Towards abolition of immunogenic structures in insect cells: characterization of a honey-bee (*Apis mellifera*) multi-gene family reveals both an allergy-related core α1,3-fucosyltransferase and the first insect Lewis-histo-blood-group-related antigen-synthesizing enzyme

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Glycoproteins from honey-bee (*Apis mellifera*), such as phospholipase A_2 and hyaluronidase, are well-known major bee-venom allergens. They carry N-linked oligosaccharide structures with two types of α1,3-fucosylation: the modification by α1,3-fucose of the innermost core GlcNAc, which constitutes an epitope recognized by IgE from some bee-venom-allergic patients, and an antennal Lewis-like GalNAcβ1,4(Fucα1,3)GlcNAc moiety. We now report the cloning and expression of two cDNAs encoding the relevant active α1,3-FucTs (α1,3-fucosyltransferases). The first sequence, closest to that of fruitfly (*Drosophila melanogaster*) FucTA, was found to be a core α1,3-FucT (EC 2.4.1.214), as judged by several enzyme and biochemical assays. The second cDNA encoded an enzyme, most related to *Drosophila* FucTC, that was shown to be capable of generating the Le^x [Galβ1-4(Fucα1-3)GlcNAc] epitope *in vitro* and is the first Lewis-type α1,3-FucT (EC 2.4.1.152) to be described in insects. The transcription levels of 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 characterization of these honey-bee gene products not only accounts for the observed α1,3-fucosylation of bee-venom glycoproteins, but is expected to aid the identification and subsequent down-regulation of the FucTs in insect cell lines of biotechnological importance.

Key words: allergy, anti-(horseradish peroxidase), fucosyltransferase (FucT), honey-bee (*Apis mellifera*), Lewis X, N-glycan.

INTRODUCTION

The glycosylation potential of insect species is increasingly being investigated not only to understand the wide range of glycotypes in nature, but also 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 that are 'foreign' to mammalian immune systems (Figures 1A and 1B). However, the 'humanization' 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 α1,3-fucose bound to the proximal *N*-acetylglucosamine of an N-glycan (i.e. core α1,3-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 α1,3-fucose constitutes a major epitope recognized by antisera raised in rabbits against insect and plant glycoproteins, such as bee (*Apis mellifera*) venom or HRP (horseradish peroxidase) respectively [13,14]. Furthermore, in one study, the sera of approx. 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 α1,3-fucose in both plants and insects [11]. Similar findings have been reported for patients allergic to fire ant [15] and wasp [16] venoms.

Detailed analysis of the N-glycans of two components of bee venom, phospholipase A_2 and hyaluronidase (designated respectively as allergens Api m 1 and Api m 2) showed that these

Abbreviations used: AMPD, 2-amino-2-methyl-1,3-propanediol; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; EST, expressed sequence tag; FucT, fucosyltransferase; GalGal, Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-Asn; βGNβGN, GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-Asn; GnGn, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-Asn; $GnGnF^6$, GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4(Fucα1-6)GlcNAcβ1-Asn; MM, Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-Asn; HRP, horseradish peroxidase; anti-HRP, antiserum raised against HRP; LacdiNAc, *N*-acetylgalactosaminyl-β1,4-*N*-acetylglucosamine (GalNAcβ1-4GlcNAc); LacNAc, galactosyl-β1,4-*N*-acetylglucosamine (Galβ1-4GlcNAc); lacto-*N*-fucopentaose III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA; Le^x, Galβ1-4(Fucα1-3)GlcNAc; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; NBT, Nitro Blue Tetrazolium; ORF, open reading frame; RP-HPLC, reverse-phase HPLC; RT, reverse transcriptase; pyridylaminated lacto-*N*-neo-tetraose, Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA; pyridylaminated lacto-*N*-tetraose, Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA.

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The DNA sequences described in this paper will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AM279411 (FucTA), AM279412 (FucTB) and AM279413 (FucTC).

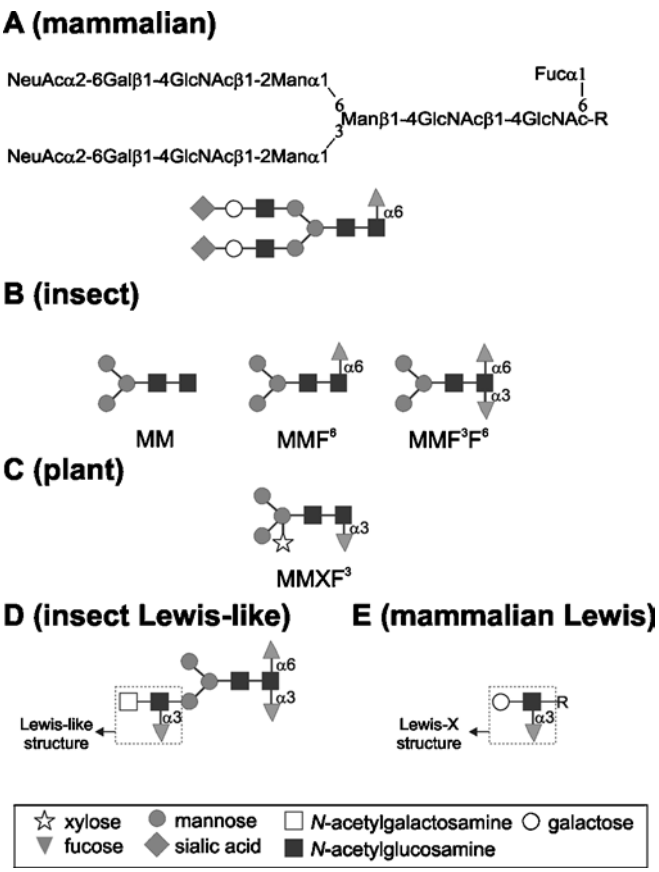

Figure 1 Comparison of N-linked oligosaccharides from mammals, insects and plants

(**A**) Typical mammalian 'complex' N-glycan structure containing terminal sialic acids and α1,6-fucose linked to the proximal *N*-acetylglucosamine. (**B**) N-Glycan structures characteristic for insects. The MM and MMF^6 structures are abundant, whereas the immunogenic MMF^3F^6 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 α1,3-linked fucose. (**D**) Most complex N-glycan structure found on bee-venom glycoproteins; apart from the immunogenic α1,3-fucose linked to the proximal *N*-acetylglucosamine, this structure contains a second α1,3-fucose linked to the distal *N*-acetylglucosamine, creating a Lewis-like structure. As shown in the present 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.

glycoproteins possess a number of non-mammalian features (see Figure 1D for the most complex structure observed). Besides the core α1,3-fucosylation mentioned above, some glycoforms of both these proteins carry a Lewis-type fucosylated LacdiNAc [*N*-acetylgalactosaminyl-β1,4-*N*-acetylglucosamine (GalNAcβ1-4GlcNAc)] structure [17,18], which resembles the Lewis X structure {Le^x [Galβ1-4(Fucα1-3)GlcNAc]; 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. (blood fluke) and *Haemonchus contortus* (the barber's-pole worm)] 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].

On the basis of the aforementioned data, we hypothesized that at least two α1,3-FucTs (α1,3-fucosyltransferases) would be encoded by the honey-bee genome. To date, all enzymes required for addition of α1,3-linked fucose residues to glycans have been found to be members of one enzyme family, CAZy glycosyltransferase family 10 [24], which includes both core α1,3-FucTs from plants, nematodes and insects as well as enzymes from bacteria and 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 α1,3-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 FucTs pertains to *Drosophila melanogaster*: its genome encodes four α1,3-FucT homologues (DmFucTA, DmFucTB, DmFucTC and DmFucTD). Of these, only DmFucTA, a core α1,3-FucT 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 honey-bee enzyme with a Lewis-type FucT may not be so closely related to the three 'orphan' FucTs (FucTB, FucTC and FucTD) from the fruitfly.

To test the hypothesis that a honey-bee FucT 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 honey-bee genome and EST (expressed sequence tag) databases for α1,3-FucT homologues. Of three FucT 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

Cloning of FucT cDNAs from the honey-bee

Male honey-bee 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 honey-bee 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 ORFs (open reading frames) were prepared by PCR using, respectively, the primers BFTB1/KpnI (GGGGTACCGATGCTCGGAATTTCACAA) 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 (AAACTGCAGTTTATAAAGATGTATTTAT) with BFTB2/XbaI or BFTC3/PstI (AAACTGCAGGTACCCCTCGACATATAATAC) with BFTC2/XbaI and ligated into a form of the pPICZαB vector (Invitrogen) modified to encode a FLAG tag.

Expression of honey-bee putative FucTs

Constructs in the pIZT-V5/His and pPICZα-type vectors were transformed respectively into insect Sf9 [*Spodoptera frugiperda* (fall armyworm)] and *Pichia pastoris* GS115 cells as previously described [27,28]. In *P. pastoris*, a methylotrophic yeast, the recombinant enzymes were expressed at 16 °C. Various strategies, as described below, were used to verify enzyme activities. Briefly, core α1,3-FucT activity was tested either by Western blotting of transfected Sf9 cells using rabbit anti-HRP (antiserum raised against HRP) or *in vitro* using various dansyl- and dabsyl-glycopeptides as substrates as used in previous studies [28,29]. The Lewis-type FucT was also assayed with dabsyl-glycopeptides as well as with pyridylaminated lacto-*N*-neo-tetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA) and pyridylaminated lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA). Where stated, *Pichia* supernatants were concentrated 10-fold using UltraFree centrifugal concentration devices (Millipore; relative molecular-mass cutoff 30 000).

MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) FucT assay

For analysis by MALDI–TOF-MS, the activity of recombinant α1,3-FucTs from concentrated *Pichia* supernatants was measured using 40 mM Mes buffer (pH 6.5), 10 mM $MnCl_2$, 1 mM GDP-fucose and 80 μM each of dabsyl-MM [Manα1-6(Manα1-3)-Manβ1-4GlcNAcβ1-Asn], -GnGn [GlcNAcβ1-2Manα1-6-(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-Asn], -GalGal [Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-Asn] and -βGNβGN [GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3)-Manβ1-4GlcNAcβ1-Asn] glycopeptides derived from bovine fibrin, and possessing the common peptide sequence GENR (Gly-Glu-Asn-Arg) (see Figure 2 for oligosaccharide structures and [28]). Reaction tubes were incubated at 30 °C and analysed after 12 h. Following incubation, 0.5 μl of a 1:10 dilution of the reaction mixture was mixed with 0.5 μl 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 (reverse-phase)-HPLC-based FucT assays

For analysis by HPLC, the activity of concentrated supernatants of *Pichia* expressing honey-bee FucTA was measured using 40 mM AMPD (2-amino-2-methyl-1,3-propanediol) and Mes buffer (pH 6.5–9.5) (Mes buffer, pH 7.0, for temperature and cation dependency), 10 mM $MnCl_2$, 1 mM GDP-fucose and 25 μM dansylated $GnGnF^6$ [GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4(Fucα1-6)GlcNAcβ1-Asn] glycopeptide derived from human IgG, with NS (Asn-Ser) as the peptide sequence (see Figure 2 for oligosaccharide structure). Reaction tubes were incubated at 23 °C and analysed after 2 h. Following incubation, an aliquot of the reaction mixture was analysed on an RP-HPLC system (Hypersil™ ODS) under isocratic (at constant concentration) conditions [9.5 % (v/v) acetonitrile and 0.045 % trifluoroacetic acid].

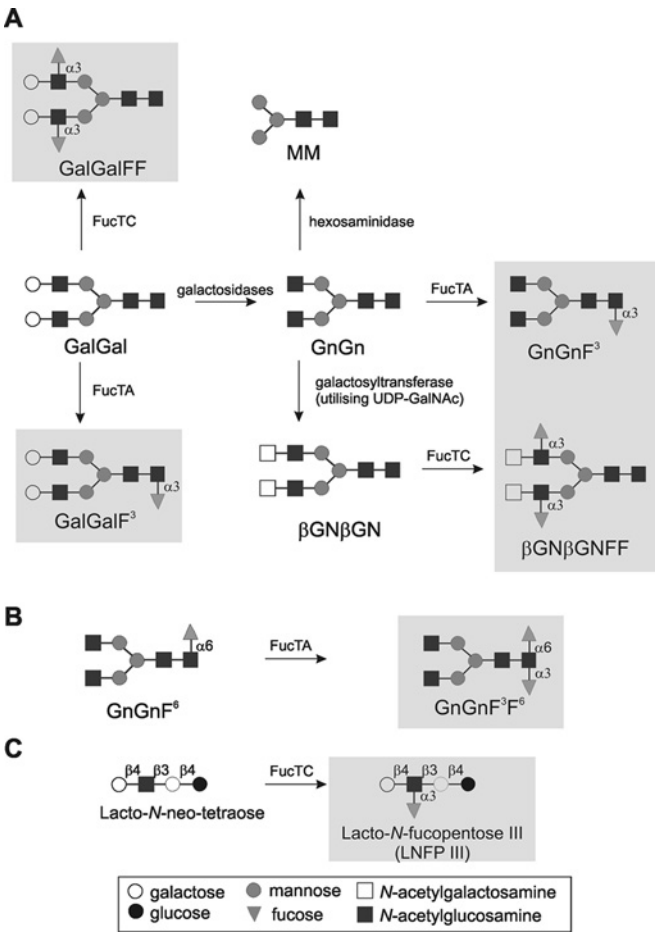

Figure 2 Synthesis and subsequent modifications of glycan structures used as substrates in enzymatic assays

The various substrates used in the present 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 βGNβGN) were synthesized from the dabsylated GalGal-glycopeptide using relevant enzymes. β1,4- and β1,3-galactosidases were used to remove galactose residues, whereas jack-bean (*Canavalia ensiformis*) hexosaminidase was used to remove the GlcNAc residues. Bovine β1,4-galactosyltransferase I, an enzyme known to also utilize UDP-GalNAc, was used to the synthesize the dabsylated βGNβGN-glycopeptide. (**B**) Dansyl-$GnGnF^6$, produced from an asialoagalacto-IgG glycopeptide, has been previously shown to be a substrate for core α1,3-FucTs from plants and insects [1,32]. (**C**) Lacto-*N*-neo-tetraose is a Lewis-type FucT substrate.

The activity of unconcentrated supernatants of *Pichia* expressing honey-bee 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 μg/μl BSA, 12.5 mM $MnCl_2$, 1.25 mM GDP-fucose and 125 μM pyridylaminated lacto-*N*-neo-tetraose. Reaction tubes were incubated at 23 °C and analysed after 2 h. Following incubation, an aliquot of the reaction mixture was analysed on an RP-HPLC system (Nucleosil C18) under isocratic conditions (0.094 M ammonium-formate, pH 4.0, 1.8 % methanol).

Western blotting

GnGn, GalGal and βGNβGN isoforms of human apo-transferrin were prepared by enzymatic remodelling using neuraminidase from the bacterium *Clostridium perfringens* (yielding GalGal-transferrin), β-galactosidase from the ascomycetous fungus

Aspergillus oryzae (yielding GnGn- from GalGal-transferrin) and bovine β1,4-galactosyltransferase I (yielding βGNβGN- from GnGn-transferrin, since the enzyme also utilizes UDP-GalNAc as a sugar donor [28,30]). Recombinant honey-bee FucTA (concentrated) and FucTC (unconcentrated) were incubated with 20 μg of the transferrin neoglycoforms in 40 μM Mes (pH 7.0), 10 mM $MnCl_2$, 1 mM GDP-Fuc and 1 mM PMSF in a total volume of 5 μl. The reactions were performed at 23 °C for 48 h. One-fifth of each incubation was mixed with an equal volume of 2× Laemmli buffer (a 10× concentrate contains 0.25 M Tris, 1.92 M glycine and 1 % SDS in aqueous solution) and analysed by Western blotting using 1:10000 dilutions of either rabbit 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 (5-bromo-4-chloroindol-3-yl phosphate)/NBT (Nitro Blue Tetrazolium) solution.

Sf9 cells were maintained, transfected and lysed as previously described [27]. Briefly, pIZT/V5-His vectors carrying complete ORFs of all three *A. mellifera* α1,3-FucT homologues with the native stop codon were used to transfect Sf9 cells [maintained in IPL-41 insect medium (Sigma–Aldrich) supplemented with 3 % (v/v) foetal-calf serum] 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 green fluorescent protein fluorescence within the cells as indication of a successful transfection. Cells were collected 48 h post-transfection, washed once with PBS and stored at − 80 °C. Cells were lysed using 50 mM Tris (pH 7.5), 150 mM NaCl and 0.5 % Triton X-100 supplemented with Complete™-Mini protease inhibitor cocktail without EDTA (Roche), or His-tag protease inhibitor cocktail (Sigma) for 10 min at 23 °C and 20 min on ice, followed by removal of insoluble material by centrifugation (14100 ***g***, 4 °C and 30 min). Different volumes of the buffer were used to normalize the number of cells per volume (to 1.25×10^4 cells/μl). Cell lysates (2.5 μl per lane) were mixed with 2× 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 (reverse transcriptase)–PCR analyses

Carniolan honey-bees (*A. mellifera* ssp. *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 honey-bees [thorax muscle (two individuals), brains (ten individuals), hypopharyngeal glands (12 individuals) and venom glands (17 individuals)] into RNA*later* (Ambion, Inc., Austin, TX, U.S.A.) solution and stored at − 20 °C. Total RNA from honey-bee tissues and glands was prepared with SV Total RNA isolation system (Promega) after Ultra-Turrax® (IKA Labortechnik, Staufen, Germany) 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 and actin) or 100–400 ng (FucTB) of RNAs in a volume of 25 μl. The primers used in the RT–PCRs had the same T_m ('melting' temperature) 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 mixture was 0.8 μM. 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 (30 s at 94 °C, 60 s at 54 °C and 150 s at 68 °C) followed by 7 min at 68 °C. PCR products were analysed on 1 % agarose gels.

RESULTS

Cloning of FucT cDNAs from the honey-bee

The first target was to identify which gene is responsible in the honey-bee for the occurrence of core α1,3-fucose on bee-venom glycoproteins; it seemed reasonable that the encoded protein would display homology to other core α1,3-FucTs, including the known *D. melanogaster* FucTA. During initial searches of the EST databanks in 2001, two partial bee brain cDNA sequences from the honey-bee 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 honey-bee 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 honey-bee FucTA sequence encoding a protein of 452 amino acids ($M_r = 53000$), 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 recognize the honey-bee FucTA PCR product, but not that of fruitfly FucTB, at low stringency [2× SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1 % SDS, 1 h, 55 °C; results not shown). The determined honey-bee FucTA cDNA sequence was identical with that later released for the predicted mRNA [accession no. XM_392653; NCBI (National Center for Biotechnology Information) Refseq database] based on that of honey-bee Contig1747; a comparison of the cDNA and genomic sequences also showed that the honey-bee FucTA is encoded by three exons.

Considering the presence of fucosylated LacdiNAc on bee-venom glycoproteins, we also sought a second FucT gene. Indeed, when using the fruitfly FucTC sequence to search the now available honey-bee genome, another predicted FucT was found (XM_394138; honey-bee Contig655); the confirmed cDNA sequence encodes a protein of 399 residues ($M_r = 47000$), which we designate as FucTC. Furthermore, using the amino acid sequence of a *Glossinia morsitans* (tsetse fly) putative FucT (GenBank® entry AJ582622), another potential homologous

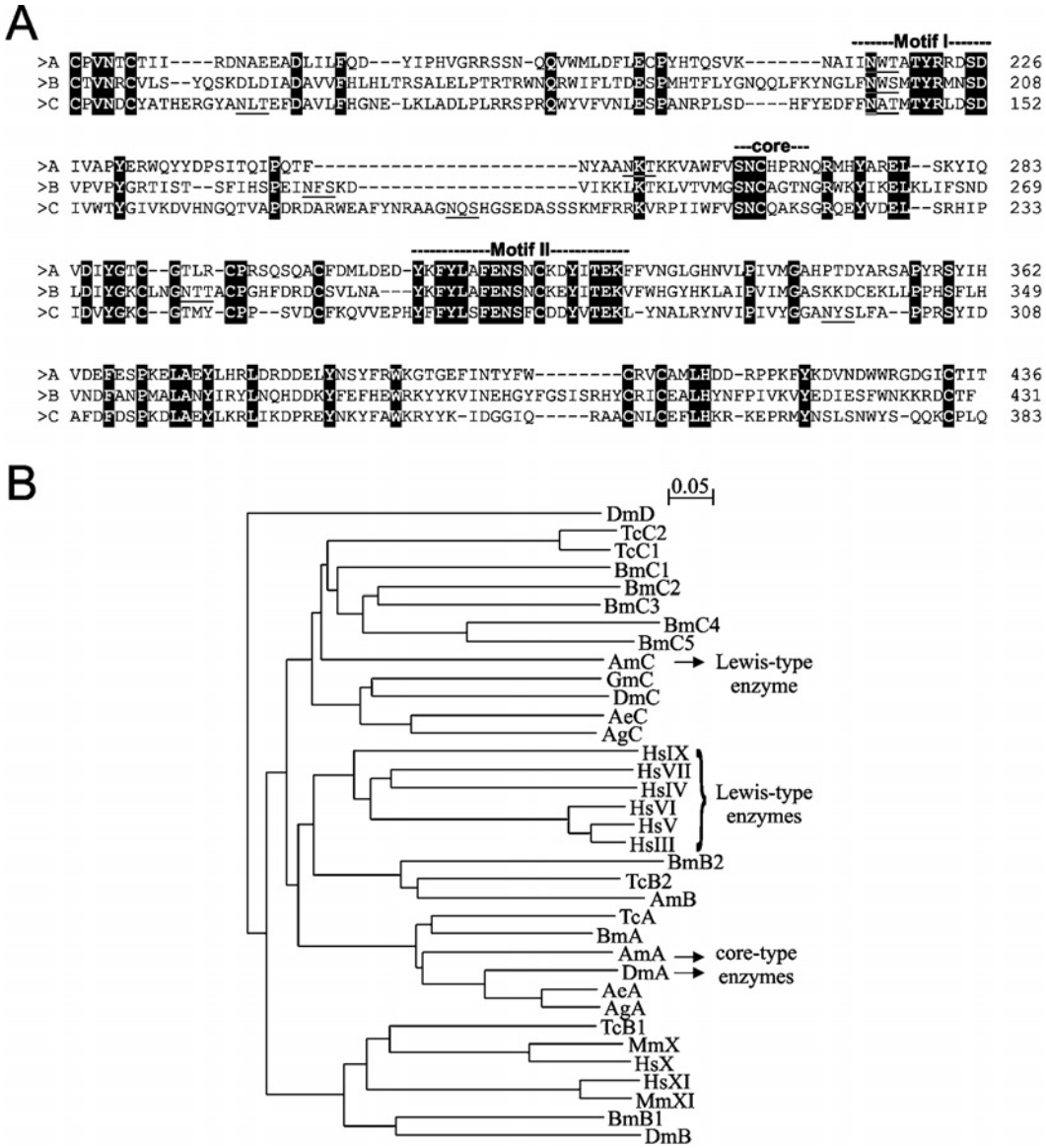

Figure 3 Homologies of honey-bee α1,3-FucTs

(**A**) Alignment of the conserved regions of honey-bee α1,3-FucT homologues. Residues identical in all sequences are highlighted. The amino acid numbering starts from the putative first methionine residue 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 α1,3-FucTs that require the prior action of *N*-acetylglucosaminyltransferase I (i.e. all plant and insect core α1,3-FucTs described to date, but not *Caenorhabditis elegans* FUT-1, which does not accept substrates with an α1,3-antennal terminal GlcNAc residue). (**B**) Phylogenetic tree of insect and mammalian α1,3-FucT homologues. The following protein sequences were analysed using ClustalW and NJPlot programs (http://www.ebi.ac.uk/clustalw/): AmA–C: *A. mellifera* FucTs A, B and C; DmA–D: *D. melanogaster* FucTs A, B, C and D; HsIII–XI: *Homo sapiens* FucTs III–VII and IX–XI; MmX–XI: *Mus musculus* (house mouse) FucTs X and XI; BmA–C5: *B. mori* manually assembled FucT homologues A (AADK01016505), B1 (AADK01001074), B2 (AADK01006340), C1 (AADK01004147), C2 (AADK01000551), C3 (AADK01017942), C4 (AADK01001565) and C5 (BAAB01004223); AeA/C: *Aedes aegypti* manually assembled FucT homologues A (AAGE02021501) and C (AAGE02000340, AAGE02000341); AgA/C: *Anopheles gambiae* manually assembled FucT homologues A (AAAB01008879) and C (AAAB01008846); GmC: *G. morsitans* FucT homologue C (AJ582622); TcA–C2: *T. castaneum* manually assembled FucT homologue A (AAJJ01000602), B1 (AAJJ01000605), B2 (AAJJ01000026), C1 (AAJJ01000008) and C2 (AAJJ01000008). GenBank® accession numbers of genomic DNA sequences that 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.

sequence was found on honey-bee 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 ($M_r = 51\,000$), which, instead of the DYI/VTEK (one-letter amino acid code) motif typical of animal α1,3-FucTs, has an EYVTEK sequence. The three honey-bee FucTs display between 28 and 34 % identity to each other over approx. 300 residues (Figure 3A).

Phylogenetic analysis (Figure 3B) groups the honey-bee 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 FucTs III–VII and IX, while the other is closer to the mammalian FucT homologues X and XI. The honey-bee 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 utilize various N-glycan substrates [1,27], whereas, as shown below, honey-bee FucTC does accept such glycans.

Expression of honey-bee FucTs in Pichia

Constructs encoding FLAG-tagged soluble forms of the three honey-bee FucT homologues were prepared for expression in the yeast *P. pastoris*. Culture supernatants were collected and concentrated prior to assaying specific FucT activities with various dabsylated N-glycopeptides (MM, GnGn, GalGal and βGNβGN; see Figure 2 for structures). MM and GnGn were used as known core FucT substrates, whereas GalGal and βGNβGN are substrates for Lewis-type enzymes [31]. As shown in Figure 4, *Pichia* transformed with the FucTA vector converted GnGn into a fucosylated form, whereas that transformed with FucTC vector converted GalGal and βGNβGN into difucosylated forms. Upon longer incubation (36 h), the honey-bee FucTA expressed in *Pichia* also converted GalGal and βGNβGN into monofucosylated forms (results 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 a HPLC-based assay with the fluorescently labelled dansylated-GnGnF^6 glycopeptide as the substrate. In agreement with previous studies on core α1,3-FucTs [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 FucTs forming, respectively, α1,3 and α1,4-fucosyl linkages. Previous studies with a plant Lewis-type α1,3/4-fucosyltransferase (tomato FucTC) showed that fucosylation of these substrates results in a shift to earlier elution times [33]. In the present study with honey-bee 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 [Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA] (Figure 5); no such shift was observed for yeast transformed with FucTA or FucTB nor when lacto-*N*-tetraose was incubated with FucTC (results not shown). These results, therefore, show that the enzyme solely acts as an α1,3-FucT. The determined activity of FucTC towards both LacNAc [galactosyl-β1,4-*N*-acetylglucosamine (Galβ1-4GlcNAc)]- and LacdiNAc-containing substrates is comparable with results showing that the mammalian FucTs 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 honey-bee FucTA and FucTC

The basic enzymological parameters of FucTA and FucTC were investigated using the aforementioned HPLC-based assays. Honey-bee FucTA is strongly activated by Mn^{2+} as compared with no-cation controls, a property common to many glycosyltransferases. On the other hand, EDTA, as well as Cu^{2+} and Zn^{2+} ions, completely abolish this enzyme's activity. The enzyme shows maximal activity in mildly alkaline conditions (pH 7.5–9.0) at room temperature (23 °C). In contrast with FucTA, FucTC is not inactivated by EDTA, although the enzyme seems to prefer Mn^{2+} and Mg^{2+} over other cations. The temperature and pH optima for the FucTC appear to be very broad; the enzyme shows 90 % of maximal activity at 4 °C and over 40 % of maximal activity at 50 °C (Figure 6).

The honey-bee FucTA and FucTC can utilize protein substrates

The assays employed to measure the activity of both core and Lewis-like α1,3-FucT utilize substrates containing predominantly carbohydrates, with little or no peptide backbone as compared with '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

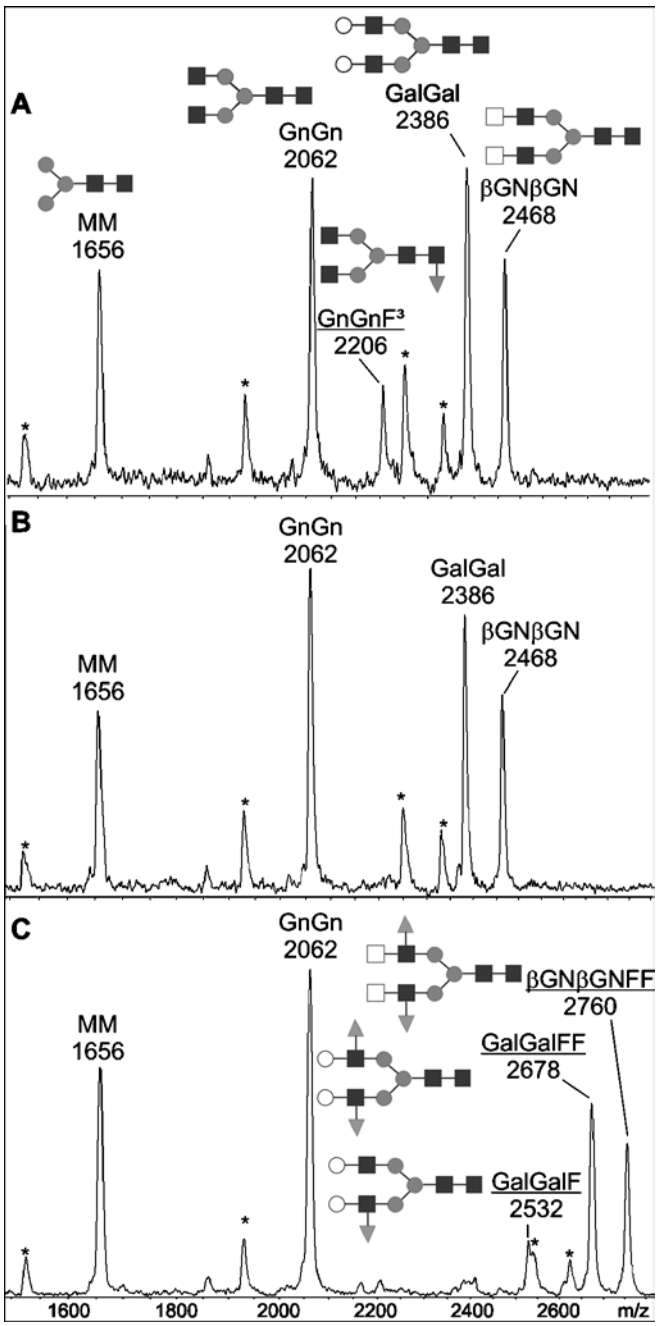

Figure 4 Activity of honey-bee FucTs expressed in *Pichia* analysed by MALDI–TOF-MS

Culture supernatants of yeast transformed with either FucTA (**A**), FucTB (**B**) or FucTC (**C**) were assayed using four dabsylated glycopeptides (MM, GnGn, GalGal and βGNβGN) 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 dabsyl group are labelled with an asterisk.

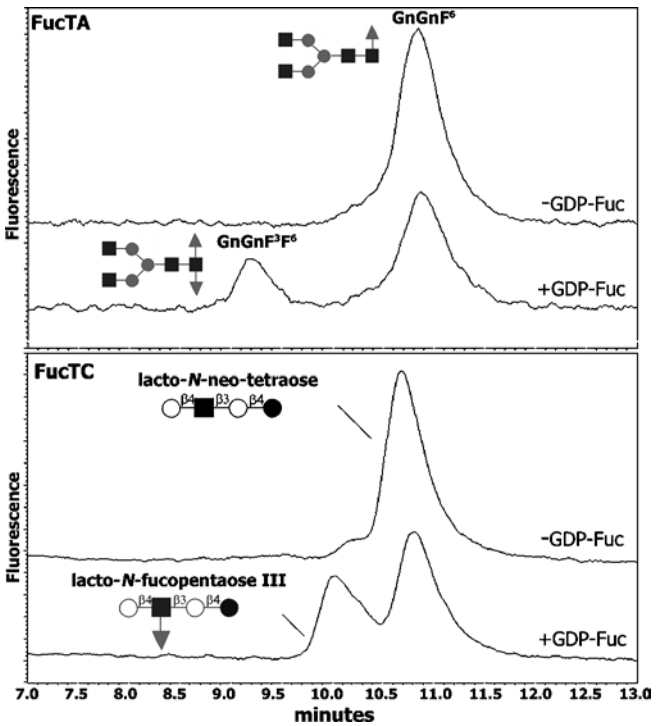

Figure 5 Activity of honey-bee FucTs expressed in *Pichia* analysed by RP-HPLC

Culture supernatants of yeast transformed with either FucTA or FucTC were assayed using either dansylated-GnGnF^6 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 (results not shown).

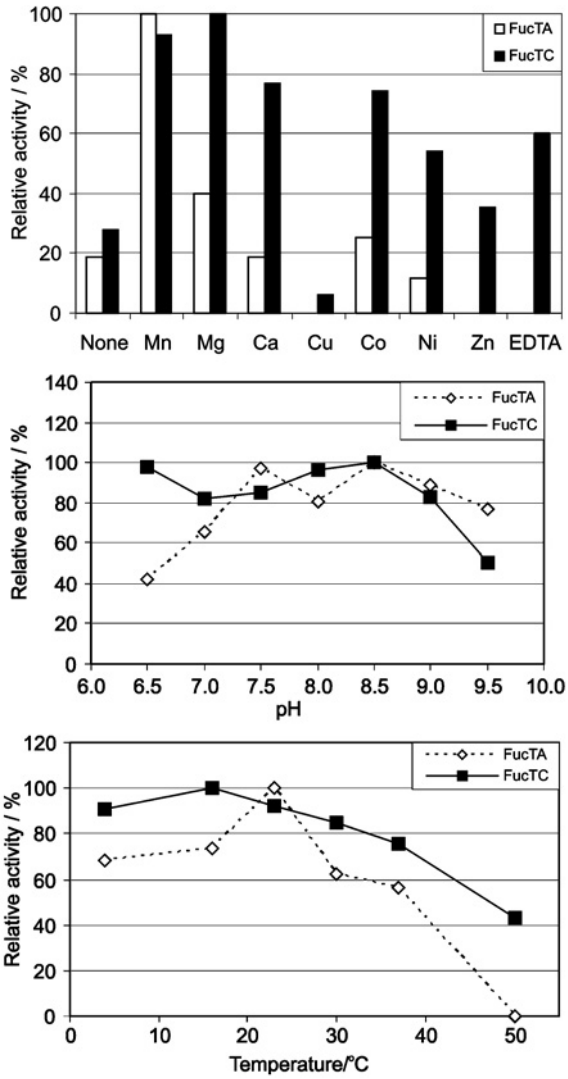

Figure 6 Properties of recombinant honey-bee FucTA and FucTC

Cation dependency, pH and temperature optima were determined using either 10-fold concentrated (FucTA) or unconcentrated (FucTC) honey-bee enzymes expressed in *Pichia*. Relative activity was normalized based on the condition giving the highest rate of fucose transfer.

utilized 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/polyacrylamide gel, transferred on to a nitrocellulose membrane and probed with anti-HRP or anti-Le^x antibodies. Whereas anti-HRP is known to bind core α1,3-linked fucose as found on many plant and insect glycoproteins [35], the L5 monoclonal antibody is apparently the only antibody capable of recognizing the Le^x epitope when attached to N-glycans [36]. The transferrin neoglycoforms carrying N-glycans predominantly terminating with β1,2-GlcNAc (i.e. GnGn-), β1,4-Gal (i.e. GalGal-) and β1,4-GalNAc (i.e. βGNβGN-transferrin) residues incubated with FucTA were recognized by anti-HRP antibodies, demonstrating that transfer of a fucose resulted in generation of the same core α1,3-fucose epitope as found on, e.g. HRP and bee-venom phospholipase. On the other hand, only GalGal-transferrin incubated with FucTC was recognized by antibodies directed against the Le^x structure, thus strongly indicating that the fucose residues were attached in α1,3-linkage to the antennal *N*-acetylglucosamine residues (Figure 7A). Even though FucTC can transfer fucose to βGNβGN, the specificity of the L5 antibody is obviously absolute for Le^x and therefore this antibody does not recognize fucosylated LacdiNAc.

Analysis of honey-bee FucT activity *in vivo*

In previous studies, we have used insect cells as hosts for core α1,3-FucT expression in order to perform screening for the ability of an ORF to confer anti-HRP binding *in vivo*, i.e. to prove the specific modification of glycoproteins of cells transfected with core α1,3-FucTs [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 α1,3-fucosylation upon transfection of relevant ORFs 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 honey-bee α1,3-FucT homologues and employed *Drosophila* FucTA as a positive control; vectors containing the entire ORF encoding each enzyme, with no tag, were used. As shown in Figure 7(B), of the honey-bee FucTs, only the FucTA conferred the ability to bind anti-HRP to glycoproteins of Sf9 cells. The lanes with the cells transfected with the honey-bee FucTB and FucTC showed no difference as compared with the empty vector control, corroborating the other results suggesting that they are not core FucTs. Furthermore, *in vitro* assays also showed that only the FucTA-transfected cells had an activity capable of transferring a fucose to GnGnF^6, thereby generating a difucosylated structure, above the otherwise very

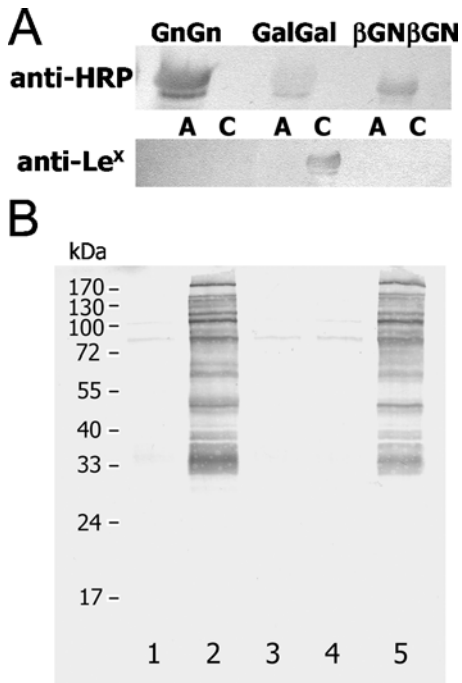

Figure 7 Epitope formation by honey-bee FucTA and FucTC

(**A**) Honey-bee FucTA and FucTC expressed in *Pichia* confer anti-HRP and anti-Le^x staining on glycoproteins. Recombinant FucTA and FucTC were incubated with modified transferrin carrying N-glycans predominantly terminating with β1,2-GlcNAc (i.e. GnGn-), β1,4-Gal (i.e. GalGal-) or β1,4-GalNAc (i.e. βGNβGN-transferrin) residues. FucTA confers anti-HRP staining on the used transferrin glycoforms (upper panel). FucTC but not FucTA confers anti-Le^x (anti-L5) staining only on GalGal-transferrin (lower panel). (**B**) Expression of honey-bee FucTs in Sf9 insect cells. Cells transfected with either an empty pIZTV5/His vector (1), honey-bee FucTA (2), honey-bee FucTB (3), honey-bee FucTC (4) or *Drosophila* FucTA (5) were lysed and subjected to SDS/PAGE and Western blotting with anti-HRP.

low background levels displayed by control transfectants (results not shown). The overall conclusion is that, as judged by results indicating that this enzyme can form the anti-HRP epitope *in vitro* and *in vivo*, the fucose transferred by FucTA is α1,3-linked to the proximal GlcNAc of N-glycans.

Expression of FucTs in different honey-bee tissues

To appraise whether the tissue-specific expression of α1,3-FucT homologues in *A. mellifera* is compatible with the synthesis of the previously observed N-glycans containing both core and antennal α1,3-linked fucose [17], total RNA from several honey-bee tissues was prepared. Following RNA normalization against actin transcripts, expression pattern of the FucT homologues in honey-bees 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 nt shorter and has a premature stop codon. Finally, the third α1,3-FucT homologue, FucTB, appears to be weakly expressed in venom

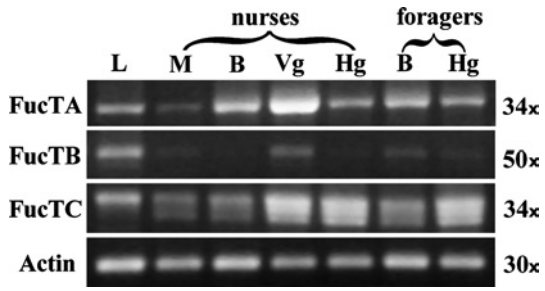

Figure 8 Expression patterns of FucTs in different tissues and glands of *A. mellifera*

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 honey-bees or of foragers from the same colony. RT–PCR was performed with the primers specific for individual FucTs (see the Experimental procedures) using different numbers of cycles. In the case of FucTB, the conditions of reaction were partially distinct from those used for FucTA, FucTC and actin. The amplified fragments of FucTA, FucTB, FucTC and actin with the respective sizes of 1350, 1160, 1200 and 600 bp are shown.

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 honey-bee 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 α1,3-fucosylated N-glycan structures on bee-venom glycoproteins.

DISCUSSION

The honey-bee 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], and 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 α1,3-fucose and α1,3-fucosylated LacdiNAc structures on an insect glycoprotein. Thus we expected to find at least two genes in the honey-bee encoding enzymes able to synthesize these structures.

In actual fact, the *A. mellifera* genome encodes three α1,3-FucT homologues, which include the consensus sequence common to all α1,3-FucTs [38,39]. As indicated by the predicted amino acid sequences, one of the honey-bee sequences (FucTA) is phylogenetically close to *Drosophila* and other proven or putative N-glycan core α1,3-FucTs (Figure 3B). Indeed, when expressed in *P. pastoris* and insect cells, this honey-bee sequence encodes an active core α1,3-FucT with a substrate specificity similar to that of the recombinant *Drosophila* FucTA [1], the native honey-bee venom gland and *Mamestra brassicae* (cabbage moth) MB-0503 cell line core α1,3-FucTs [25,26] and various plant N-glycan core α1,3-FucTs [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 with the preference of the *Caenorhabditis* (nematode worm) FUT-1 for this substrate [29]. This suggests that the substrate specificity of invertebrate core α1,3-FucTs (i.e. FucTA and FUT-1) is somewhat more variable and flexible than that of invertebrate core

α1,6-fucosyltransferases, which require a terminal unsubstituted GlcNAc residue [31].

The second homologue found in the present study (FucTC) resembles a number of other related sequences from various insects, including the *Drosophila* FucTC sequence. Unlike DmFucTC [27], the honey-bee FucTC encodes an active Lewis α1,3-FucT and is indeed the first such enzyme to be characterized 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 honey-bee FucTC seems to be more related to *Bombyx mori* (silk moth) and *Tribolium castaneum* (red flour beetle) sequences, whereas the *Drosophila* FucTC is more similar to *Anopheles gambiae* (the major African malaria mosquito), *Aedes aegypti* (the yellow-fever mosquito) and *G. 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 honey-bee 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 honey-bee α1,3-FucT homologues and fucosylated N-glycan structures found on bee-venom glycoproteins [17,18] by performing expression studies of all three honey-bee α1,3-FucT 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 α1,3-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 honey-bee FucTA also appears to be expressed in hypopharyngeal glands that produce the royal jelly proteins in honey-bee nurses [44–46] and carbohydrate-metabolizing enzymes after the bees adopt the forager role [47–49]. This result is in contrast to 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 α1,3-linked fucose appear also to bind other tissues in *Drosophila* (e.g. the garland gland) [43,51,52], indicating that α1,3-linked fucosylated N-glycan structures might occur in insect tissues other than, as generally accepted, neurons [1,27] and venom glands [11,17,18].

Honey-bee 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 honey-bee 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 honey-bee glycoproteins. It is indeed probable that the honey-bee only expresses a β1,4-*N*-acetylgalactosaminyltransferase, akin to those previously described from *Trichoplusia ni* (cabbage looper) [54] and *D. melanogaster* [55], and lacks a β1,4-galactosyltransferase of the type found in mammals. Therefore presumably only the substrate with terminal GalNAc, required to generate a fucosylated LacdiNAc, is present in honey-bees *in vivo*.

The expression panel suggests that honey-bee 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 honey-bee FucTA and FucTC. Interestingly, honey-bee FucTB appears to be phylogenetically closer to known N-glycan core α1,3-FucTs like *Drosophila* FucTA and honey-bee FucTA than to the *Drosophila* FucTB and the putative human and murine FucTs X and XI. However, extensive attempts with both fruitfly and honey-bee FucTB homologues to demonstrate an activity towards potential substrates (MM, GnGn, GalGal, βGNβGN, 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 α1,3-FucT, from an insect other than *Drosophila*, as well as the first insect Lewis-type FucT. These enzymes account for the N-glycan α1,3-fucosylation events previously hypothesized on the basis of N-glycan analysis of honey-bee glycoproteins and both generate products associated with immune responses to glycans from invertebrates [11,13,19,20]. Despite both being members of the same enzyme family, honey-bee 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 with those commonly used baculovirus hosts such as *T. ni* (HiFive®) and Sf21 cells [57,58] known to express core α1,3-fucosylated N-glycans, but for which we lack relevant DNA sequence information. Indeed, of the various non-*Drosophila* insect species for which such data are currently publicly available, only *B. mori* is of direct biotechnological importance, with silkworm BmN cells sometimes being used for baculovirus-based expression. However, the relatively high homology between the fruitfly and honey-bee FucTA core α1,3-FucT sequences, and the observed ability of these sequences to cross-hybridize, may be useful in screening cDNA libraries from baculovirus-host species for which no genomic data is currently available. Identification of the core α1,3-FucTs 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 α1,3-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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