collected and concentrated prior to assaying specific fucosyltransferase activities with various dabsylated N-glycopeptides (MM, GnGn, GalGal and β GN β GN; see Figure 2 for structures). MM and GnGn were used as known core fucosyltransferase substrates, whereas GalGal and β GN β GN are substrates for Lewis-type enzymes [31]. As shown in Figure 4, *Pichia* transformed with the FucTA vector converted GnGn to a fucosylated form, whereas that transformed with FucTC vector converted GalGal and β GN β GN to difucosylated forms. Upon longer incubation (36h), the honeybee FucTA expressed in *Pichia* also converted GalGal and β GN β GN to monofucosylated forms (data not shown). FucTB-transformed yeast showed no obvious activity in the initial screening, thus all further experiments were performed on FucTA and FucTC.

FucTA activity was also measured using HPLC-based assay with the fluorescently-labelled dansylated-GnGnF⁶ glycopeptide as the substrate. In agreement with previous studies on core α 1,3-fucosyltransferases [32], the addition of a fucose by FucTA lead to reduction of the substrate glycan retention time (Figure 5). Since FucTC appeared to be a Lewis-type enzyme, we performed HPLC-based assays using pyridylaminated forms of the tetrasaccharides lacto-*N*-neo-tetraose and lacto-*N*-tetraose as substrates, since these have been successfully used with Lewis-type fucosyltransferases forming, respectively, α 1,3 and α 1,4-fucosyl linkages. Previous studies with a plant Lewis-type α 1,3/4-fucosyltransferase (tomato

FucTC) showed that fucosylation of these substrates results in a shift to earlier elution times [33]. In the present study with honeybee FucTC, only in the case of lacto-*N*-neo-tetraose was there an alteration in the retention time, resulting from formation of lacto-*N*-fucopentaose III (Figure 5); no such shift was observed for yeast transformed with FucTA or FucTB nor when lacto-*N*-tetraose was incubated with FucTC (data not shown). These data, therefore, show that the enzyme solely acts as an α 1,3-fucosyltransferase. The determined activity of FucTC towards both LacNAc and LacdiNAc-containing substrates is comparable to data showing that the mammalian fucosyltransferases III, IV and IX have varying levels of activity *in vitro* towards both LacNAc (in order to generate Le^x) and LacdiNAc, whereas FucT VII displays no activity towards LacdiNAc [34].

Enzymatic properties of honeybee FucTA and FucTC

The basic enzymological parameters of FucTA and FucTC were investigated using the aforementioned HPLC-based assays. Honeybee FucTA is strongly activated by Mn²⁺ as compared to no-cation controls, a property common to many glycosyltransferases. On the other hand, EDTA, as well as Cu²⁺ and Zn²⁺ ions, completely abolish this enzyme's activity. The enzyme shows maximal activity in mildly alkaline conditions (pH 7.5-9.0) at room temperature. In contrast to FucTA, FucTC is not inactivated by EDTA, although the enzyme seems to prefer Mn²⁺ and Mg²⁺ over other cations. The temperature and pH optima for the FucTC appear to be very broad; an enzyme shows 90% of maximal activity at 4°C and over 40% of maximal activity at 50°C (Figure 6).

The honeybee FucTA and FucTC can utilise protein substrates

The assays employed to measure the activity of both core and Lewis-like α 1,3-fucosyltransferase utilise substrates containing predominantly carbohydrates, with little or no peptide backbone as compared to "real" glycoproteins. Furthermore, these assays primarily indicate transfer of fucose but do not give information about the epitopes formed. To address this major difference between utilised substrates and the ones present *in vivo*, we made use of the human glycoprotein apo-transferrin whose glycans were modified to be potential substrates for the two recombinant enzymes. After incubation with GDP-fucose and *Pichia* culture supernatants containing either FucTA or FucTC, the glycoprotein preparations were separated on a standard SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with anti-HRP or anti-Le^x antibodies. Whereas anti-HRP is known to bind core α 1,3-linked fucose as found on many plant and insect glycoproteins [35], the L5 monoclonal antibody is

apparently the only antibody capable of recognising the Le^x epitope when attached to N-glycans [36]. The transferrin neoglycoforms carrying N-glycans predominantly terminating with β 1,2-GlcNAc (*i.e.*, GnGn-), β 1,4-Gal (*i.e.*, GalGal-) and β 1,4-GalNAc (*i.e.*, β GN β GN-transferrin) residues incubated with FucTA were recognised by anti-HRP antibodies demonstrating that transfer of a fucose resulted in generation of the same core α 1,3-fucose epitope as found on, *e.g.*, horseradish peroxidase and bee venom phospholipase. On the other hand, only GalGal-transferrin incubated with FucTC was recognised by antibodies directed against the Le^x structure, thus strongly indicating that the fucose residues were attached in α 1,3-linkage to the antennal *N*-acetylglucosamine residues (Figure 7A). Even though FucTC can transfer fucose to β GN β GN, the specificity of the L5 antibody is obviously absolute for Le^x and therefore this antibody does not recognise fucosylated LacdiNAc.

Analysis of honeybee fucosyltransferases activity in vivo

In previous studies, we have used insect cells as hosts for core α 1,3-fucosyltransferase expression in order to perform screening for the ability of an open reading frame to confer anti-HRP binding *in vivo*, *i.e.* to prove the specific modification of glycoproteins of cells transfected with core α 1,3-fucosyltransferases [27, 29]. In the present study, we chose Sf9 cells (a typical biotechnological lepidopteran cell line), which naturally display only a low degree of binding to anti-HRP. Induction of core α 1,3-fucosylation upon transfection of relevant open reading frames in this cell line is expected to result in the creation of the anti-HRP epitope, which can then be detected by Western blotting. We, therefore, transfected these cells with the honeybee α 1,3-fucosyltransferase homologues and employed *Drosophila* FucTA as a positive control; vectors containing the entire open reading frame encoding each enzyme, with no tag, were used. As shown in Figure 7B, of the honeybee fucosyltransferases, only the FucTA conferred the ability to bind anti-HRP to glycoproteins of Sf9 cells. The lanes with the cells transfected with the honeybee FucTB and FucTC showed no difference as compared to the empty vector control corroborating the other data suggesting that they are not core fucosyltransferases. Furthermore, *in vitro* assays also showed that only the FucTA-transfected cells had an activity capable of transferring a fucose to GnGnF⁶, thereby generating a difucosylated structure, above the otherwise very low background levels displayed by control transfectants (data not shown). The overall conclusion is that, as judged by data indicating that this enzyme can form the anti-HRP

epitope *in vitro* and *in vivo*, the fucose transferred by FucTA is α 1,3-linked to the proximal GlcNAc of N-glycans.

Expression of fucosyltransferases in different honeybee tissues

To appraise whether the tissue-specific expression of α 1,3-fucosyltransferase homologues in *Apis mellifera* is compatible with the synthesis of the previously observed N-glycans containing both core and antennal α 1,3-linked fucose [17], total RNA from several honeybee tissues was prepared. Following RNA normalisation against actin transcripts, expression pattern of the fucosyltransferase homologues in honeybees was estimated by RT-PCR using gene specific primers. As expected, the expression panel indicates that there is a very strong presence of FucTA transcripts in venom glands, followed by strong expression of FucTA in bee brain and weaker expression in the other analysed samples (Figure 8). FucTC, on the other hand, appears to be expressed more equally in various samples, with an apparently higher expression rate in venom and hypopharyngeal glands. The reproducible appearance of the triplet band in FucTC RT-PCR reactions for most tissues could indicate alternative splicing of the FucTC transcripts. Indeed TA cloning and subsequent sequencing confirmed the existence of two alternatively spliced forms of FucTC; one corresponded to that expressed in *Pichia*, whereas the second form is 90 nucleotides shorter and has a premature stop codon. Finally, the third α 1,3-fucosyltransferase homologue, FucTB, appears to be weakly expressed in venom glands, foragers' brain tissue and in larvae; it should be noted that it was necessary to increase the number of RT-PCR cycles to 50 and use increased amounts of RNA in order to detect FucTB transcripts in honeybee RNA preparations. Overall, the expression panel data indicating the presence of both the core-modifying FucTA and the Lewis-like FucTC in venom glands is in accordance with the previous studies indicating the presence of both core and antennal α 1,3-fucosylated N-glycan structures on bee venom glycoproteins.

Discussion

The honeybee *Apis mellifera* is an object of scientific study for a number of reasons; in particular, the search for genetic clues as to its social behaviour has led to the recent sequencing of its genome and the examination of bee brain EST sequences [37], but also the anaphylaxis induced in some individuals, as an allergenic reaction to bee stings, is a focus of allergological interest [11]. The present study has its origins in previous enzymological and structural analyses related to bee venom glycoproteins [17], which then showed, for the first time, the presence of both core α 1,3-fucose and α 1,3-fucosylated LacdiNAc structures on an insect glycoprotein. Thus, we expected to find at least two genes in the honeybee encoding enzymes able to synthesise these structures.

In actual fact, the *Apis mellifera* genome encodes three α 1,3-fucosyltransferase homologues which include the consensus sequence common to all α 1,3-fucosyltransferases [38, 39]. As indicated by the predicted amino acid sequences, one of the honeybee sequences (FucTA) is phylogenetically close to *Drosophila* and other proven or putative N-glycan core α 1,3-fucosyltransferases (Figure 3B). Indeed, when expressed in *Pichia pastoris* and insect cells, this honeybee sequence encodes an active core α 1,3-fucosyltransferase with a substrate specificity similar to that of the recombinant *Drosophila* FucTA [1], the native honeybee venom gland and *Mamestra brassicae* MB-0503 (cabbage moth cell line) core α 1,3-fucosyltransferases [25, 26] and various plant N-glycan core α 1,3-fucosyltransferases [40-42]. The observed transfer of fucose to GalGal, in addition to GnGn, has also been found with the fruitfly FucTA [1]. On the other hand, no transfer was seen towards MM by either insect FucTA, which contrasts to the preference of the *Caenorhabditis* FUT-1 for this substrate [29]. This suggests that the substrate specificity of invertebrate core α 1,3-fucosyltransferases (*i.e.*, FucTA and FUT-1) is somewhat more variable and flexible than that of invertebrate core α 1,6-fucosyltransferases which require a terminal unsubstituted GlcNAc residue [31].

The second homologue found in this study (FucTC) resembles a number of other related sequences from various insects, including the *Drosophila* FucTC sequence. Unlike DmFucTC [27], the honeybee FucTC encodes an active Lewis α 1,3-fucosyltransferase and is indeed the first such enzyme to be characterised from an insect; it is very likely that this enzyme is involved in synthesis of fucosylated LacdiNAc N-glycans found on bee venom glycoproteins [17]. It appears that, the FucTC homologues form two distinct subgroups; the

honeybee FucTC seems to be more related to *Bombyx mori* and *Tribolium castaneum* sequences, whereas the *Drosophila* FucTC is more similar to *Anopheles gambiae*, *Aedes aegypti* and *Glossina morsitans* sequences. However, it remains to be determined whether this genetic 'split' is generally reflected in the presence or absence of fucosylated LacdiNAc.

Apart from demonstrating that both honeybee FucTA and FucTC can act on glycoproteins and that FucTA is able to confer fucose-dependent anti-HRP staining in an *in vivo* system, albeit heterologous, we sought to strengthen the link between the honeybee α 1,3-fucosyltransferase homologues and fucosylated N-glycan structures found on bee venom glycoproteins [17, 18] by performing expression studies of all three honeybee α 1,3-fucosyltransferase homologues in different bee tissues. According to the RT-PCR expression panel, FucTA appears to be strongly expressed in venom glands, a result that correlates with the presence of the core α 1,3-linked fucose on the N-glycans of bee venom glycoproteins. The occurrence of FucTA transcripts in bee brain tissue is also in agreement with expression of DmFucTA in *Drosophila* heads and in a *Drosophila* neuronal cell line [27] and in the use of anti-HRP as a neuronal marker in insects [43]. The honeybee FucTA also appears to be expressed in hypopharyngeal glands that produce the royal jelly proteins in honeybee nurses [44-46] and carbohydrate-metabolising enzymes after the bees adopt the forager role [47-49]. This result contrasts the apparent lack of fucosylated N-glycan structures on major royal jelly glycoproteins [50]. The explanation could be that transcripts detected in muscles and the hypopharyngeal gland might originate from neighbouring neuronal tissue which is expected to express the FucTA. On the other hand, antibodies detecting N-glycan core α 1,3-linked fucose appear also to bind other tissues in *Drosophila* (*e.g.*, the garland gland) [43, 51, 52], indicating that α 1,3-linked fucosylated N-glycan structures might occur in insect tissues other than, as generally accepted, neurons [1, 27] and venom glands [11, 17, 18].

Honeybee FucTC appears to be expressed in all tissues analysed and the reproducible appearance of the triplet band in FucTC RT-PCR reactions (where the largest band is of the expected size) for most tissues could indicate alternative splicing of transcripts of this gene. The higher expression rate of FucTC in venom glands correlates well with the occurrence of LacdiNAc structures present on bee venom glycoproteins. However, even though honeybee FucTC is expressed to a high degree in the hypopharyngeal glands, the N-glycans of major royal jelly glycoproteins appear, as mentioned above, not to be fucosylated. It was also observed that the expression of FucTC is similar in both nurse's and forager's glands that produce different proteins and show morphological and ultrastructural differences [53]. On the other hand, the dual *in vitro* activities of FucTC, towards both LacNAc and LacdiNAc

structures, may not have relevance *in vivo*, since only fucosylated forms of the latter have been previously described on honeybee glycoproteins. It is indeed probable that the honeybee only expresses a β 1,4-*N*-acetylgalactosaminyltransferase, akin to those previously described from *Trichoplusia ni* [54] and *Drosophila melanogaster* [55], and lacks a β 1,4-galactosyltransferase of the type found in mammals. Therefore, presumably only the substrate with terminal GalNAc, required to generate a fucosylated LacdiNAc, is present in honeybees *in vivo*.

The expression panel suggests that honeybee FucTB is very weakly expressed in larvae, venom glands and foragers' brain tissue but not at all in nurses' brain tissue; indeed, its expression is several orders of magnitude lower than that of honeybee FucTA and FucTC. Interestingly, honeybee FucTB appears to be phylogenetically closer to known N-glycan core α 1,3-fucosyltransferases like *Drosophila* FucTA and honeybee FucTA than to the *Drosophila* FucTB fucosyltransferase homologue and the putative human and murine fucosyltransferases X and XI. However, extensive attempts with both fruitfly and honeybee FucTB homologues to demonstrate an activity towards potential substrates (MM, GnGn, GalGal, β GN β GN, lacto-*N*-neo-tetraose and lacto-*N*-tetraose) were not successful. This, in conjunction with the fact that we were able to account for the major fucosylated structures present in N-glycans on bee venom and fruitfly glycoproteins, still leaves an open question as regards the *in vivo* function of FucTB proteins. However, any results with an insect FucTB might prove valuable for the understanding of the function of the homologous mammalian FucT X and XI, for which also, to date, no activity has been detected [56]. Indeed, it may be that the assumption, that standard 'glycoprotein' substrates are applicable, is false.

In conclusion, we have identified a core α 1,3-fucosyltransferase, from an insect other than *Drosophila*, as well as the first insect Lewis-type fucosyltransferase. These enzymes account for the N-glycan α 1,3-fucosylation events previously hypothesised on the basis of N-glycan analysis of honeybee glycoproteins and both generate products associated with immune responses to glycans from invertebrates [11, 13, 19, 20]. Despite being both members of the same enzyme family, honeybee FucTA and FucTC have different substrate specificities; indeed, as with other members of CAZy glycosyltransferase family 10, the only feature in common is the transfer of fucose, from GDP-Fuc, to GlcNAc residues. The wider significance of our findings is, however, in connection to those commonly-used baculovirus hosts such as *Trichoplusia ni* (HiFive[®]) and Sf21 cells [57, 58] known to express core α 1,3-fucosylated N-glycans, but for which we lack relevant DNA sequence information. Indeed, of

the various non-*Drosophila* insect species for which such data is currently publicly-available, only *Bombyx mori* is of direct biotechnological importance with BmN cells sometimes being used for baculovirus-based expression. However, the relatively-high homology between the fruitfly and honeybee FucTA core α 1,3-fucosyltransferase sequences and the observed ability of these sequences to cross-hybridise may be useful in screening cDNA libraries from baculovirus-host species for which no genomic data is currently available. Identification of the core α 1,3-fucosyltransferases from such cell lines would pave the way for knocking-out/down the corresponding gene, thus resulting in abolition/reduction of the expression of the immunogenic core α 1,3-fucose moiety. This approach would complement other efforts to re-engineer insect cell lines [9, 59] in order to generate 'human-like' glycans on proteins of pharmaceutical interest.

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The abbreviations used are: AMPD, 2-amino-2-methyl-1,3-propanediol; anti-HRP, antiserum raised against horseradish peroxidase; dabsyl, 4-dimethylaminoazobenzene-4-sulfonyl; dansyl, 5-dimethylaminonaphthalene-1-sulfonic acid; EST, expressed sequence tag; FucT, fucosyltransferase; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 4-morpholinoethanesulfonic acid; ORF, open reading frame; RP-HPLC, reverse phase high performance liquid chromatography; RT, reverse transcription; LacNAc, galactosyl- β 1,4-*N*-acetylglucosamine (Gal β 1-4GlcNAc); LacdiNAc, *N*-acetylgalactosaminyl- β 1,4-*N*-acetylglucosamine (GalNAc β 1-4GlcNAc); pyridylaminated lacto-*N*-neo-tetraose, Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc-PA; pyridylaminated lacto-*N*-tetraose, Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc-PA; lacto-*N*-fucopentaose III, Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4Glc-PA; Le^x, Gal β 1-4(Fuc α 1-3)GlcNAc; GalGal, Gal β 1-4GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4-Asn; β GN β GN, GalNAc β 1-4GlcNAc β 1-2Man α 1-6(GalNAc β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4-Asn; GnGn, GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4-Asn; GnGnF⁶, GlcNAc β 1-2Man α 1-6(GlcNAc β 1-2Man α 1-3)Man β 1-4(Fuc α 1-6)GlcNAc β 1-4-Asn; MM, Man α 1-6(Man α 1-3)Man β 1-4GlcNAc β 1-4-Asn.

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Figure legends

Figure 1: Comparison of N-linked oligosaccharides from mammals, insects and plants:

A. Typical mammalian ‘complex’ N-glycan structure containing terminal sialic acids and α 1,6-fucose linked to the proximal N-acetylglucosamine. **B.** N-glycan structures characteristic for insects. The MM and MMF⁶ structures are abundant, whereas the immunogenic MMF³F⁶ structure accounts for 1% of the N-glycans from *Drosophila*. **C.** Typical structure found in plants. The plant N-glycans share some features with insect N-glycans, especially the α 1,3-linked fucose. **D.** Most complex N-glycan structure found on bee venom glycoproteins; apart from the immunogenic α 1,3-fucose linked to the proximal N-acetylglucosamine, this structure contains a second α 1,3-fucose linked to the distal N-acetylglucosamine creating a Lewis-like structure. As shown in this study, the synthesis of these structures is catalysed by FucTA and FucTC, respectively. **E.** Blood group related Lewis X structure present in mammals. “-R” represents an oligosaccharide backbone of a glycoconjugate. The glycans are depicted following the glycan nomenclature of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>) with the mammalian structure also displayed in an extended IUPAC/IUBMB-type form.

Figure 2: Synthesis and subsequent modifications of glycan structures used as substrates in enzymatic assays:

The various substrates used in this study, except the dabsylated MM-glycopeptide, were acceptors for either FucTA or FucTC. Products of these enzymes are shaded; glycans are depicted following the glycan nomenclature of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>). **A.** Dabsylated glycopeptides (MM, GnGn and β GN β GN) were synthesised from the dabsylated GalGal-glycopeptide using relevant enzymes. β 1,4- and β 1,3-galactosidases were used to remove galactose residues, whereas jack bean hexosaminidase was used to remove the GlcNAc residues. Bovine β 1,4-galactosyltransferase I, an enzyme known to also utilise UDP-GalNAc, was used to synthesise the dabsylated β GN β GN-glycopeptide. **B.** Dansyl-GnGnF⁶, produced from an asialoagalacto-IgG glycopeptide, has been previously shown to be a substrate for core α 1,3-fucosyltransferases from plants and insects. **C.** Lacto-N-neo-tetraose is a Lewis-type fucosyltransferase substrate.

Figure 3: Homologies of honeybee α 1,3-fucosyltransferases: **A.** Alignment of the conserved regions of honeybee α 1,3-fucosyltransferase homologues. Residues identical in all sequences are highlighted. The amino acid numbering starts from the putative first methionine and potential N-glycosylation sites are underlined. The motifs I and II defined by Oriol et al. [39] are also shown. The ‘core’ motif, containing the sequence SNCXXRN, appears to be present in those core α 1,3-fucosyltransferases which require the prior action of *N*-acetylglucosaminyltransferase I (*i.e.*, all plant and insect core α 1,3-fucosyltransferases described to date, but not *Caenorhabditis elegans* FUT-1 which does not accept substrates with an α 1,3-antennal terminal GlcNAc residue). **B.** Phylogenetic tree of insect and mammalian α 1,3-fucosyltransferase homologues. The following protein sequences were analysed using ClustalW and NJPlot programs (<http://www.ebi.ac.uk/clustalw/>): AmA-C: *Apis mellifera* fucosyltransferases A, B and C; DmA-D: *Drosophila melanogaster* fucosyltransferases A, B, C and D; HsIII-XI: *Homo sapiens* fucosyltransferases III, IV, V, VI, VII, IX, X and XI; MmX-XI: *Mus musculus* fucosyltransferases X and XI; BmA-C5: *Bombyx mori* manually assembled fucosyltransferase homologues A (AADK01016505), B1 (AADK01001074), B2 (AADK01006340), C1 (AADK01004147), C2 (AADK01000551), C3 (AADK01017942), C4 (AADK01001565) and C5 (BAAB01004223); AeA/C: *Aedes aegypti* manually assembled fucosyltransferase homologues A (AAGE02021501) and C (AAGE02000340, AAGE02000341); AgA/C: *Anopheles gambiae* manually assembled fucosyltransferase homologues A (AAAB01008879) and C (AAAB01008846); GmC: *Glossina morsitans* fucosyltransferase homologue C (AJ582622); TcA-C2: *Tribolium castaneum* manually assembled fucosyltransferase homologue A (AAJJ01000602), B1 (AAJJ01000605), B2 (AAJJ01000026), C1 (AAJJ01000008) and C2 (AAJJ01000008). GenBank accession numbers of genomic DNA sequences which were used to manually assemble sequences are in brackets. Proteins, for which activity was demonstrated *in vitro* and/or *in vivo*, are appropriately labelled as core or Lewis-like enzymes.

Figure 4: Activity of honeybee fucosyltransferases expressed in *Pichia* analysed by MALDI-TOF-MS. Culture supernatants of yeast transformed with either FucTA (A), FucTB (B) or FucTC (C) were assayed using four dabsylated glycopeptides (MM, GnGn, GalGal and β GN β GN) in the presence of GDP-Fuc. The glycans are depicted following the glycan nomenclature of the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>), *i.e.*, mannose residues are shown as grey circles,

galactose residues as white circles, glucose residues as black circles, *N*-acetylglucosamines as black squares, *N*-acetylgalactosamines as white squares and fucose residues as grey triangles (as shown in Figure 2). Structures appearing due to enzymatic activity are underlined. Peaks resulting from laser-induced cleavage of a part of the dansyl group are labelled with an asterisk.

Figure 5: Activity of honeybee fucosyltransferases expressed in *Pichia* analyzed by RP-HPLC. Culture supernatants of yeast transformed with either FucTA or FucTC were assayed using either dansylated-GnGnF⁶ glycopeptide (FucTA) or lacto-*N*-neo-tetraose (FucTC) in the presence or absence of GDP-Fuc. The FucTC was also tested using lacto-*N*-tetraose but did not display any activity towards that substrate (data not shown).

Figure 6: Properties of recombinant honeybee FucTA and FucTC. Cation dependency, pH and temperature optima were determined using either ten-fold concentrated (FucTA) or unconcentrated (FucTC) honeybee enzymes expressed in *Pichia*. Relative activity was normalised based on the condition giving the highest rate of fucose transfer.

Figure 7: Epitope formation by honeybee FucTA and FucTC: A. Honeybee FucTA and FucTC expressed in *Pichia* confer anti-HRP and anti-Le^x staining to glycoproteins. Recombinant FucTA and FucTC were incubated with modified transferrin carrying N-glycans predominantly terminating with β 1,2-GlcNAc (*i.e.*, GnGn-), β 1,4-Gal (*i.e.*, GalGal-) or β 1,4-GalNAc (*i.e.*, β GN β GN-transferrin) residues. FucTA confers the anti-HRP staining to the used transferrin glycoforms (first row). FucTC but not FucTA confers anti-Le^x (anti-L5) staining only to GalGal-transferrin (second row). **B.** Expression of honeybee fucosyltransferases in Sf9 insect cells. Cells transfected with either an empty pIZTV5/His vector (1), honeybee FucTA (2), honeybee FucTB (3), honeybee FucTC (4) and *Drosophila* FucTA (5) were lysed and subject to SDS-PAGE and Western blotting with anti-horseradish peroxidase.

Figure 8: Expression patterns of fucosyltransferases in different tissues and glands of *A. mellifera*. Total RNA was isolated from the whole larva (L) as well as from thorax muscles (M), brains (B), venom glands (Vg) and hypopharyngeal glands (Hg) of nurse honeybees or of foragers from the same colony. RT-PCR was performed with the primers specific for individual FucTs (see Experimental procedures) using different numbers of cycles. In the

case of FucTB, the conditions in reaction were partially distinct from these used at FucTA, FucTC and actin. The amplified fragments of FucTA, FucTB, FucTC and actin with the respective sizes of 1350 bp, 1160 bp, 1200 bp and 600 bp are shown.

Figure 1

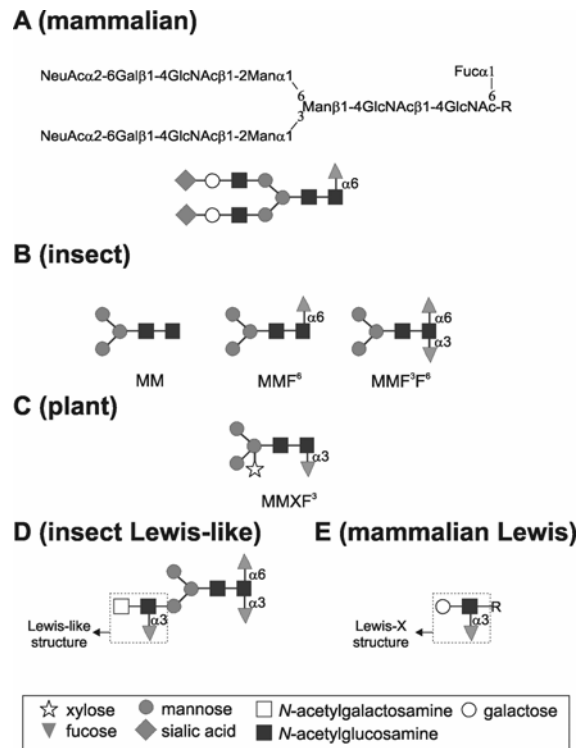


Figure 2

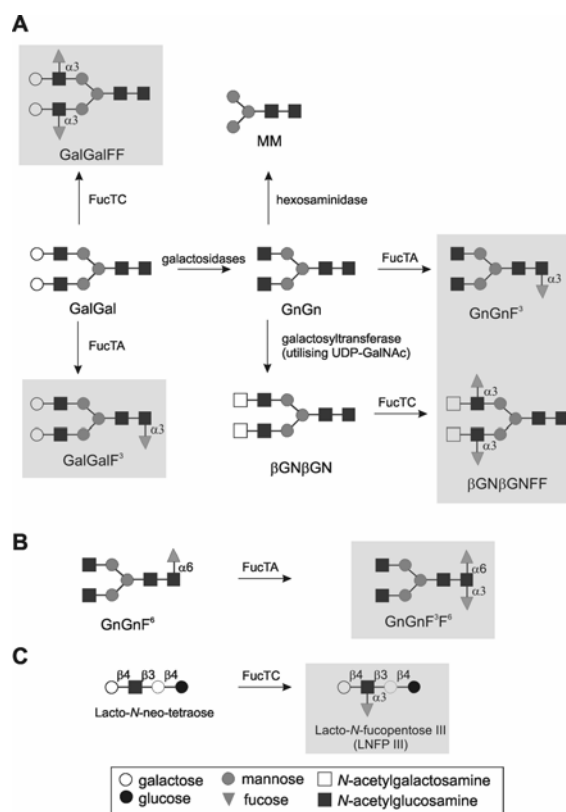


Figure 3

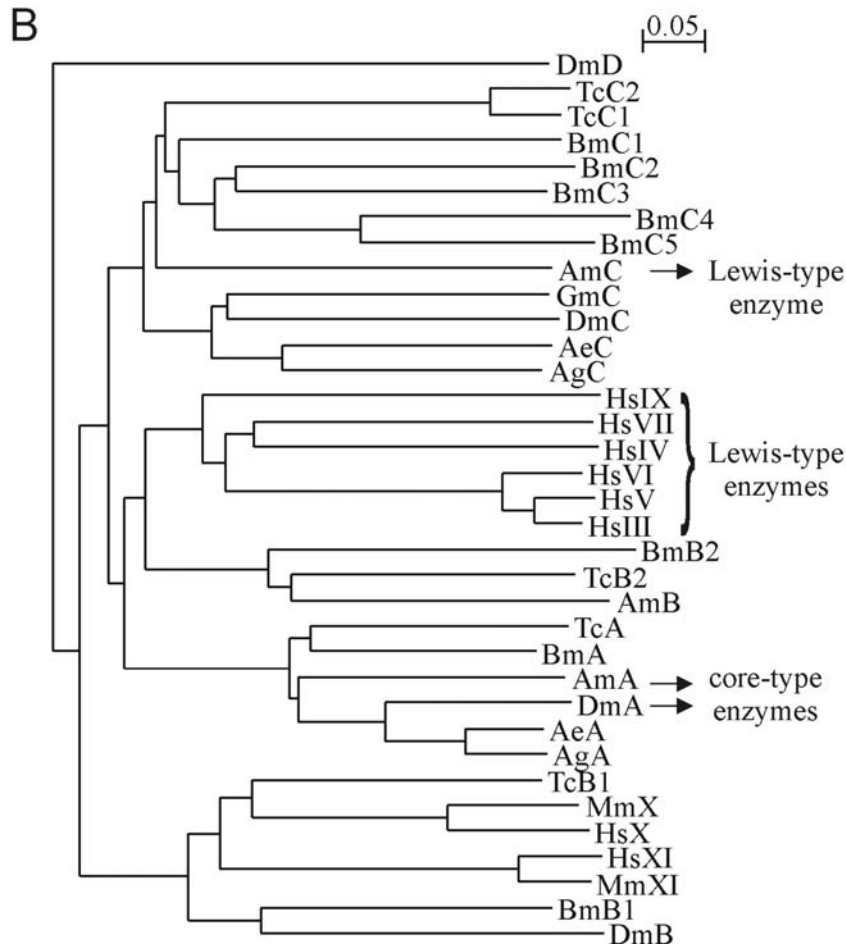
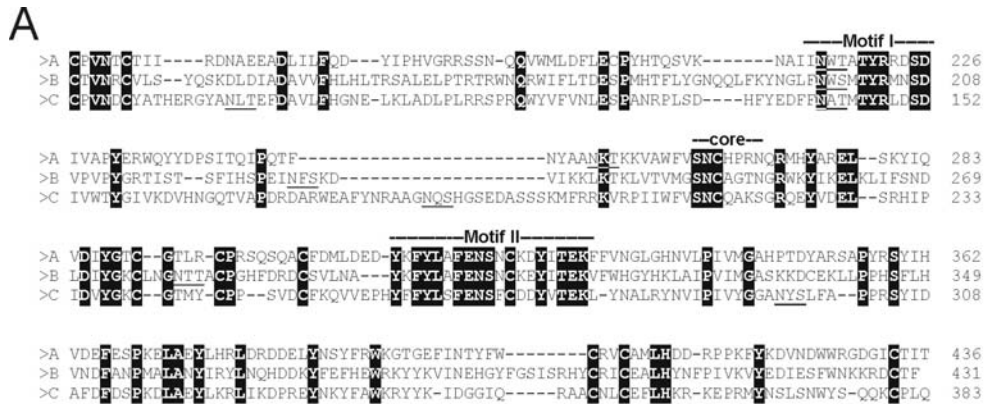


Figure 4

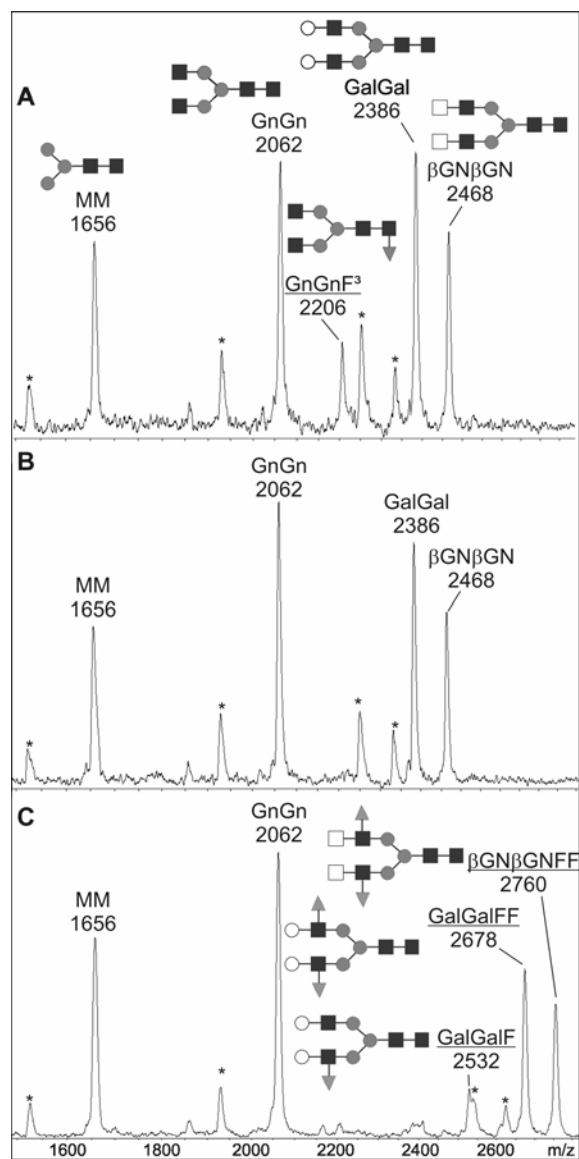


Figure 5

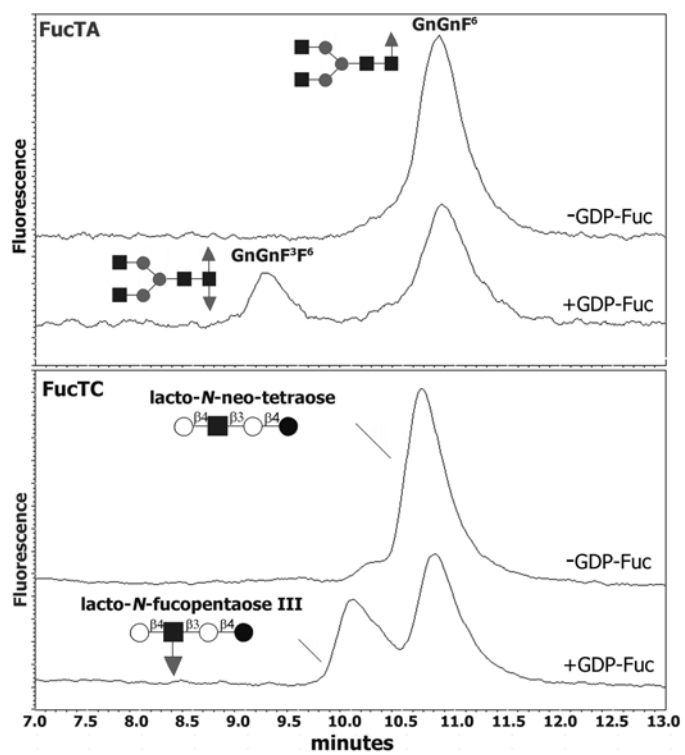


Figure 6

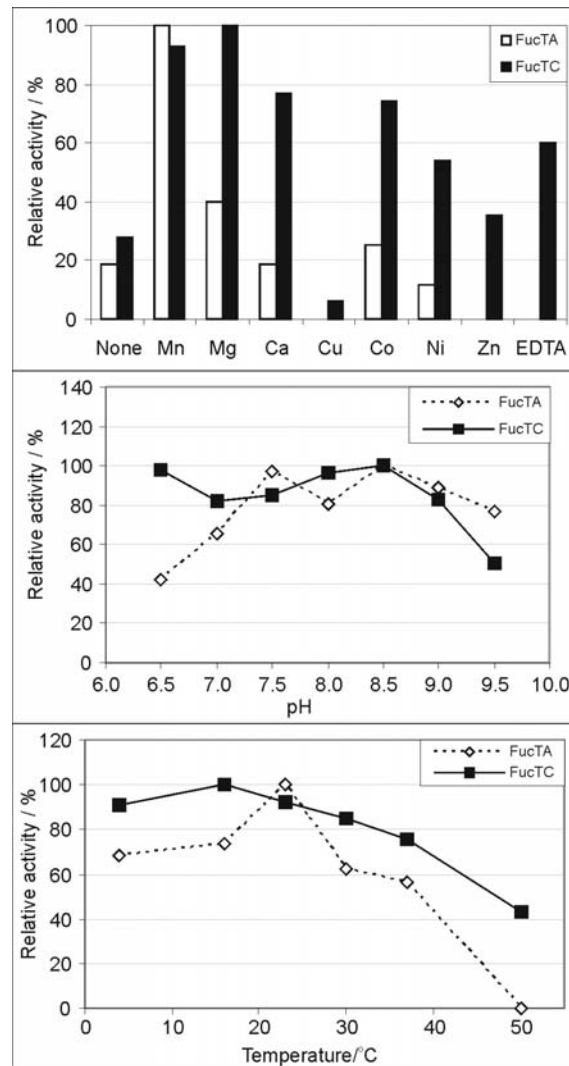


Figure 7

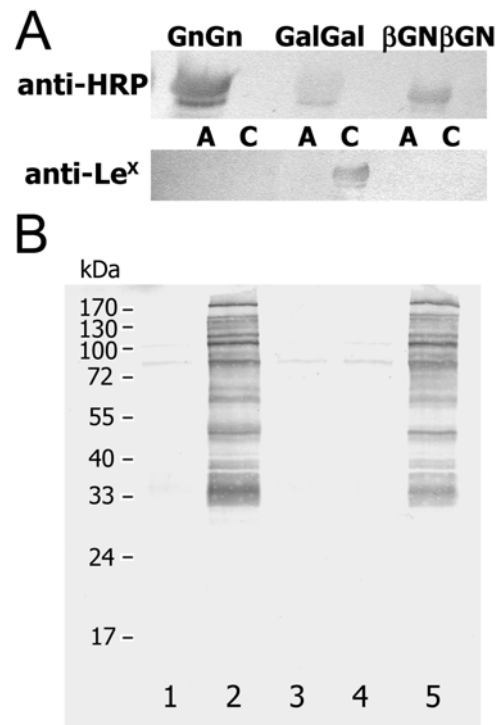


Figure 8

