Glycoconj J (2015) 32:141–151
DOI 10.1007/s10719-015-9585-7

ORIGINAL ARTICLE

Identification of β1,3-galactosyltransferases responsible for biosynthesis of insect complex-type *N*-glycans containing a T-antigen unit in the honeybee

Tomomi Ichimiya[1] · Megumi Maeda[2,3] · Shou Sakamura[3] · Masato Kanazawa[3] · Shoko Nishihara[1] · Yoshinobu Kimura[2,3]

Received: 3 February 2015 / Revised: 20 March 2015 / Accepted: 24 March 2015 / Published online: 1 May 2015

Abstract Honeybees (*Apis mellifera*) produce unique complex-type *N*-glycans bearing a Galβ1-3GalNAc (T-antigen) unit, and honeybee-specific *N*-glycans are linked to royal jelly glycoproteins. In this study, we identified two novel honeybee β1,3-galactosyltransferase (*β1,3-GalT*) genes responsible for biosynthesis of the T-antigen in insect *N*-glycans. The products of the two putative *β1,3-GalT* genes (*β1,3-GalT1* and *β1,3-GalT2*), which were expressed in *Sf21* insect cells, transferred galactose (Gal) residues to GalNAc2GlcNAc2Man3GlcNAc2-PA to form the Galβ1-3GalNAc unit, indicating that the identified genes were involved in biosynthesis of the β1-3 Gal-containing *N*-glycan. Therefore, using biochemistry and molecular biology techniques, we revealed a unique *N*-glycan biosynthesis mechanism in the cephalic region of honeybees, which has not previously been found in other animal or plant cells.

Keyword Insect β1,3-galactosyltransferase · Insect *N*-glycan · *N*-glycan biosynthesis · Thomsen-Friedenreich antigen · *Apis mellifera*

✉ Shoko Nishihara
shoko@soka.ac.jp

✉ Yoshinobu Kimura
yosh8mar@cc.okayama-u.ac.jp

[1] Department of Bioinformatics, Faculty of Engineering, Soka University, 1-236 Tangi-cho, Tokyo, Japan

[2] Department of Biofunctional Chemistry, Graduate School of Environmental and Life Science, Okayama University, Tsushima Naka 1-1-1, Okayama 700-8530, Japan

[3] Department of Biofunctional Chemistry, Graduate School of Natural Science and Technology, Okayama University, Tsushima Naka 1-1-1, Okayama 700-8530, Japan

Abbreviations

β1,3-GalT	β1,3-galactosyltransferase
GalNAc α-*p*Nph	*p*-nitrophenyl-*N*-acetyl-α-galactosaminide
MRJP	Major royal jelly protein
PA-	Pyridylamino
SF-HPLC	Size-fractionation HPLC
Man	D-mannose
Gal	D-galactose
GlcNAc	*N*-acetyl-D-glucosamine
GalNAc	*N*-acetyl-D-galactosamine
LacdiNAcM3	GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA
GalLacdiNAcM3	Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA or GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA
Gal2LacdiNAcM3	Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA
T-antigen	Thomsen-Friedenreich antigen (Galβ1-3GalNAc)

Introduction

In a previous study [1], from royal jelly glycoproteins, we identified new complex-type *N*-glycans bearing the Galβ1-3GalNAc (Thomsen-Friedenreich antigen: T-antigen) unit at their non-reducing ends. Based on this discovery, we proposed

that a unique *N*-glycan processing mechanism was functioning in the cephalic region of honeybees (*Apis mellifera*), and that this indicated the involvement of a new β1,3-galactosyltransferase (β1,3-GalT) in the *N*-glycan processing pathway (Fig. 1). Subsequently, we confirmed the activity of β1,3-GalT in a microsomal fraction prepared from the cephalic portion of honeybees [2], and found that a major royal jelly glycoprotein (MRJP1) harbors the insect complex-type *N*-glycan at its N-terminal region [3]. Several core 1 β1,3-galactosyltransferases (core 1 β1,3-GalTs), which transfer the galactose residue by β1-3-linkage to α-*N*-acetylgalactosamine (GalNAc) linked to serine or threonine residues, have been characterized and their corresponding genes identified in mammals [4, 5], nematodes [6], and insects [7, 8]. However, the gene encoding the β1,3-GalT involved in the biosynthesis of the T-antigen unit in insect complex-type *N*-glycans has yet to be identified.

Currently, the physiological significance or function of the honeybee complex-type *N*-glycans containing the T-antigen unit is unknown. However, a previous report [9] indicated that MRJP1 [10] (designated RJP-3 in reference [9]) was expressed in a subset of Kenyon cells in the mushroom bodies of the honeybee brain. The mushroom bodies, which are the memory center in the insect brain, are involved in learning and the formation and storage of memory. Honeybees are highly social insects that exhibit complex forms of communication, and these behaviors indicate that sophisticated brain functions must be integrated in the mushroom bodies. Various *N*-glycans linked to proteins also play critical roles in cell-cell communication and tissue construction; hence, it is possible that unique honeybee brain-specific *N*-glycans (or *N*-glycoproteins such as MRJP1) are involved in the construction of brain tissues, including the mushroom body. In order to reveal the physiological significance of complex-type *N*-glycans, it will be necessary to identify the *β1,3-GalT* genes involved in biosynthesis of the T-antigen unit. Although many *β1,3-GalT* genes (including putative genes) have been identified in the *Drosophila* gene database, the genes that encode the β1,3-GalT proteins responsible for processing insect *N*-glycans remain unknown.

In this study, based on homology and expression patterns of human, *Drosophila melanogaster*, and honeybee *β1,3-GalT* genes, we identified two novel honeybee genes encoding β1,3-GalTs, namely *β1,3-GalT1* and *β1,3-GalT2*, which are responsible for the synthesis of the T-antigen unit in insect complex-type *N*-glycans. Subsequently, we constructed a heterologous expression system using an insect cell line (*Sf21* cells). Because honeybee β1,3-GalT1 and β1,3-GalT2 but not honeybee core 1 β1,3-GalT were capable of transferring the galactose (Gal) residue to GalNAc2GlcNAc2Man3GlcNAc2-PA, we suggest that β1,3-GalT1 and β1,3-GalT2 must be specialized in insect *N*-glycan biosynthesis.

Experimental procedures

Vector construction and expression of honeybee β1,3-GalT proteins

The putative catalytic domains of honeybee core 1 β1,3-GalT (amino acid 52–372, DDBJ accession number: LC017708) [8], honeybee β1,3-GalT1 (amino acid 27–412, DDBJ accession number: LC017705), and honeybee β1,3-GalT2 (amino acid 11–396, DDBJ accession number: LC017706)were expressed as secreted proteins fused with a FLAG peptide in insect cells according to the GATEWAY™ Cloning Technology (Invitrogen, Carlsbad, CA, USA) instruction manual [11, 12]. The DNA fragments of honeybee *core 1 β1,3-GalT*, honeybee *β1,3-GalT1*, and honeybee *β1,3-GalT2* were amplified by two-step PCR. The first PCR used cDNA from honeybee adult heads as a template for amplification and the following primer sets: for honeybee *core 1 β1,3-GalT*, the forward primer was 5′-AAAAAGCAGGCTATATGTTTAATTGGTTTTCTGGTGG-3′ and the reverse primer was 5′-AGAAAGCTGGGTCTAGTATTCAGTCAATACAGTAATTG-3′; for honeybee *β1,3-GalT1*, the forward primer was 5′-AAAAGCAGGCTATTATTATCGCACTACTTATTACGATGTCC-3′ and the reverse primer was 5′-GAAAGCTGGGTTCATTTACATTTACTTTTACCATCAAATAATTTTTTCC-3′; and for honeybee *β1,3-GalT2*, the forward primer was 5′-AAAAAGCAGGCTATATGGACAAATTGCAATTATTGCCATTGG-3′ and the reverse primer was 5′-AGAAAGCTGGGTTCAAATATTTCTACTATTTCTACTTAATGTAACTG-3′. The second PCR used the first PCR product as a template, with the forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ and the reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′. The amplified fragments were recombined with the pDONR™201 vector (Invitrogen). Subsequently, each insert was transferred between the attR1 and attR2 sites of pVL1393-FLAG to yield pVL1393-FLAG-honeybee *core 1 β1,3-GalT*, pVL1393-FLAG-honeybee *β1,3-GalT1*, and pVL1393-FLAG-honeybee *β1,3-GalT2*, and these were cotransfected with BaculoGold viral DNA (BD Biosciences, San Jose, CA, USA) into *Sf21* insect cells according to the manufacturer's instructions. The cells were then incubated for 5 d at 25 °C to produce recombinant viruses. Subsequently, *Sf21* cells were infected with each recombinant virus and incubated for 5 d to express FLAG-honeybee core 1 β1,3-GalT, FLAG-honeybee β1,3-GalT1, and FLAG-honeybee β1,3-GalT2 as secreted proteins. A mixture containing 30 mL culture medium, 100 μL of anti-FLAG M1 affinity gel (Sigma, St. Louis, MO, USA), 10 mM $CaCl_2$, and 2 mM $MnCl_2$ was then produced. Each protein-gel mixture was washed twice with wash buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, 2 mM $MnCl_2$), and 0.2 mg/mL FLAG peptide was eluted in wash buffer.

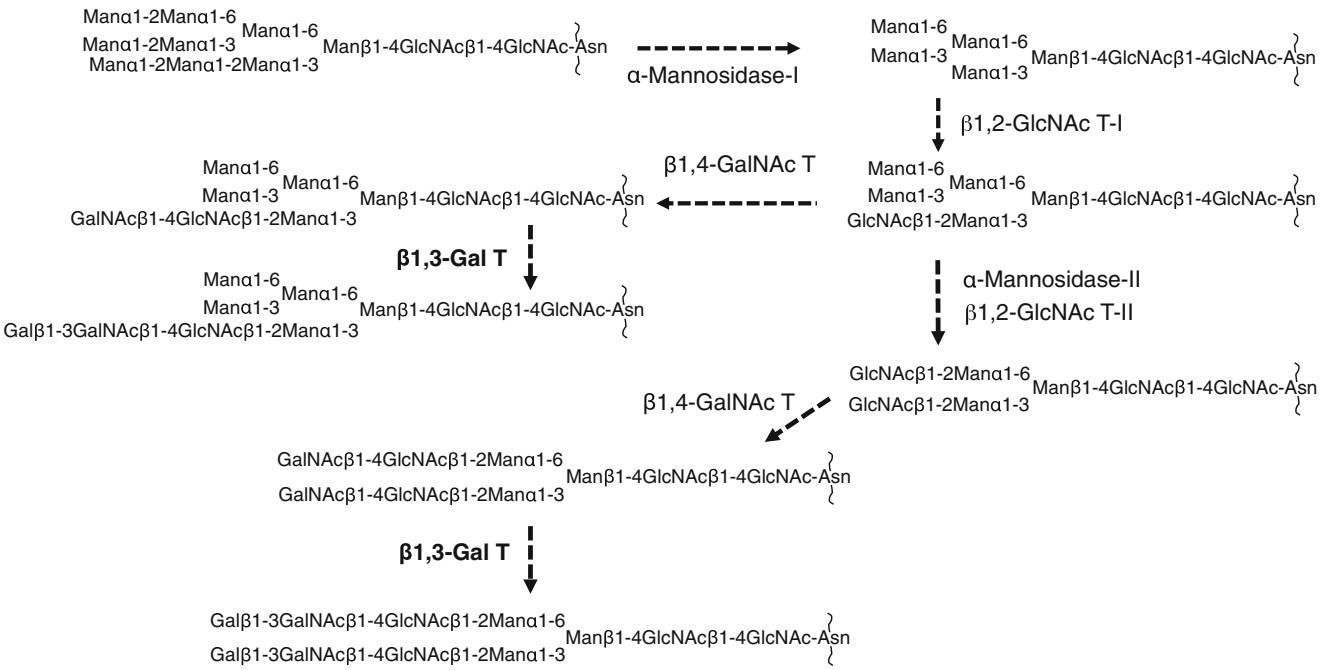


Fig. 1 Proposed biosynthetic pathway of a unique insect *N*-glycan harboring the Galβ1-3GalNAc (T-antigen) unit. Two novel honeybee (*Apis mellifera*) β1,3-GalTs, namely β1,3-GalT1 and β1,3-GalT2, were identified in this study.

Western blot analysis

Purified FLAG-honeybee core 1 β1,3-GalT, FLAG-honeybee β1,3-GalT1, and FLAG-honeybee β1,3-GalT2 were subjected to 10 % SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a membrane, which was probed with anti-FLAG-peroxidase-conjugated monoclonal antibody (Sigma, St. Louis, MO, USA) and stained with Konica Immunostaining HRP-1000 kit (Konica, Tokyo, Japan). The intensity of bands was measured with a densitometer to determine the amount of the purified enzyme using FLAG-BAP Control Protein (Sigma).

Assay of β1,3-GalT activity

Uridine diphosphate-[^{3}H]galactose (UDP-[^{3}H]Gal) (20 Ci/mmol) was supplied by American Radiolabeled Chemicals (Saint Louis, MO, USA) and *p*-nitrophenyl-*N*-acetyl-α-galactosaminide (GalNAc α-*p*Nph) was purchased from Calbiochem Merck Millipore (Billerica, MA, USA). An assay of galactosyltransferase activity was performed as previously described [8]. The reaction mixture contained 3.17 mg of purified FLAG-honeybee core 1 β1,3-GalT, 100 mM MES (pH 6.8), 2 mM ATP, 20 mM MnCl2, 0.2 % triton X-100, 250 μM GalNAc α-*p*Nph, and 0.4 mM UDP-Gal (including 1.25 μM UDP-[^{3}H]Gal) to a total volume of 40 μL. After incubation at 25 °C for 1 h, the reaction was terminated with the addition of 200 μL of distilled water. The reaction mixture was applied to Sep-Pak C18 cartridges and unreacted UDP-[^{3}H]Gal was washed out with water. The products on the column were eluted with methanol and their radioactivity was measured using a liquid scintillation counter.

Pyridylaminated *N*-glycan substrate (LacdiNAcM3, GalNAc2GlcNAc2Man3GlcNAc2-PA) was prepared from royal jelly glycoproteins as previously described [2]. Purified FLAG-honeybee core 1 β1,3-GalT, FLAG-honeybee β1,3-GalT1, or FLAG-honeybee β1,3-GalT2 (3.7 μg, 4.0 μg, and 2.7 μg, respectively) was incubated with 680 pmol of LacdiNAcM3, 20 mM UDP-Gal, and in 100 mM MOPS buffer (pH 6.8) containing 2 mM ATP, 20 mM MnCl2, and 0.2 % Triton X-100 at 37 °C for 16 h. After boiling the reaction mixture for 3 min and subjecting it to centrifugation at 12, 000*g* for 5 min., an 18 μL aliquot was extracted and then analyzed by size fractionation HPLC (SF-HPLC) using a Shodex Asahipak NH2P-50 column (10×250 mm; Showa Denko, Tokyo, Japan) as previously described [1]. The occurrence of β1,3-Gal residue was confirmed by β1,4-galactosidase (EC number; 3.2.1.23, cloned from *Streptococcus pneumonia* and expressed in *E. coli*; Sigma) and β1,3-galactosidase (95–150 mU, cloned from *Xanthomonas manihotis* and expressed in *E. coli*; New England Biolabs, Code No. P0726S) digestions, as described in a previous paper [2]. Although the *Xanthomonas* β-galactosidase hydrolyzes both β1-3-galactosidic and β1-6-galactosidic, it has been reported that this *Xanthomonas* β-galacatosidase preferentially hydrolyses β1-3 galactosyl linkage [13] and shows about 100-fold preference for β1-3 over β1-6 linkages (manufacture's instruction sheet).

Results

Molecular cloning of honeybee core 1 β1,3-GalT and GalT activity assay

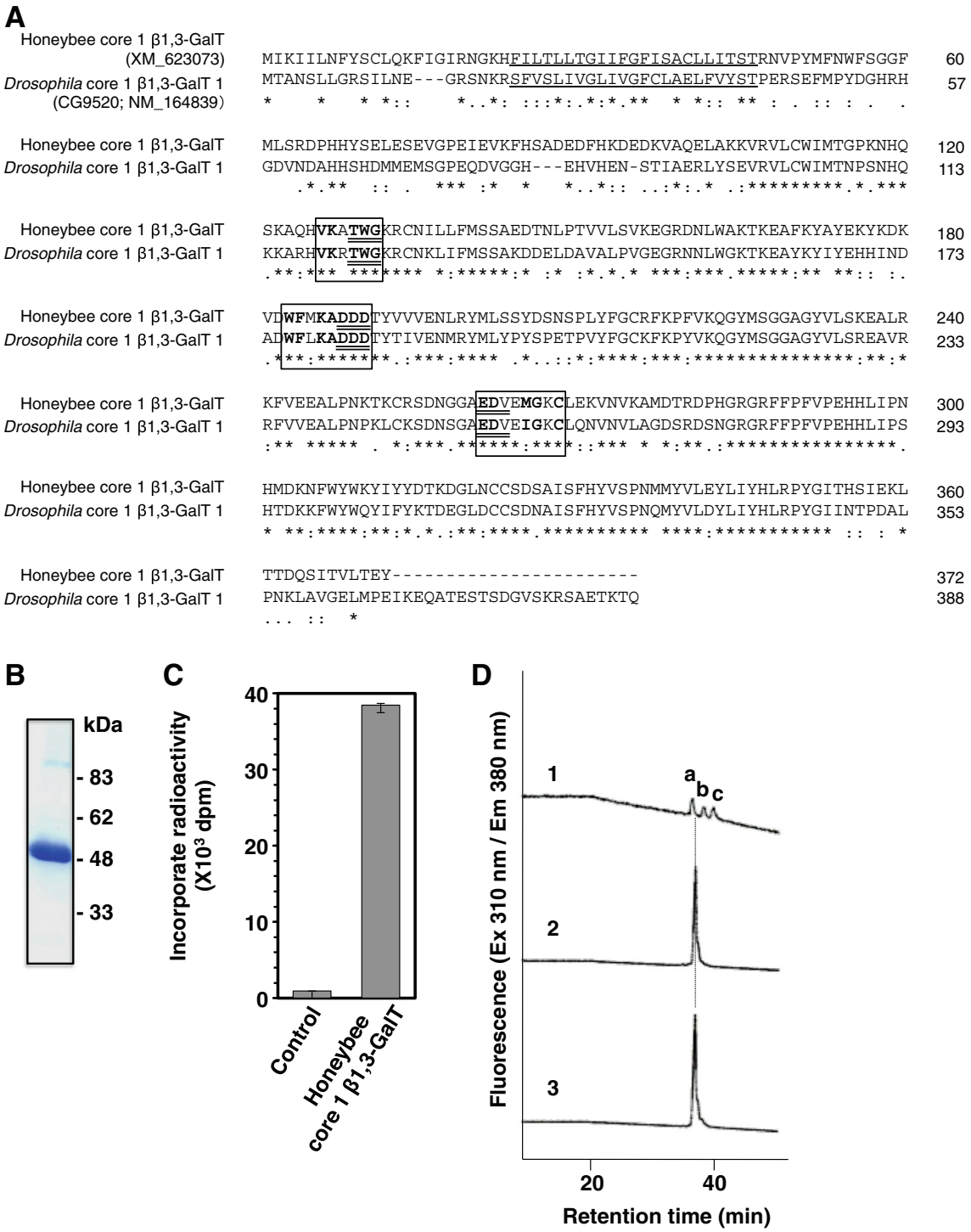
The complex-type *N*-glycans that we previously identified [1–3] had Galβ1-3GalNAc (T-antigen) units at their non-reducing ends. The core 1 β1,3-GalTs reportedly synthesize the T-antigen unit on *O*-glycan in several species, including *D. melanogaster* [7, 8]. To date, four *Drosophila core 1 β1,3-GalT* genes have been reported [7]. Here, we cloned the honeybee ortholog of *Drosophila core 1 β1,3-GalT1* (CG9520), which encodes a protein that has the highest β1,3-GalT activity toward the GalNAc residue of all proteins produced by the four reported genes [7, 8] (Fig. 2A). The overall nucleic acid or amino acid sequence identity of the honeybee ortholog to *Drosophila core 1 β1,3-GalT1* was 55 % or 58 %, respectively. However they showed high amino acid sequence similarity (86 %), indicating the similar transferase activity. The honeybee ortholog also had TWG, DDD and EDV motifs (double underlines in Fig. 2A) that have been reported in *Drosophila* core 1 β1,3-GalTs as conserved motifs [7]. By comparing with the human and the other three *Drosophila* core 1 β1,3-GalTs, we found three core 1 β1,3-GalT motifs, VKxTW(G/A), WFxKADDD, and EDxx(I/L/M)GxC (rectangles in Fig. 2A). The core 1 β1,3-GalT motifs included TWG, DDD and EDV motifs. Orthologs for the other three *Drosophila core 1 β1,3-GalT*s were not found in the honeybee genome, indicating that the honeybee ortholog of *Drosophila core 1 β1,3-GalT1* was the only candidate for honeybee *core 1 β1,3-GalT*. The protein was expressed as a FLAG-tagged protein and purified using anti-FLAG M1 affinity gel (Fig. 2B). The purified honeybee ortholog of *Drosophila* core 1 β1,3-GalT1 showed GalT activity toward GalNAc α-*p*Nph, demonstrating that we had cloned the actual honeybee *core 1 β1,3-GalT*. Subsequently, we investigated the β1,3-GalT activity toward LacdiNAcM3 (GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA). However, as shown in Fig. 2D, GalT activity toward the *N*-glycan could not be detected. This result strongly indicated that core 1 β1,3-GalT could not synthesize the Galβ1-3GalNAc unit on *N*-glycans.

Estimation and molecular cloning of honeybee β1,3-GalT involved in the synthesis of insect *N*-glycans

To date, β1,3-GalTs that can transfer Gal with β1-3-linkage on *N*-glycans have not been reported in insects. However, two types of human β1,3-GalTs, namely β1,3-GalT1 and β1,3-GalT2, can transfer Gal with β1-3-linkage to synthesize type I structures (Galβ1-3GlcNAc), probably on *N*-glycan in the brain [14, 15], although they cannot synthesize the Galβ1-3GalNAc (T-antigen). We performed a BLAST search of the honeybee and *Drosophila* database using the human β1,3-GalT1 or β1,3-GalT2 amino acid sequences as a query, and we found three putative honeybee β1,3-GalTs (Fig. 3). Among these, two types of putative β1,3-GalTs (which we designated as β1,3-GalT1 and β1,3-GalT2) had *Drosophila* orthologs, CG8668 and CG33145, respectively, which are expressed in the adult brain. Therefore, we predicted that these putative honeybee β1,3-GalTs might synthesize the Galβ1-3GalNAc (T-antigen) unit on *O*-glycan.

We cloned two types of cDNA, *β1,3-GalT1* (GenBank accession number: XM_001122156; DDBJ accession number: LC017705) and *β1,3-GalT2* (DDBJ accession number: LC017706), as novel *β1,3-GalT* gene candidates. Alignments of their putative amino acid sequences are shown in Figs. 4A and 5A. The putative honeybee β1,3-GalT1 comprised 412 amino acids and had a DxD sequence, an apparent metal binding site of glycosyltransferase (Fig. 4A). Both honeybee β1,3-GalT1 and its *Drosophila* ortholog CG8668 also had three motifs similar to human β1,3 glycosyltransferase (β3GT) motifs, (I/L)RxxWG, (F/Y)(L/V/M)xxxDxD, and (E/D)D(V/A)(Y/F)xGxC/S [16], suggesting that the putative honeybee β1,3-GalT1 transfers Gal, GlcNAc, or GalNAc with β1-3-linkage. The putative honeybee β1,3-GalT2 comprised 396 amino acids and had a DxD sequence (Fig. 5A). As

Fig. 2 Activity of honeybee (*Apis mellifera*) core 1 β1,3-GalT towards GalNAc α-*p*Nph. **A** Alignment of amino acid sequences of *Drosophila* core 1 β1,3-GalT1 (CG9520) and the honeybee core 1 β1,3-GalT, which is the honeybee ortholog of *Drosophila* core 1 β1,3-GalT1. Alignment was performed using the ClustalX program. Introduced gaps are indicated by hyphens. Asterisks indicate identical amino acids. Colons and dots indicate fully conserved amino acids defined by a score >0.5 and ≤0.5, respectively. Hydropathy analysis and prediction of transmembrane helices of the amino acid sequence were performed using the SOSui program (Mitsui Knowledge Industry). The putative transmembrane domain is underlined. The DDD sequence (bold letters) is the supposed divalent cation binding site. The double underlines indicate TWG, DDD and EDV motifs that have been reported in *Drosophila* core 1 β1,3-GalTs as conserved motifs [7]. Rectangles indicate three core 1 β1,3-GalT motifs found in human and insects core 1 β1,3-GalTs. TWG, DDD and EDV motifs were included in three core 1 β1,3-GalT motifs. *Drosophila* core 1 β1,3-GalT1: annotation symbol of FlyBase, CG9520; GenBank accession number, NM_164839. Honeybee core 1 β1,3-GalT: GenBank accession number, XM_623073. **B** Western blot analysis of purified FLAG-tagged honeybee core 1 β1,3-GalT using anti-FLAG mAb. **C** Activity of honeybee core 1 β1,3-GalT, the ortholog of *Drosophila* core 1 β1,3-GalT1, toward GalNAc α-pNph. Specific activity toward GalNAc α-*p*Nph was 82 pmol/μg/h. **D** SF-HPLC of LacdiNAcM3 (GalNAc2GlcNAc2Man3GlcNAc2-PA) treated with honeybee core β1,3-GalT1. (1) The elution profiles of authentic LacdiNAcM3 (peak a), Gal1LacdiNAcM3 (peak b), and Gal2LacdiNAcM3 (peak c). (2) Non-treated LacdiNAcM3 (substrate only). (3) Treated with the honeybee core β1,3-GalT. The PA-sugar chains were eluted by increasing the water content from 80 % acetonitrile/water to 20 % acetonitrile/water in a linear gradient



with β1,3-GalT1, both honeybee β1,3-GalT2 and its *Drosophila* ortholog CG33145 had three motifs similar to human β3GT motifs. Both honeybee proteins expressed in the *Sf21* insect cells were purified by anti-FLAG M1 affinity gel as shown in Figs. 4B and 5B.

β1,3-galactosyltransferase activity of putative β1,3-GalT1 and β1,3-GalT2

β1,3-Galactosyltransferase activity of putative β1,3-GalT1 and β1,3-GalT2 were analyzed using UDP-Gal as a donor substrate

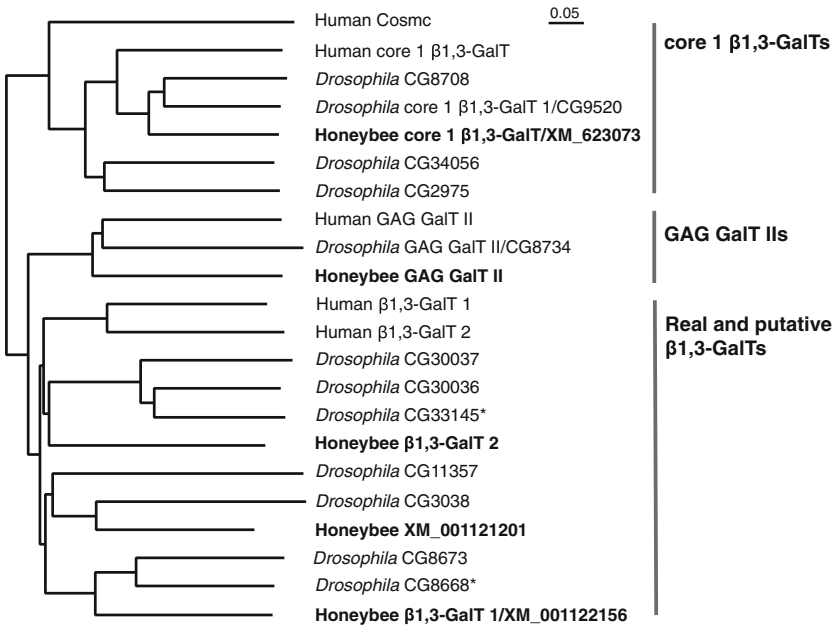


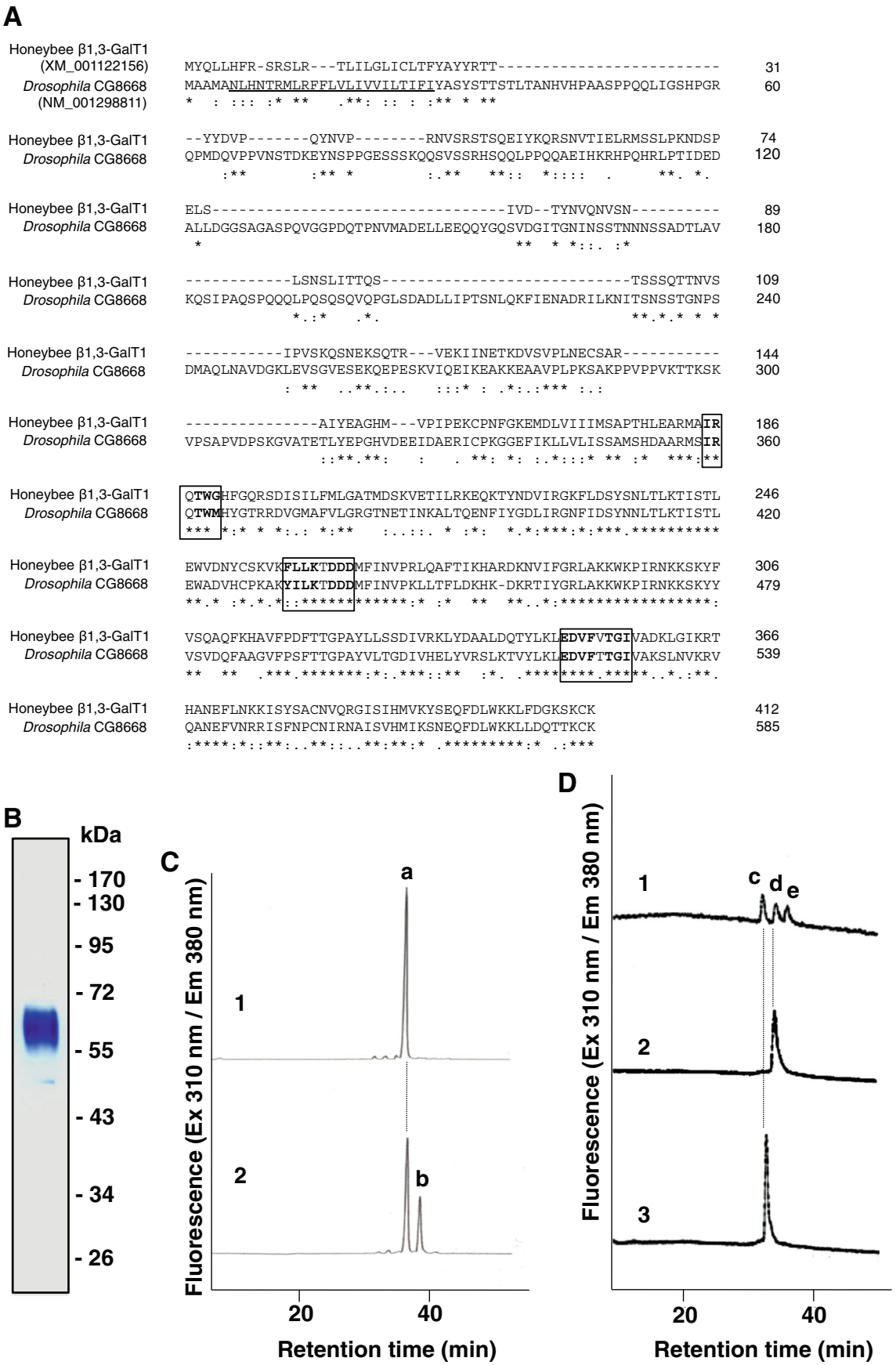
Fig. 3 Phylogenetic tree of β1,3-GalTs. The phylogenetic tree was constructed using the amino acid sequences and the ClustalX program. The branch length indicates the evolutionary distance between the members. The scale bar (0.05) represents the evolutionary distance. Insect β1,3-GalTs were clustered into three groups: (1) core 1 β1,3-GalTs, (2) GAG GalT IIs, and (3) other β1-3-GalTs. Core 1 β1,3-GalTs transfer Gal to GalNAc residues on mucin type *O*-glycans. GAG GalT IIs transfer Gal with β1-3-linkage to Galβ1-4Xyl in glycosaminoglycans. The asterisk indicates where expression of the gene occurs in the adult head

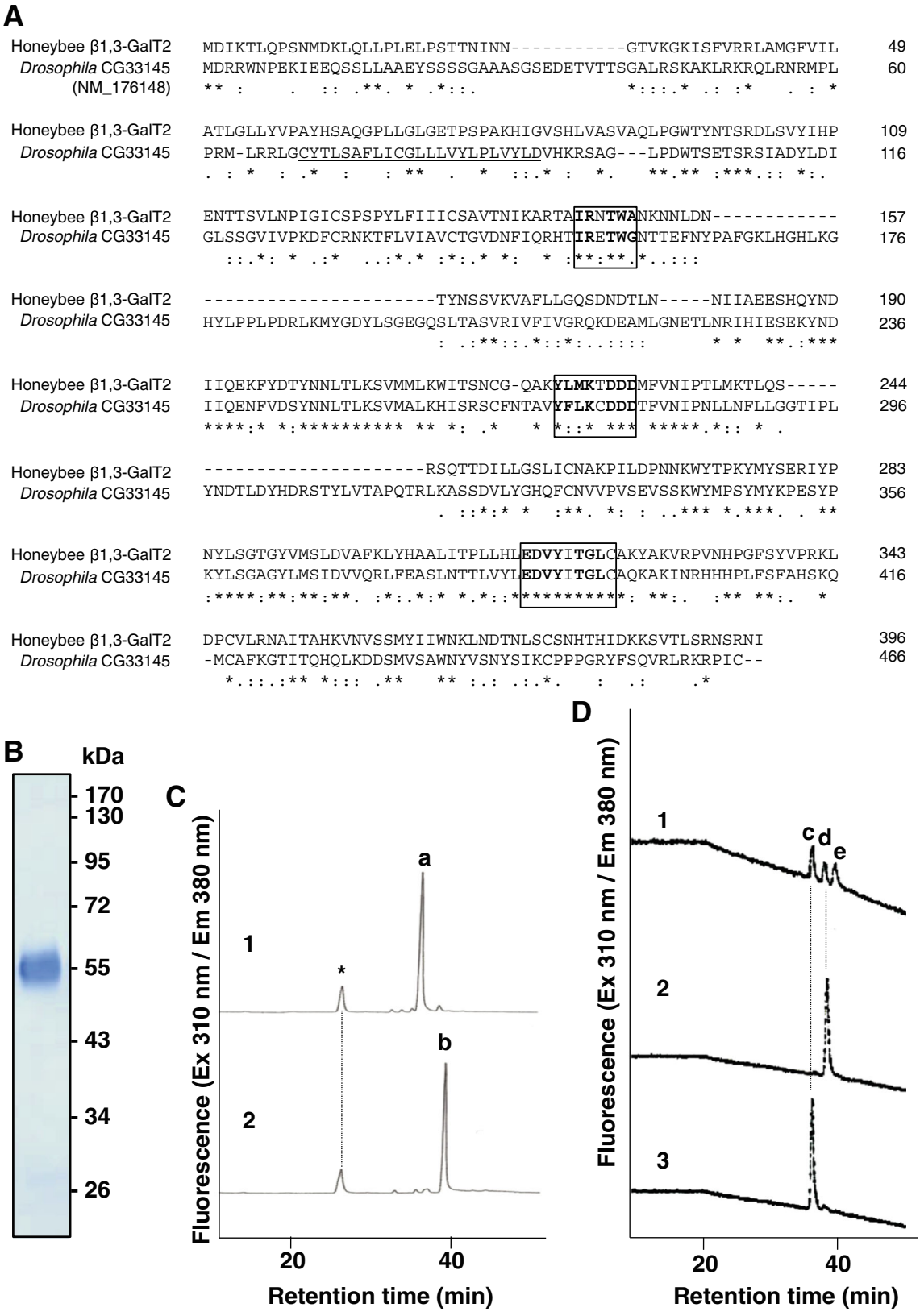
and LacdiNAcM3 (GalNAcβ1-4GlcNAcβ1-2Manα1-6(GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA) as an acceptor substrate. Affinity-purified putative β1,3-GalT1 (18.8 μg) and β1,3-GalT2 (7.96 μg) were incubated separately with 680 pmol of GalNAc2GN2M3, and 20 mM UDP-Gal in 100 mM MOPS buffer (pH 6.8) containing 2 mM ATP, 20 mM MnCl2, and 0.2 % Triton X-100 at 37 °C for 24 h. As shown in Fig. 4C, by incubation with putative β1,3-GalT1, a new product (peak b) was detected in addition to the substrate (peak a), and the elution position corresponded to that of Gal1LacdiNAcM3, suggesting that a Gal residue was transferred. To confirm the transfer of the Gal residue with β1-3-linkage, the product was pooled and treated with β1,3-galactosidase. As shown in Fig. 4D, the elution position of the β1-3 galactosidase digest of the product corresponded to that of the original substrate (LacdiNAcM3), indicating that the galactosyl residue was transferred by β1-3-linkage but not by β1-4-linkage. The product was not digested by β1-4-linkage-specific diplococcal β-galactosidase (data not shown). Therefore, we demonstrated that honeybee β1,3-GalT1 could synthesize the T-antigen unit in complex-type *N*-glycans. For putative β1,3-GalT2 activity (Fig. 5C), a new product (peak b), for which the elution position was coincident with that of Gal1LacdiNAcM3, was also detected by incubation with putative β1,3-GalT2. Although the amount of β1,3-GalT2 used for the assay was less than half that of β1,3-GalT1, the acceptor substrate (LacdiNAcM3) almost completely disappeared, suggesting that the specific activity of the β1,3-GalT2 was much higher than that of the β1,3-GalT1. In order to confirm the transfer of the galactosyl residue with β1-3-linkage, the product obtained by treatment with β1,3-GalT2 was pooled and treated with β1,3-galactosidase. As shown in Fig. 5D, the elution position for the β1,3-galactosidase digest of the product corresponded to that of the original substrate (LacdiNAcM3), indicating that the galactosyl residue was transferred by β1-3-linkage. These results showed that honeybee β1,3-GalT1 and β1,3-GalT 2 are responsible for the synthesis of the T-antigen unit in the complex-type *N*-glycans.

Discussion

To our knowledge, this is the first study to identify β1,3-GalTs that can transfer Gal residues via a β1-3-linkage mode to β1,

Fig. 4 Activity of honeybee β1,3-GalT1 toward LacdiNAcM3. **A** Alignment of amino acid sequences of honeybee β1,3-GalT1 (XM_001122156) and its *Drosophila* ortholog CG8668 (NM_001298811). Alignment was performed using the ClustalX program. Introduced gaps are indicated by hyphens. Asterisks indicate identical amino acids. Colons and dots indicate fully conserved amino acids defined by a score >0.5 and ≤0.5, respectively. Hydropathy analysis and prediction of transmembrane helices of the amino acid sequence were performed using the SOSui program (Mitsui Knowledge Industry). The putative transmembrane domain is underlined. The DDD sequence (bold letters) is the supposed divalent cation binding site. Rectangles indicate three β1,3 glycosyltransferase (β3GT) motifs. **B** Western blot analysis of purified FLAG-tagged honeybee β1,3-GalT1 using anti-FLAG mAb. **C** SF-HPLC of LacdiNAcM3 (GalNAc2GlcNAc2Man3GlcNAc2-PA) treated with honeybee β1,3-GalT1. (1) LacdiNAcM3, and (2) LacdiNAcM3 incubated with the recombinant honeybee β1,3-GalT1. **D** SF-HPLC of β1,3-galactosidase digest of peak b in C. (1) The elution profile of authentic LacdiNAcM3 (peak c), Gal1 LacdiNAcM3 (peak d), and Gal2LacdiNAcM3 (peak e). (2) Galactosylated LacdiNAcM3, galactosylated by the action of honeybee β1,3-GalT1 (peak b in C). (3) β1,3-galactosidase digest of peak b. Peak b in C was incubated with the recombinant β1,3-galactosidase (95 mU) in Na-acetate buffer (pH 5.5) at 37 °C overnight


A
Honeybee β1,3-GalT1 (XM_001122156)
Drosophila CG8668 (NM_001298811)
MYQLLHFR-SRSLR---TLILGLICLTFYAYYRTT----------
MAAMANLHNTRMLRFFLVLIVVILTIFIYASYSTTSTLTANHVHPAASPPQQLIGSHPGR
--YYDVP-------QYNVP--------RNVSRSTSQEIYKQRSNVTIELRMSSLPKNDSP
QPMDQVPPVNSTDKEYNSPPGESSSKQQSVSSRHSQQLPPQQAEIHKRHPQHRLPTIDED
ELS----------IVD--TYNVQNVSN----------
ALLDGGSAGASPQVGGPDQTPNVMADELLEEQQYGQSVDGITGNINSSTNNNSSADTLAV
LSNSLITTQS----------TSSSQTTNVS
KQSIPAQSPQQQLPQSQSQVQPGLSDADLLIPTSNLQKFIENADRILKNITSNSSTGNPS
IPVSKQSNEKSQTR---VEKIINETKDVSVPLNECSAR----------
DMAQLNAVDGKLEVSGVESEKQEPESKVIQEIKEAKKEAAVPLPKSAKPPVPPVKTTKSK
AIYEAGHM---VPIPEKCPNFGKEMDLVIIIMSAPTHLEARMAIR
VPSAPVDPSKGVATETLYEPGHVDEEIDAERICPKGGEFIKLLVLISSAMSHDAARMSIR
QTWGHFGQRSDISILFMLGATMDSKVETILRKEQKTYNDVIRGKFLDSYSNLTLKTISTL
QTWMHYGTRRDVGMAFVLGRGTNETINKALTQENFIYGDLIRGNFIDSYNNLTLKTISTL
EWVDNYCSKVKFLLKTDDDMFINVPRLQAFTIKHARDKNVIFGRLAKKWKPIRNKKSKYF
EWADVHCPKAKYILKTDDDMFINVPKLLTFLDKHK-DKRTIYGRLAKKWKPIRNKKSKYY
VSQAQFKHAVFPDFTTGPAYLLSSDIVRKLYDAALDQTYLKLEDVFVTGIVADKLGIKRT
VSVDQFAAGVFPSFTTGPAYVLTGDIVHELYVRSLKTVYLKLEDVFTTGIVAKSLNVKRV
HANEFLNKKISYSACNVQRGISIHMVKYSEQFDLWKKLFDGKSKCK
QANEFVNRRISFNPCNIRNAISVHMIKSNEQFDLWKKLLDQTTKCK
B
kDa
- 170
- 130
- 95
- 72
- 55
- 43
- 34
- 26
C
a
b
1
2
Fluorescence (Ex 310 nm / Em 380 nm)
20
40
Retention time (min)
D
c
d
e
1
2
3
Fluorescence (Ex 310 nm / Em 380 nm)
20
40
Retention time (min)



4-GalNAc residues in complex-type *N*-glycans. The amino acid sequence identity and similarity of the two β1,3-GalTs that we identified in honeybees were 33 % and 77 %, respectively (Fig. 6A). As shown in Fig. 3, insect β1,3-GalTs were

◀ **Fig. 5** Activity of honeybee (*Apis mellifera*) β1,3-GalT2 toward LacdiNAcM3. **A** Alignment of amino acid sequences of honeybee β1,3-GalT2 and its *Drosophila* ortholog CG33145 (NM_176148). Alignment was performed using the ClustalX program. Introduced gaps are indicated by hyphens. Asterisks indicate identical amino acids. Colons and dots indicate fully conserved amino acids defined by a score >0.5 and ≤0.5, respectively. Hydropathy analysis and prediction of transmembrane helices of the amino acid sequence were performed using the SOSui program (Mitsui Knowledge Industry). The putative transmembrane domain is underlined. The DDD sequence (bold letters) is the supposed divalent cation binding site. Rectangles indicate three β3GT motifs. **B** Western blot analysis of purified FLAG-tagged honeybee β1,3-GalT2 using anti-FLAG mAb. **C** SF-HPLC of LacdiNAcM3 treated with honeybee β1,3-GalT2. (1) LacdiNAcM3 and (2) LacdiNAcM3 incubated with the recombinant honeybee β1,3-GalT2. The asterisk indicates the elution positions of the pyridylaminated *N*-acetylchitopentaose (used as an internal standard). **D** SF-HPLC of β1,3-galactosidase digest of peak b in C. (1) The elution profile of authentic LacdiNAcM3 (peak c), Gal1 LacdiNAcM3 (peak d), and Gal2LacdiNAcM3 (peak e). (2) Galactosylated LacdiNAcM3, galactosylated by the action of honeybee β1,3-GalT2 (peak b in C). (3) β1,3-galactosidase digest of peak b. Peak b in C was incubated with the recombinant β1,3-galactosidase (95 mU) in Na-acetate buffer (pH 5.5) at 37 °C overnight

clustered into three sub-groups: (1) core 1 β1,3-GalTs, (2) GAG GalT IIs, and (3) other β1,3-GalTs. The newly identified β1,3-GalTs belong to the third sub-group and share the β3GT motif [16, 17] with human β1,3-glycosyltransferases (Fig. 6A and B), because the members of the third group have relatively higher homology to human β1,3- *N*-acetylglucosaminyltransferase (β1,3-GlcNAcT) 2 – 8 and human β1,3-GalT 4 and 5, as well as human β1,3-GalT 1 and 2, than core 1 β1,3-GalTs. Thus, we propose the extended β3GT motifs including insects, (I/L/V)Rx(T/S)WG/A/M, (Y/F)(L/F/I/V/M)(L/M/F) xx(D/Q)xD, and (E/D)D(V/A)(Y/F)x(T)GL/I/M/V (Fig. 6B). We also found three core 1 β1,3-GalT motifs, VKxTW(G/A), WFxKADDD, and EDxx(I/L/M)GxC in human and insects core 1 β1,3-GalTs (Fig. 2 and 6B).

For human β1,3-glycosyltransferases, core 1 β1,3-GalT and β1,3-GalT4 transfer the Gal residue to GalNAc residue but not to GlcNAc by β1-3-linkage [16, 17]. However, the former enzyme is involved in the biosynthesis of *O*-glycans and the latter enzyme is involved in gangliosides (GD1β/GM1/GA1), and a mammalian β1,3-GalT that has transferase activity towards *N*-glycans has yet to be found. Compared with the human β3GT motif or core 1 β1,3-GalT motif of domain 3, the putative honeybee β3GT motif has one inserted amino acid residue (Thr) at the sixth position. For insect β3GT motifs (*Drosophila* and honeybees, Fig. 6), the hydroxyl amino acid (Thr) occurs commonly in the third domain, suggesting that the insertion of the Thr residue could be critical for β1,3-GalT activity toward *N*-glycans. In order to test this working hypothesis in further research, it will be necessary to construct a mutated honeybee β1,3-GalT, in which the Thr residue in the third domain is deleted. It will also be necessary to check whether the human β1,3-GalT4 carries transferase activity toward *N*-glycans as well as gangliosides.

It is noteworthy that prolonged incubation of the acceptor substrate with recombinant β1,3-GalT1/2 and UDP-Gal did not produce the di-galactosylated product, namely Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-6(Galβ1-3GalNAcβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-PA (Gal2LacdiNAcM3). This suggests that another β1,3-GalT might be necessary for transfer of the second galactosyl residue, or that the first transferred galactosyl residue causes steric hindrance to glycosyltransferase activity related to the free form oligosaccharide substrate. To clarify the mechanism by which the second β1,3 Gal residue is transferred, other putative β1,3-GalT genes should be expressed and their activity analyzed using mono-galactosylated substrates.

In previous studies [1–3], we identified a unique insect complex-type *N*-glycan containing the T-antigen unit (Galβ1-3GalNAc) in a royal jelly glycoprotein (Asn 28 of MRJP1). Furthermore, we observed β1,3-GalT activity in a microsomal fraction prepared from a honeybee cephalic portion, in which various royal jelly proteins are biosynthesized. One royal jelly glycoprotein, MRJP1, is expressed in a subset of Kenyon cells in the mushroom bodies of the honeybee brain [9]. Additionally, various *N*-glycans linked to proteins expressed on the cell surface are known to play critical roles in cell-cell communication and tissue construction; hence, the unique honeybee brain-specific *N*-glycans might be responsible for construction of brain tissues. Therefore, in further research, controlling the expression of the unique insect complex-type *N*-glycans in the honeybee brain may provide insights into the development of brain tissue. To suppress the biosynthesis of the insect specific *N*-glycans in honeybee brain, information of glycosyltransferase genes responsible for insect *N*-glycan-processing has been required. In this study, we succeeded in identifying two β1,3-GalT genes involved in the processing of insect complex-type *N*-glycans, but the physiological differences between these two β1,3-GalTs, including their expression sites or the timing of expression in the cephalic region of the honeybee, are currently unknown. Therefore, expression analysis and identification of expression sites for the two β1,3-GalT genes is the next step toward elucidating the physiological function of the insect complex-type *N*-glycans that contain the T-antigen unit.

Since similar complex type *N*-glycans containing the T-antigen unit was found on hemocyanins of gastropods, *Helix pomatia* [18] and *Lymnaea stagnalis* [19], β1,3-GalTs responsible for the synthesis of the T-antigen unit might commonly occur and function in the processing pathway of invertebrate *N*-glycoproteins. Although the physiological function of these non-mammal *N*-glycans

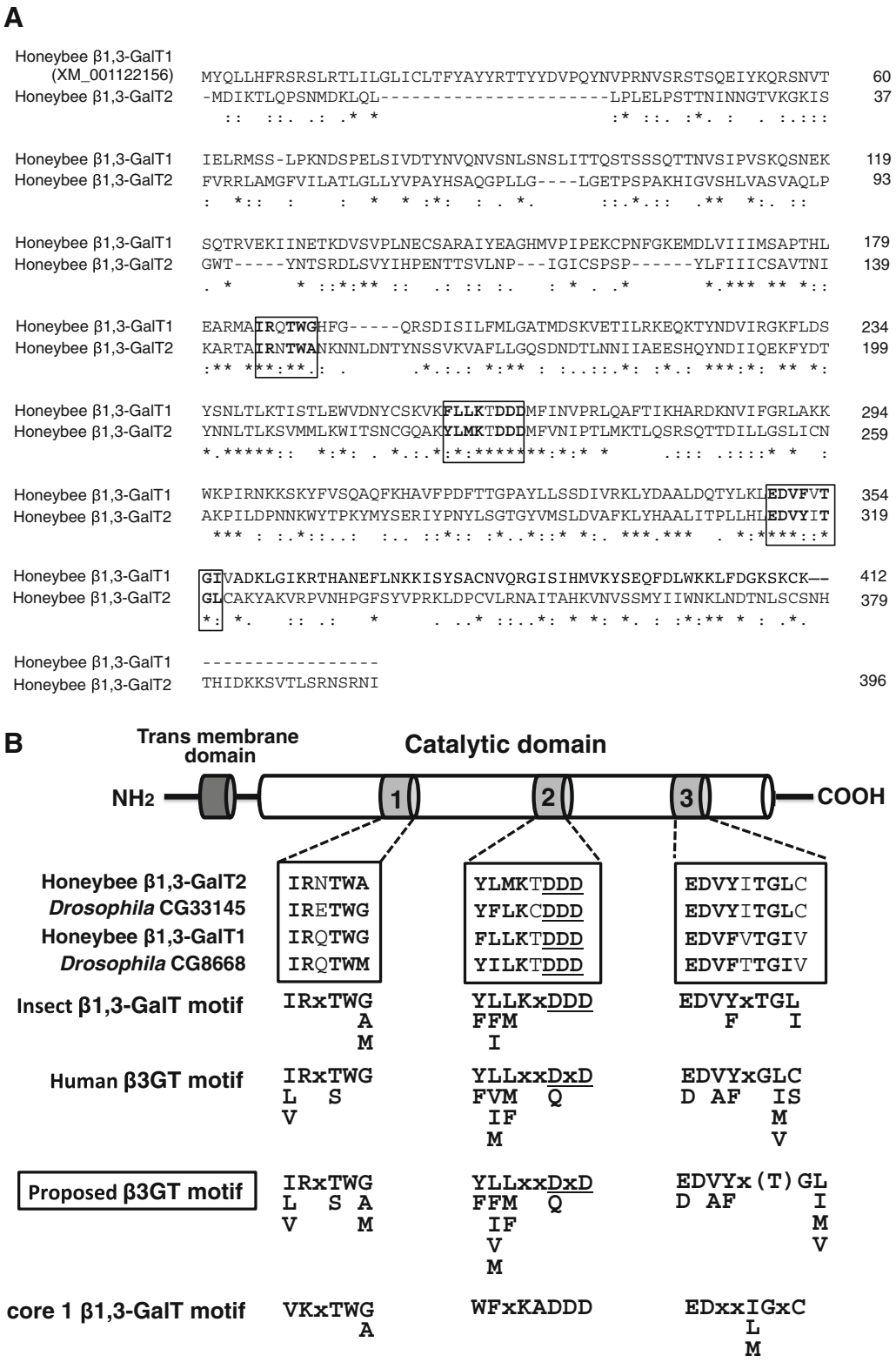


Fig. 6 Three proposed β3GT motifs in human and insect β1,3 glycosyltransferases. **A** Alignment of amino acid sequences of honeybee β1,3-GalT1 (XM_001122156) and honeybee β1,3-GalT2. Alignment was performed using the ClustalX program. Introduced gaps are indicated by hyphens. Asterisks indicate identical amino acids. Colons and dots indicate fully conserved amino acids defined by a score >0.5 and ≤0.5, respectively. The DDD sequence (bold letters) is the supposed divalent cation binding site. Rectangles indicate three β3GT motifs. **B** Honeybee (*Apis mellifera*) β1,3-GalT1 and β1,3-GalT2 share the β3GT motifs with human β1,3-glycosyltransferases. Where letters are underlined, this indicates the binding sites of divalent cations. Humanβ3GT motifs were amended to include human β1,3-GlcNAcT8

remains obscure, these unique structural feature may give an immunological activity to stimulate the vertebrate immune system.

References

1. Kimura, Y., Ushijima, T., Maeda, M., Hama, Y., Kimura, M., Okihara, K., Sugimoto, H., Yamada, H.: Tumor antigen occurs in *N*-glycan of royal jelly glycoproteins honeybee cells synthesize T-antigen unit in *N*-glycan moiety. Biosci. Biotechnol. Biochem. **70**, 2583–2587 (2006)
2. Kimura, Y., Sakamura, S., Ushijima, T., Hama, Y., Kajiura, H., Fujiyama, K., Okihara, K., Sugimoto, H., Yamada, H.: Evidence for new beta1-3 galactosyltransferase activity involved in biosynthesis of unusual *N*-glycan harboring T-antigen in *Apis mellifera*. Biosci. Biotechnol. Biochem. **71**, 1111–1114 (2007)
3. Kimura, Y., Nagai, H., Miyamoto, M., Kimura, M., Yonekura, M.: Identification of a royal jelly glycoprotein that carries unique complex-type *N*-glycans harboring the T-antigen (Galβ1-3GalNAc) unit. Biosci. Biotechnol. Biochem. **74**, 2148–2150 (2010)
4. Ju, T., Brewer, K., D'Souza, A., Cummings, R.D., Canfield, W.M.: Cloning and expression of human core β1,3-galactosyltransferase. J. Biol. Chem. **277**, 178–186 (2002)
5. Kudo, T., Iwai, T., Kubota, T., Takayama, Y., Hiruma, T., Inaba, N., Zhang, Y., Gotoh, M., Togayachi, A., Narimatsu, H.: Molecular cloning and characterization of a novel UDP-Gal : GalNacα peptide β1,3-galactosyltransferase (C1Gal-T2), an enzyme synthesizing a core 1 structure of *O*-glycan. J. Biol. Chem. **277**, 47724–47731 (2002)
6. Ju, T., Zheng, Q., Cummings, R.D.: Identification of core 1 *O*-glycan T-synthase from *Caenorhabditis elegans*. Glycobiology **16**, 947–958 (2006)
7. Müller, R., Hülsmeier, A.J., Altman, F., Hagen, K.T., Tiemeyer, M., Hennet, T.: Characterization of mucin-type core-1 β1-3 galactosyltransferase homologous enzyme *Drosophila melanogaster*. FEBS J. **272**, 4295–4305 (2005)
8. Yoshida, H., Fuwa, T.J., Arima, M., Hamamoto, H., Sasaki, N., Ichimiya, T., Osawa, K., Ueda, R., Nishihara, S.: Identification of the *Drosophila* core 1 β1,3-galactosyltransferase gene that synthesizes T antigen in the embryonic central nervous system and hemocytes. Glycobiology **18**, 1094–1104 (2008)
9. Kucharski, R., Malwszka, R., Hayward, D.C., Ball, E.E.: A royal jelly protein is expressed in the subset of Kenyon cells in the mushroom bodies of the honey bee brain. Naturwissenschaften **85**, 343–346 (1998)
10. Schmitzová, J., Klaudiny, J., Albert, S., Schröder, W., Hanes, J., Júdová, J., Simúth, J.: A family of major royal jelly proteins of the honeybee *Apis mellifera L*. Cell. Mol. Life Sci. **54**, 1020–1030 (1998)
11. Takemae, H., Ueda, R., Okubo, R., Nakato, H., Izumi, S., Saigo, K., Nishihara, S.: Proteoglycan UDP-galactose:β-xylose β1,4-galactosyltransferase I is essential for viability in *Drosophila melanogaster*. J. Biol. Chem. **278**, 15571–15578 (2003)
12. Ueyama, M., Takemae, H., Ohmae, Y., Yoshida, H., Toyoda, H., Ueda, R., Nishihara, S.: Functional analysis of proteoglycan galactosyltransferase II RNA interference mutant flies. J. Biol. Chem. **283**, 6076–6084 (2008)
13. Wong-Madden, S.T., Landry, D.: Purification and characterization of novel glycosidases from the bacterial genus *Xanthomonas*. Glycobiology **5**, 19–28 (1995)
14. Kolbinger, F., Streiff, M.B., Katopodis, A.G.: Cloning of a human UDP-galactose:2-acetamido-2-deoxy-D-glucose 3β-galactosyltransferase catalyzing the formation of type 1 chains. J. Biol. Chem. **273**, 433–440 (1998)
15. Amado, M., Almeida, R., Carneiro, F., Levery, S.B., Holmes, E.H., Nomoto, M., Hollingsworth, M.A., Hassan, H., Schwientek, T., Nielsen, P.A., Bennett, E.P., Clausen, H.: A family of human β3-galactosyltransferases. Characterization of four members of a UDP-galactose:β-N-acetyl-glucosamine/β-N-acetyl-galactosamine β-1, 3-galactosyltransferase family. J. Biol. Chem. **273**, 12770–12778 (1998)
16. Togayachi, A., Kozono, Y., Kuno, A., Ohkura, T., Sato, T., Hirabayashi, J., Ikehara, Y., Narimatsu, H.: β3GnT2 (B3GNT2), a major polylactosamine synthase: analysis of B3GNT2-deficient mice. Methods Enzymol. **479**, 185–204 (2010)
17. Togayachi, A., Sato, T., Iwai, T., Narimatsu, H.: Cloning and characterization of β1,3-glycosyltrasferase family with a β3GT motifs. Trends Glycosci. Glycotechnol. **19**, 29–40 (2007)
18. Van Kuik, J.A., Sijbesma, R.P., Kamerling, J.P., Vliegenthart, J.F.G.: Primary structures determination of seven novel *N*-linked carbohydrate chains derived from hemocyanin of *Lymnaea stangnails*. Eur. J. Biochem. **169**, 399–411 (1987)
19. Lommerse, J.P.M., Thomas-Oates, J.E., Gielens, C., Préaux, G., Kamerling, J.P., Vliegenthart, J.F.G.: Primary structure of 21 novel monoantennary and diantennary N-linked carbohydrate chains from alpha-D-hemocyanin of *Helix pomatia*. Eur. J. Biochem. **249**, 195–222 (1997)