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Biochemical characterization and mutational analysis of silkworm *Bombyx mori* β-1,4-*N*-acetylgalactosaminyltransferase and insight into the substrate specificity of β-1,4-galactosyltransferase family enzymes

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ABSTRACT

Silkworm *Bombyx mori* is one of the insect hosts for recombinant protein production at academic and industrial levels. *B. mori* and other insect cells can produce mammalian proteins with proper posttranslational modifications, such as *N*-glycosylation, but the structures of *N*-glycans in *B. mori* are mainly high mannose- and paucimannose-type, while mammals also produce hybrid- and complex-type glycans. Recently, complex-type *N*-glycans whose structures are different from mammalian ones have been identified in some insect cell *N*-glycomes at very low levels compared with levels of high mannose- and paucimannose-type glycans. However, their functions and the enzymes involved in the biosynthesis of insect complex-type *N*-glycans are not clear, and complex-type *N*-glycans, except for *N*-acetylglucosamine-terminated glycans, are still not identified in the *B. mori N*-glycome. Here, we focused on the β-1,4-galactosyltransferase family (also known as glycosyltransferase family 7, GT7) that contains mammalian β-1,4-galactosyltransferase and insect β-1,4-*N*-acetylgalactosaminyltransferase. A gene for a GT7 protein (BmGalNAcT) from *B. mori* was cloned, expressed in a soluble form using a silkworm expression system, and the gene product showed strict β-1,4-*N*-acetylgalactosaminyltransferase activity but not β-1,4-galactosyltransferase activity. A mutation in Ile298 or Ile310, which are predicted to be located in the active site, reduced its glycosyltransferase activity, suggesting that these residues and the corresponding residues are responsible for substrate specificity of GT7. These results suggested that BmGalNAcT may be involved in the complex-type *N*-glycans, and moreover, bioinformatics analysis revealed that *B. mori* might have an extra gene for a GT7 enzyme with different specificity in addition to the known insect GT7 glycosyltransferases.

1. Introduction

Glycoconjugates play important roles in numerous biological processes, including development, differentiation, cellular interaction, and viral infection (Varki, 2017). In eukaryotes, protein glycosylation is one of the most common co- and posttranslational modifications of proteins and is involved in protein folding, function, stability, and trafficking (Apweiler et al., 1999; Helenius and Aebi 2001; Moremen and Molinari 2006). Glycosylation of proteins is divided into several types based on linkages between carbohydrates and amino acid residues, e.g., *N*-glycosylation and *O*-glycosylation. *N*-glycosylation is a relatively well-studied protein modification that occurs in the endoplasmic reticulum (ER), where a precursor of tetradecasaccharide $Glc_3Man_9GlcNAc_2$ (Glc, glucose; Man, mannose; GlcNAc, *N*-acetylglucosamine) is transferred to an asparagine residue in the Asn-X-Ser/Thr motif (where X is any amino acid residue except for Pro) of a nascent polypeptide. *N*-glycans are further processed by glycoside hydrolases and glycosyltransferases that are located in the ER and Golgi apparatus to produce many types of *N*-glycans, such as high mannose-type, hybrid-type, and complex-type glycans. The sequential monosaccharide trimming from the glycan

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Abbreviations: β2GnTII, β-1,2-*N*-acetylglucosaminyltransferase II; β4GalNAcT, β-1,4-*N*-acetylgalactosaminyltransferase; β4GalT, β-1,4-galactosyltransferase; BmNPV, *Bombyx mori* nucleopolyhedrovirus; ER, endoplasmic reticulum; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GT7, glycosyltransferase family 7; Man, mannose; PA, pyridylamino; PNGase F, peptide:*N*-glycanase F; pNP, *p*-nitrophenyl; UDP, uridine-5′-diphosphate

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precursor by α-glucosidase I, α-glucosidase II and α-mannosidase in the ER produces oligomannosidic *N*-glycans, which are extremely conserved among eukaryotes (Miyazaki et al., 2011; Moremen et al., 1994). In contrast, in the later steps of glycan processing, several Golgi-resident enzymes modify the structure of *N*-glycans, which diverge significantly among different phyla and kingdoms (Wilson, 2002).

Insects and their cultured cells produce mainly high mannose-type and paucimannose-type *N*-glycans that contain 2–9 mannose residues and are partially core-fucosylated with α-1,3 and α-1,6 linkages (Kajiura et al., 2015; Koles et al., 2007; Kurz et al., 2015; Liu et al., 2019; Stanton et al., 2017; Scheys et al., 2019). Insect cells are widely used to produce recombinant proteins because of the high productivity and capability of proper posttranslational modifications, including *N*-glycosylation, but structural differences in *N*-glycans between insect and mammalian cells influence the biological activity of recombinant glycoproteins, especially when they are applied to biopharmaceuticals. For instance, core fucosylation and degalactosylation of immunoglobulin G1 *N*-glycans negatively affect antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, respectively (Hodoniczky et al., 2005; Sakae et al., 2017; Shinkawa et al., 2003). To address this problem, several research groups have established approaches for the conversion of the *N*-glycan structure of recombinant glycoproteins expressed in insect cultured cells into mammalian-like *N*-glycan structures (Aumiller et al., 2003, 2012; Breitbach and Jarvis, 2001; Geisler and Jarvis, 2012a; Hollister et al., 1998, Hollister and Jarvis, 2001; Hollister et al., 2002; Jarvis et al., 2001; Kim et al., 2011; Mabashi-Asazuma et al., 2013, 2014; Palmberger et al., 2012; Palomares et al., 2003; Suganuma et al., 2018; Tomiya et al., 2003; Toth et al., 2014).

Recent studies have revealed that insects produce a variety of complex-type *N*-glycans at much lower levels compared to high mannose- and paucimannose-type *N*-glycans (reviewed in Paschinger and Wilson, 2019). Not only neutral sugars (Gal, GlcNAc, and GalNAc) but also anionic sugars (glucuronic acid) and anionic and zwitterionic modifications (sulfate, phosphorylcholine, and phosphoethanolamine) have been observed in the *N*-glycomes of dipterans (*Drosophila melanogaster* [Aoki and Tiemeyer, 2010], *Anopheles gambiae* and *Aedes aegypti* [Kurz et al., 2015]), lepidopterans (*Trichoplusia ni* and *Lymantria dispar* [Stanton et al., 2017]), and royal jelly glycoproteins (Hykollari et al., 2018). Though causing controversy, terminally sialylated *N*-glycans were found only in *D. melanogaster* embryos (Aoki et al., 2007; Koles et al., 2007). Moreover, in the past two decades, glycosyltransferases, which were suggested to be involved in complex-type glycan biosynthesis, including β-1,2-*N*-acetylglucosaminyltransferase II (β2GnTII) (Geisler and Jarvis, 2012b; Miyazaki et al., 2019), β-1,4-*N*-acetylgalactosaminyltransferase (β4GalNAcT) (Haines and Irvine, 2005; Vadaie and Jarvis, 2004) and sialyltransferase (Kajiura et al., 2015; Koles et al., 2004), have been identified in insects. Some glycosyltransferases have been reported to affect the development of the pest beetle *Tribolium castaneum* (Walski et al., 2016); however, the details of the physiological function of insect complex-type glycans are not clear.

Domestic silkworm *Bombyx mori* is used for the production of recombinant proteins *via* baculovirus vector and transgenic techniques (Kato et al., 2010; Maeda et al., 1985). We have also expressed several glycoproteins using the *B. mori* nucleopolyhedrovirus (BmNPV) bacmid (Ishikiriyama et al., 2009; Miyazaki et al., 2018; Motohashi et al., 2005; Ogata et al., 2009; Sasaki et al., 2009), which is a derived from BmNPV genomic DNA, and converted *N*-glycans of immunoglobulin G1 expressed in silkworm pupae into the mammalian-like structure by transfecting human β2GnTII and β-1,4-galactosyltransferase (β4GalT) genes related to the complex-type *N*-glycan biosynthesis (Kato et al., 2017). In a previous study, recombinant human β-1,3-glucosaminyltransferase 2 expressed in silkworm larvae had an unusual *N*-glycan with bisecting GlcNAc and terminal Gal without introducing mammalian GTs (Dojima et al., 2009). However, there are fewer studies on glycan-related enzymes in *B. mori* than in *D. melanogaster* and other lepidopterans, and glycosyltransferases that catalyze the formation of the unusual glycan structure are not identified.

Here, we focused on the β4GalT family, also known as glycosyltransferase family 7 (GT7), in the CAZy database (http://www.cazy.org/) (Lombard et al., 2014). The GT7 family contains mammalian β4GalT that elongates the complex-type *N*-glycan by adding a β-1,4-Gal residue whereas its orthologs from the insects *D. melanogaster* and *T. ni* prefer GalNAc to Gal (Haines and Irvine, 2005; Vadaie and Jarvis, 2004). In this study, we cloned and enzymatically characterized the *B. mori* β4GalT ortholog and identified it as β4GalNAcT. Mutational analysis of the enzyme revealed that two amino acid residues are important for the recognition of GalNAc in donor substrate, and moreover, the distribution of β4GalT and β4GalNAcT in the GT7 family was discussed based on bioinformatics analysis.

2. Materials and Methods

2.1. Chemicals

p-Nitrophenyl β-D-glucopyranoside (Glcβ-pNP), *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (GlcNAcβ-pNP), *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide (GalNAcβ-pNP, *p*-nitrophenyl α-D-mannopyranoside (Manα-pNP), uridine-5′-diphosphogalactose (UDP-Gal), and uridine-5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc) were purchased from Merck (Darmstadt, Germany). GlcNAcβ1–3GalNAcα-pNP and GalNAcβ1–4GlcNAcβ-pNP were obtained from Tokyo Chemical Industry (Tokyo, Japan). Galβ1–4GlcNAcβ-pNP was synthesized previously (Usui et al., 1993). The fluorescent pyridylaminated (PA) glycans used were purchased from Masuda Chemical Industries (Takamatsu, Japan). All other reagents were of analytical grade and purchased from Wako Pure Chemical Industry (Osaka, Japan) or Merck unless otherwise stated.

2.2. Transcriptional analysis

First-to fifth-instar larvae, pupae, and adults (Ehime Sanshu, Ehime, Japan) were flash-frozen in liquid nitrogen and ground to powder. Total RNA was extracted from the resulting powder using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The first-strand cDNA was synthesized with a PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol. The expression of the *BmGalNAcT* gene was analyzed using quantitative PCR (qPCR) using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka Japan) and the gene-specific primer set BmGalNAcT_F and BmGalNAcT_R (Table 1). The program for thermal cycling was performed using the Mx3000P system (Agilent Technologies, Santa Clara, CA, USA); the cycling conditions were 15 s denaturation at 95 °C and 60 s annealing/extension at 60 °C. The gene for glyceraldehyde-3-phosphate dehydrogenase (*BmGAPDH*) was also amplified as a reference using the BmGAPDH_F and BmGAPDH_R primers (Table 1). The entire experiment was performed in three independent biological replicates. Quantification of the relative transcript abundance was achieved using the ΔΔCq method. The primers for qPCR were designed using the Primer-BLAST server (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012).

2.3. Construction of the BmNPV bacmid and mutagenesis

DNA fragments encoding full-length BmGalNAcT were amplified by PCR using the synthesized cDNA from a fifth-instar larva as a template and a pair of primer sets, BmGalNAcT_EcoRI_F and BmGalNAcT_XbaI_R (Table 1), followed by subcloning into the pFastBac1 vector (Thermo Fisher Scientific). The transmembrane region of BmGalNAcT was predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001). DNA fragments encoding the N-terminally FLAG (DYKDDDDK)-tagged BmGalNAcT (Asp38–Ser420) without

Table 1
Primers used in this study.

Primer	Sequence (5′ to 3′)
BmGAPDH_F	GCTGGAATTTCTTTGAATGAC
BmGAPDH_R	CAATGACTCTGCTGGAATAACC
BmGalNAcT_F	GGCCGTATTCCTGAACCACA
BmGalNAcT_R	CCGACGTTCATCAGTTTGGC
BmGalNAcT_EcoRI_F	TTTTGAATTCATGGGCAGCGGCGCGAGCGG
FLAG-BmGalNAcTΔ37_F	GATTACAAGGATGACGACGATAAGGACGCCTCGCCGCTCAAAACC
BmGalNAcT_XbaI_ R	GCTCTAGATCAGCTGCGCTCGTCGATGTTC
EcoRI-Bx-FLAG_F	GCGAATTCATGAAGATACTCCTTGCTATTGCATTAATGTTGTCAACAGTAATGTGGGTGTCAACAGATTACAAGGATGACGACGATAAG
BmGalNAcT-I298M_F	GCTTCCATGGACAAACTTAATTTCAAA
BmGalNAcT-I298M_R	TTTGTCCATGGAAGCGGACATGTGCCT
BmGalNAcT-I310Y_F	GAAGATTACTTCGGTGGCGTGTCGGCT
BmGalNAcT-I310Y_R	ACCGAAGTAATCTTCATATGGTAATTT

The restriction enzyme sites are underlined.

putative cytosolic and transmembrane regions were amplified by PCR using the synthesized cDNA as a template and a pair of primers, FLAG-BmGalNAcTΔ37_F and BmGalNAcT_XbaI_R (Table 1). A sequence encoding bombyxin secretion signal peptide (MKILLAIALMLSTVMWVST) was added by PCR using the resultant DNA fragment as a template and a pair of primers, EcoRI-Bx-FLAG_F and BmGalNAcT_XbaI_R (Table 1), and then the products were ligated into the pFastBac1 vector using EcoRI and XbaI restriction sites. The constructs were verified by DNA sequencing. *Escherichia coli* BmDH10Bac-*CP*$^-$-*Chi*$^-$ competent cells, which contain cysteine protease- and chitinase-deficient BmNPV bacmid (Park et al., 2008), were transformed with the resultant plasmid and cultured on LB agar plate medium containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 40 μg/mL isopropyl β-D-1-thiogalactopyranoside, and 100 μg/mL 5-bromo-4-chloro-3-indolyl-4-galactopyranoside at 37 °C for 2 days. The recombinant BmNPV bacmid containing the *BmGalNAcT* gene was extracted from a white positive colony after blue-white selection.

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with the desired primers (Table 1) and the pFastBac1 vector harboring the gene coding the soluble form of BmGalNAcT as a template according to the manufacturer's protocol. The recombinant BmNPV bacmids for BmGalNAcT mutants were constructed using the resultant plasmids in the same manner as the wild-type enzyme.

2.4. Expression and purification of recombinant BmGalNAcT

To express the recombinant BmGalNAcT (rBmGalNAcT), chitosan/rBmGalNAcT bacmid complexes were prepared as described previously (Kato et al., 2016) and then injected into fifth-instar silkworm larvae. The bacmid-injected larvae were reared on an artificial diet (Silkmate S2, Nohsan Corp., Yokohama, Japan) at 26 °C for 6 days. Hemolymph was collected by cutting a caudal leg of larvae in a tube and then mixed with 1 mM 1-phenyl-2-thiourea. The fat body was harvested by cutting and dissection. The hemolymph and fat body samples were stored at −80 °C until further analysis. The hemolymph was mixed with 9 vol of 50 mM Tris-HCl buffer (pH 7.4) containing 300 mM NaCl and 16% polyethylene glycol 4000 and incubated at 4 °C overnight. The insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant was applied onto DDDDK-tagged Protein Purification Gel (Medical and Biological Laboratories, Nagoya, Japan). The column was washed with 50 mM Tris-HCl buffer (pH 7.4) containing 300 mM NaCl and 0.1% Triton X-100, and then the FLAG-tagged protein was eluted with 100 μg/mL FLAG-tag peptide (Medical and Biological Laboratories). The fraction containing rBmGalNAcT was buffer-exchanged into 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH buffer (pH 6.0) using an Amicon Ultra centrifugal device (Merck). Protein expression and purity were confirmed by SDS-PAGE with Coomassie brilliant blue (CBB) staining and western blotting using an anti-DDDDK-tag monoclonal antibody (Medical and Biological Laboratories) as the primary antibody and an anti-mouse IgG antibody labeled with horseradish peroxidase (Medical and Biological Laboratories) as the secondary antibody. The protein concentration was determined by the bicinchoninic acid method using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and bovine serum albumin as a standard.

2.5. Enzyme assay

To measure the glycosyltransferase activity, a reaction mixture containing 50 μg/mL rBmGalNAcT, 1 mM acceptor substrate (0.1 μM in the case of PA-glycans), 1 mM donor substrate, 10 mM $MnCl_2$, 1 mg/mL bovine serum albumin, and 50 mM HEPES-NaOH (pH 6.0) was prepared and incubated at 37 °C. To examine the effect of pH on glycosyltransferase activity, the same reaction mixtures, with the exception that 50 mM sodium acetate (pH 4.0–5.0) and HEPES-NaOH (pH 5.0–8.0) buffers were used, were incubated at 37 °C for 1 min. The effect of temperature was examined at 20–50 °C using 50 mM HEPES-NaOH buffer (pH 6.0). The metal dependency was investigated at 37 °C in 50 mM HEPES-NaOH buffer (pH 6.0) with 10 mM $MgCl_2$, $CaCl_2$, or $CoCl_2$ instead of $MnCl_2$. After incubation, the reaction mixtures were boiled for 5 min to quench the enzymatic reaction.

The amounts of products were measured by reverse phase high-performance liquid chromatography (RP-HPLC) using an HPLC system equipped with LC-10AD VP pumps (Shimadzu, Kyoto, Japan). The reaction mixtures containing pNP sugars were applied to a Mightysil RP-18 GP column (4.6 mm × 250 mm, Kanto Chemical Co., Tokyo, Japan) and eluted using 10% (vol/vol) methanol, followed by detection using an SPD-10AVvp UV-VIS detector at a wavelength of 300 nm. The products of PA-glycans were applied to a TSKgel ODS-80T$_M$ column (4.6 mm × 250 mm, Tosoh, Tokyo, Japan), eluted with 100 mM ammonium acetate buffer (pH 4.0), and monitored using an RF-10A XL fluorescence detector (Shimadzu) at 320 nm excitation and 400 nm emission. The flow rate was 1.0 mL/min, and the column temperature was 40 °C in both cases.

2.6. Glycan digestion

The recombinant protein was denatured and deglycosylated with peptide:*N*-glycanase F (PNGase F, Takara Bio) according to the manufacturer's protocol. For the activity measurement, rBmGalNAcT was treated with PNGase F in native conditions.

2.7. Bioinformatics, phylogenetics, and homology modeling

The DELTA-BLAST server (https://blast.ncbi.nlm.nih.gov/) (Boratyn et al., 2012) was used to search for homologous proteins, and the dbCAN server (http://bcb.unl.edu/dbCAN2/) was used to annotate uncharacterized proteins based on the CAZy classification (Zhang et al., 2018). Sequence alignment was performed using the MUSCLE algorithm (Edgar, 2004), and phylogenetic analysis was carried out with the maximum likelihood method using MEGA X software (Kumar et al., 2018). A phylogenetic tree was described using the iTOL v4 server (Letunic and Bork 2018). The homology model of BmGalNAcT was produced using the SWISS-MODEL server (https://swissmodel.expasy.org) (Biasini et al., 2014) using the amino acid sequence of BmGalNAcT and the coordinates of human β4GalT1 (PDB: 2AGD) as a template. Figures were prepared using PyMOL (http://www.pymol.org/).

3. Results and discussion

3.1. Identification and expression of the BmGalNAcT gene

The DELTA-BLAST search using the amino acid sequence of human β4GalT1 (GenBank: NP_001488.2) found eleven putative protein sequences from *B. mori* with more than 20% sequence identity (Supplementary Fig. S1). These can be isoforms of seven putative glycosyltransferases, which have been annotated as β-1,4-*N*-acetylgalactosaminyltransferase bre-4-like (XP_021205072.1, XP_021205073.1, XP_021205074.1), β-1,4-galactosyltransferase 7 (XP_004933019.1, XP_004933020.1), β-1,4-*N*-acetylgalactosaminyltransferase bre-4 (XP_021203993.1), β-1,4-galactosyltransferase 1-like (XP_004926306.3), uncharacterized protein LOC101738741 (XP_004926305.1), glycosyltransferase (XP_012545893.1, XP_012545894.1, NP_001243988.1), and chondroitin sulfate synthase 1 (XP_012545235.1). The dbCAN annotation server indicated that all the proteins are classified into the glycosyltransferase family 7 (GT7), which is based on the CAZy classification (Lombard et al., 2014), as well as mammalian β4GalT1, which is involved in the complex-type *N*-glycan biosynthesis and lactose biosynthesis (Ramakrishnan and Qasba, 2001).

GT7 enzymes have been reported to transfer Gal and/or GalNAc residues from UDP-Gal and/or UDP-GalNAc donors to other oligosaccharides with a β-1,4 linkage. Among *B. mori* GT7 proteins, putative glycosyltransferase (XP_012545894.1), named BmGalNAcT, has the highest sequence identity (36.5%) with human β4GalT1 and phylogenetically belongs to the same clade, including insect β4GalNAcTs. The expression profile of *BmGalNAcT* was investigated by quantitative reverse transcription PCR using *BmGalNAcT*-specific primers and cDNA derived from *B. mori* larvae, pupae, and adults. The *BmGalNAcT* gene was ubiquitously expressed in all the stages tested, and the expression level particularly increased in the pupal and adult stages (Fig. 1). Walski et al. reported that the highest expression level of beetle *T. castaneum* β4GalNAcTA, which is orthologous to BmGalNAcT, was observed in the pupal stage (Walski et al., 2016). Recently, differences between males and females of the planthopper *Nilaparvata lugens N*-glycome were reported (Scheys et al., 2019). The amount of high mannose-type *N*-glycans was increased in female insects compared to that in male insects, while the complex-type *N*-glycan profiles were similar. Moreover, the expression levels of several *N*-glycan-related genes were different between male and female adults of *N. lugens*. However, no remarkable difference was observed in the expression levels of *BmGalNAcT* in male and female adults of *B. mori* (Fig. 1).

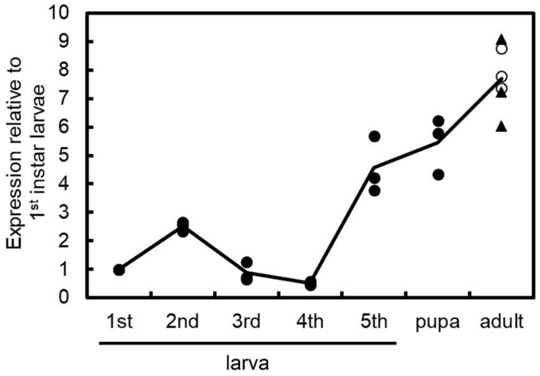

Fig. 1. Expression of the *BmGalNAcT* gene in silkworm developmental stages. Transcript levels were analyzed by qPCR using the gene-specific primers (Table 1) in larval (first-to fifth-instar), pupal, and adult stages (n = 3). Female (*open circle*) and male (*filled triangle*) adults were analyzed independently. Lines connect the average values. *BmGAPDH* was used as a reference gene to normalize the expression level.

The DNA encoding the full-length BmGalNAcT was cloned and then sequenced (DDBJ/EMBL/GenBank accession number is LC481643). BmGalNAcT comprises 420 amino acids, and its theoretical molecular weight is 47,722; it was predicted to be a transmembrane protein with a hydrophobic region at the N-terminus (Ala15–Leu37) by the TMHMM server, like the mammalian β4GalT1 and insect β4GalNAcT enzymes, which have been reported to be localized in the Golgi apparatus (Haines and Irvine, 2005; Teasdale et al., 1992; Vadaie and Jarvis, 2004).

3.2. Expression and purification of rBmGalNAcT

To investigate the enzymatic characteristics of BmGalNAcT, the recombinant enzyme (rBmGalNAcT), where the predicted cytosolic and transmembrane regions (Met1–Leu37) were deleted and a bombyxin signal peptide and a FLAG-tag were added at the N-terminus, was constructed and expressed under the control of the polyhedrin promoter (Fig. 2A and B) using the silkworm-BmNPV bacmid expression system. rBmGalNAcT was successfully expressed in silkworm larvae and secreted to hemolymph, and SDS-PAGE analysis showed that the molecular weight of rBmGalNAcT is 60 kDa, which is higher than its theoretical mass of 45 kDa (Fig. 2C). The polyethylene glycol precipitation and FLAG-tag affinity chromatography resulted in a single band for rBmGalNAcT observed in the SDS-PAGE analysis (Fig. 2D) and a yield of approximately 5.5 μg per larva.

The NetNGlyc server (http://www.cbs.dtu.dk/services/NetNGlyc/) analysis showed that BmGalNAcT has 10 potential *N*-glycosylation sites (Asn52, Asn70, Asn85, Asn89, Asn90, Asn101, Asn108, Asn113, Asn168, and Asn194), whereas human β4GalT1, *T. ni* and *D. melanogaster* orthologs have 1, 6 and 5 sequons, respectively. Deglycosylation of purified rBmGalNAcT by PNGase F resulted in a decrease in the molecular weight to approximately 49 kDa, which is close to the theoretical mass of rBmGalNAcT (Fig. 2D). Western blotting using an anti-FLAG tag antibody supported the fact that rBmGalNAcT was purified to homogeneity from silkworm hemolymph and was *N*-glycosylated (Fig. 2E).

3.3. Substrate specificity and enzymatic properties of rBmGalNAcT

First, the purified rBmGalNAcT was incubated with UDP-Glc, UDP-Gal, and UDP-GalNAc as donor substrates and pNP-GlcNAc as an acceptor substrate to examine its donor substrate specificity. The RP-HPLC analysis showed that a new peak appeared at 38 min of elution time when using UDP-GalNAc as a donor substrate (Fig. 3), but no product was observed in the reaction mixtures incubated with UDP-Glc and UDP-Gal for 12 h (data not shown). The elution time of the product was identical to that of authentic GalNAcβ1–4GlcNAcβ-pNP, indicating that rBmGalNAcT catalyzed the transfer of a GalNAc residue from UDP-GalNAc to GlcNAcβ-pNP with β1–4 linkage. The *T. ni* and *D. melanogaster* orthologs can utilize UDP-Gal as a donor, but its efficiency was lower than that of UDP-GalNAc (Haines and Irvine, 2005; Vadaie and Jarvis, 2004). Therefore, BmGalNAcT had strict donor substrate specificity compared with the other insect orthologs.

Then, to investigate its acceptor specificity, various *p*-nitrophenyl monosaccharides and disaccharides were used for the glycosyltransferase reaction with UDP-GalNAc as a donor. rBmGalNAcT also exhibited activity toward Glcβ-pNP and GlcNAcβ1–3GalNAcα-pNP

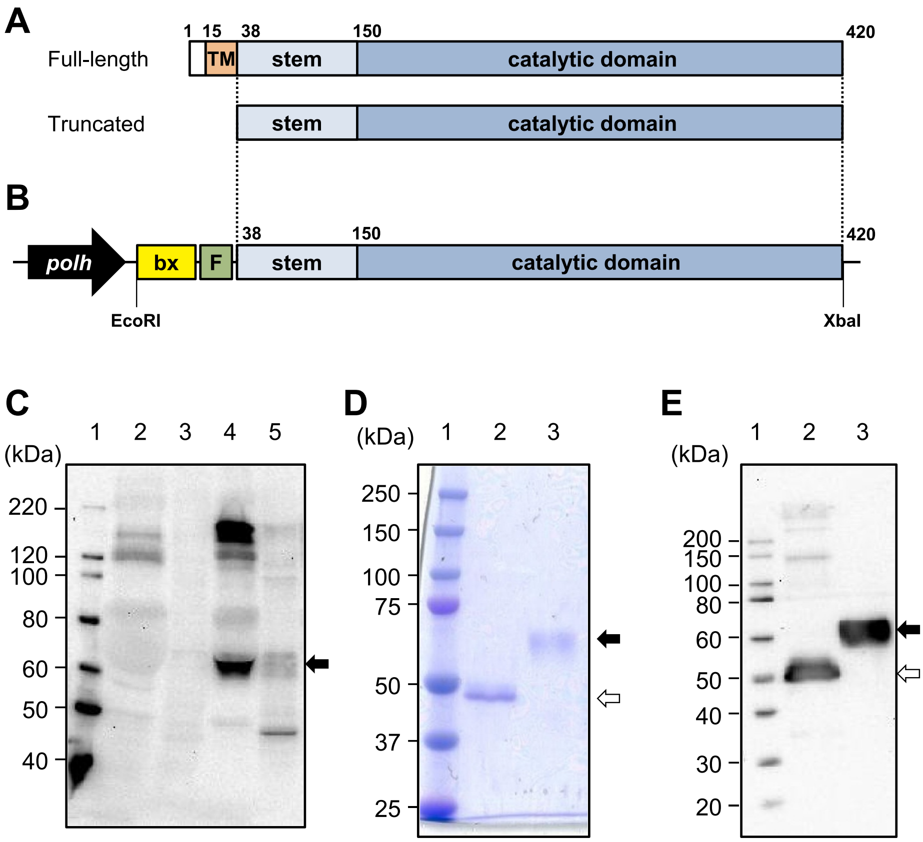

Fig. 2. Expression and purification of rBmGalNAcT. (A) Deletion of the N-terminal sequence (residues 1–37) containing the predicted cytosolic and transmembrane (TM) regions. **(B)** Construction of the expression vector for rBmGalNAcT. Abbreviations: *polh*, polyhedron promoter; bx, bombyxin secretion signal; F, FLAG-tag. EcoRI and XbaI were used for subcloning into the pFastBac1 vector. **(C)** Western blotting analysis of the expression of rBmGalNAcT in silkworm larvae using an anti-FLAG-tag antibody. Lane 1, molecular weight marker; lanes 2 and 3, hemolymph and fat body from mock silkworm, respectively; lanes 4 and 5, hemolymph and fat body from bacmid-injected silkworm, respectively. **(D and E)** SDS-PAGE analysis of purified rBmGalNAcT with CBB staining (D) and western blotting (E). Lane 1, molecular weight markers; lane 2, PNGase F-treated rBmGalNAcT; lane 3, intact rBmGalNAcT. The bands for the intact and deglycosylated enzymes are indicated with black and white arrows, respectively.

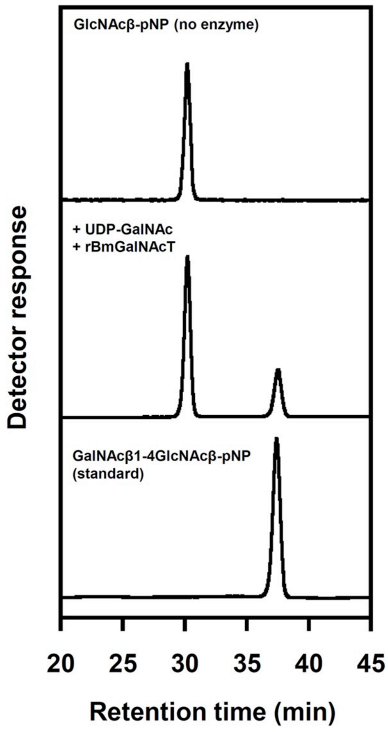

Fig. 3. HPLC analysis of the reaction products generated by rBmGalNAcT. Purified rBmGalNAcT was incubated with UDP-GalNAc and GlcNAcβ-pNP in the presence of Mn^{2+} at pH 6.0 for 5 min, and then the reaction mixtures were analyzed by reverse-phase HPLC using a Mightysil RP-18 GP column with the conditions described in the Materials and Methods section. *Upper*, the reaction mixture without rBmGalNAcT; *middle*, the reaction mixture incubated with rBmGalNAcT; *lower*, the authentic standard of GalNAcβ1-4GlcNAcβ-pNP.

with 0.26% and 32% efficiency compared with that toward GlcNAcβ-pNP (0.99 μmol/min/mg protein) (Table 2). No activity was detected with Galβ-pNP, Manα-pNP, Galβ1–4GlcNAcβ-pNP, or GalNAcβ1–4GlcNAcβ-pNP (data not shown). These results suggested that rBmGalNAcT recognized the stereochemistry of glucose but not galactose and mannose and preferred GlcNAc to glucose as an acceptor substrate. The activity toward *N*-glycans was also measured using the UDP-GalNAc donor, and all the GlcNAc-terminated glycans tested could be acceptor substrates (Table 2). Among these glycan substrates, the glycan with one GlcNAc residue on the α1–6 mannosyl arm was the best acceptor. Although the reason was not clear, it was possible that the conformation of the glycans may influence the substrate recognition of rBmGalNAcT.

The effect of pH on rBmGalNAcT activity was examined using UDP-GalNAc and GlcNAcβ-pNP as substrates over a pH range of 4.0–8.0. The enzyme exhibited the highest activity at pH 6.0 (Fig. 4A). The effect of temperature was tested at 20–50 °C, and the optimum temperature was determined to be 37 °C (Fig. 4B). Because GT7 enzymes usually require divalent metal ions as cofactors to bind the sugar nucleotide substrate (Ramakrishnan et al., 2001; Ramakrishnan and Qasba, 2010; Ramasamy et al., 2005), the activity was investigated in the presence of several divalent metal chlorides. rBmGalNAcT had the highest activity under the condition with Mn^{2+}, while the enzyme exhibited lower than 4% activity in the presence of Mg^{2+}, Ca^{2+}, and Co^{2+} ions (Fig. 4C). rBmGalNAcT has ten *N*-glycosylation sites, and its carbohydrate content relative to the molecular weight was approximately 18% (Fig. 2D and E). The effect of deglycosylation on enzymatic activity was also assayed. No effect was observed in the catalytic activity after deglycosylation by PNGase F (Fig. 4D). Although it was not clear which asparagine residue was glycosylated, eight of ten potential glycosylation sites were located in the stem region (38–150 residues, Fig. 2A). Thus, the *N*-glycans may not directly contribute to the enzymatic activity but were probably involved in the protection of the stem region against proteases.

Table 2
Enzymatic activity of rBmGalNAcT toward various sugars.

Acceptor substrates	Activity (nmol/min/mg)	Relative activity (%)[a]
pNP-glycosides (1 mM)		
Glcβ-pNP	25.6 ± 4.0	0.26
Galβ-pNP	N.D.[b]	–
GlcNAcβ-pNP	$9.92 \pm 0.40 \times 10^{3}$	100
GalNAcβ-pNP	N.D.	–
Manα-pNP	N.D.	–
GlcNAcβ1–3GalNAcα-pNP	$3.18 \pm 0.65 \times 10^{3}$	32
GalNAcβ1–4GlcNAcβ-pNP	N.D.	–
Galβ1–4GlcNAcβ-pNP	N.D.	–
PA-glycans (0.1 μM)		
GlcNAcβ1–2Manα1–6 Manβ1–4GlcNAc$_2$-PA GlcNAcβ1–2Manα1–3	$1.60 \pm 0.06 \times 10^{-1}$	61
GlcNAcβ1–2Manα1–6 Manβ1–4GlcNAc$_2$-PA Manα1–3	$2.60 \pm 0.10 \times 10^{-1}$	100
Manα1–6 Manβ1–4GlcNAc$_2$-PA GlcNAcβ1–2Manα1–3	$1.30 \pm 0.04 \times 10^{-1}$	50

[a] The activity toward GlcNAcβ-pNP and Manα1–3(GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAc$_2$-PA (pH 6.0 and 37°C) are taken to be 100% of that toward pNP-glycosides and PA-glycans, respectively.

[b] Not detected.

3.4. Mutagenesis of the residues in the active site

All the structure-determined GT7 enzymes catalyze the transfer of Gal rather than GalNAc. To identify the residues that are important for the substrate specificity of BmGalNAcT, the homology model of BmGalNAcT was generated and compared with the other GT7 glycosyltransferases. In the structure of bovine β4GalT complexed with UDP-Gal, Leu255, Met277, Gly292, and Tyr289 are located near the 2-hydroxy group of the galactose residue, whereas the corresponding residues in BmGalNAcT are Leu276, Ile298, Gly313, and Ile310, respectively (Fig. 5A and B). Multiple sequence alignment reveals that Ile298 and Ile310 in BmGalNAcT are highly conserved among the previously reported β4GalNAcTs, while the corresponding residues in β4GalTs are Met and Tyr, respectively. Therefore, three mutants of rBmGalNAcT, namely, I298M, I310Y, and I298M-I310Y, were constructed, and their activity was measured using UDP-GalNAc and pNP-GlcNAc as substrates. I298M exhibited 18.4 ± 1.1% activity compared with the wild-type enzyme, and no activity was detected in the reaction mixture with either I310Y or I298M-I310Y (Fig. 5C). These results suggest that both Ile298 and Ile310 of BmGalNAcT play important roles in the recognition of the GalNAc residue of the donor substrate and that the Ile310→Tyr mutation might reduce the affinity with the GalNAc residue more than the Leu298→Met mutation. Specificity conversion of bovine β4GalT1 into β4GalNAcT by the replacement of Tyr289 by Ile had been reported previously, and this mutation resulted in the acquisition of β4GalNAcT activity instead of β4GalT activity (Ramakrishnan and Qasba, 2002). Thus, our study supported the hypothesis that residues corresponding to Ile298 and Ile310 in BmGalNAcT are determinants of donor substrate specificity in GT7 β4GalNAcTs.

3.5. Distribution of GalT and GalNAcT in insect GT7

Several research groups have reported that lepidopteran and dipteran cells produce insect-specific complex-type *N*-glycans, including those harboring not only the β-1,4-GalNAc but also the β-1,4-Gal moiety at the nonreducing ends (Aoki et al., 2007; Koles et al., 2007; Kurz et al., 2015; Stanton et al., 2017). No galactosylated or GalNAcylated *N*-glycan was detected in the *N*-glycome of *B. mori* fifth-instar larvae (Kajiura et al., 2015); however, there has been only one report that recombinant immunoglobulin expressed in *B. mori* larvae possessed β-1,4-Gal-terminated *N*-glycan (Dojima et al., 2009). It is still unclear whether another β4GalNAcT catalyzes β-1,4-galactosylation on insect *N*-glycans as well as β-1,4-*N*-acetylgalactosaminylation *in vivo* and/or whether they possess β4GalT that transfers galactose residues. We analyzed insect GT7 protein sequences and compared them with characterized GT7 glycosyltransferases. GT7 could be divided into roughly six subgroups: vertebrate β4GalT, invertebrate β4GalNAcT, mammalian β4GalNAcT, snail β-1,4-*N*-acetylglucosaminyltransferase (LsGlcNAcT), xylosylprotein β-4-galactosyltransferase, and chondroitin sulfate-related β4GalNAcT (Fig. 6). The three-dimensional structures and sequence alignment indicate that conservation of the amino acid residues

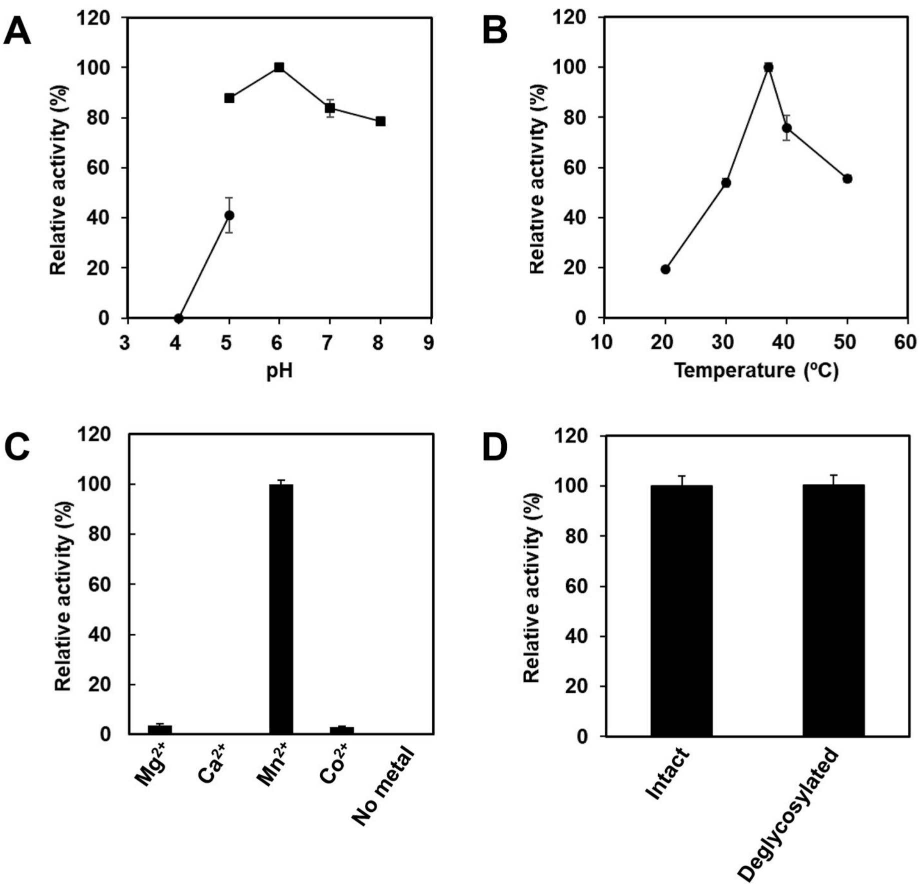

Fig. 4. General properties of rBmGalNAcT. (A) pH dependence, (B) temperature dependence, (C) metal ion dependence and (D) effect of deglycosylation. The pH dependence was measured at 37 °C using 50 mM sodium acetate (pH 4.0–5.0) or HEPES-NaOH (pH 5.0–8.0) buffer. The temperature dependence was measured at 20–50 °C using 50 mM HEPES-NaOH (pH 6.0). The effect of divalent metal ions was investigated at 37 °C using 50 mM HEPES-NaOH buffer (pH 6.0) containing 10 mM of each metal chloride. (D) Activity of the enzyme deglycosylated with PNGase F at 37 °C for 1 h. The activity of rBmGalNAcT without preincubation was taken to be 100%.

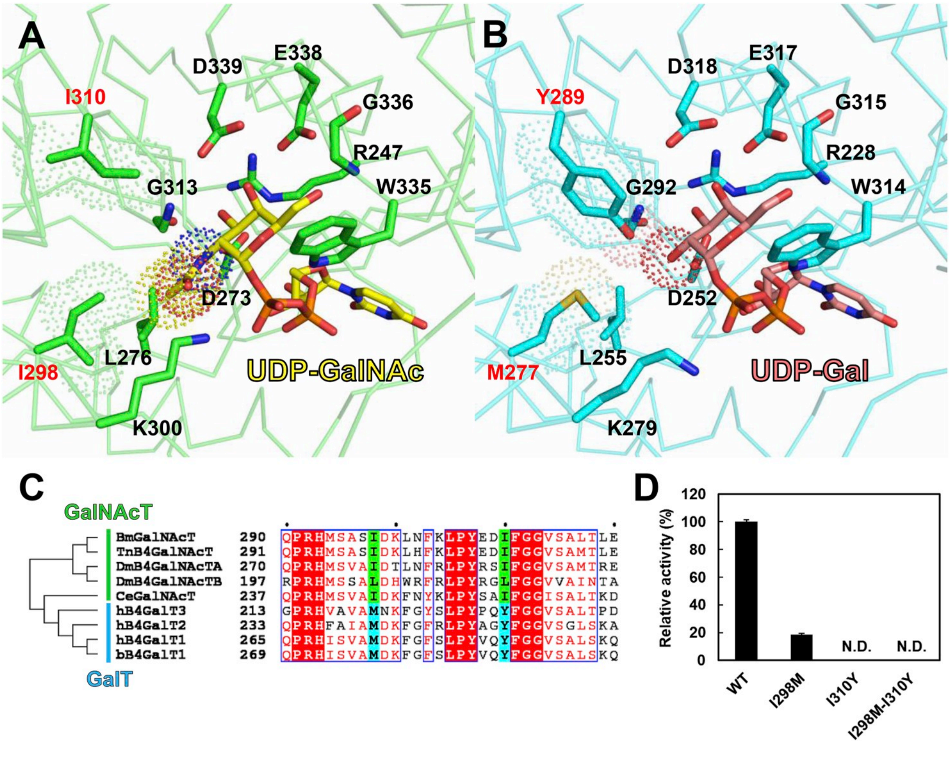

Fig. 5. Amino acid residues involved in donor substrate specificity. (A and B) Active site residues involved in the recognition of the donor substrates UDP-GalNAc (*yellow*) and UDP-Gal (*pink*) in the homology model of BmGalNAcT (*green*) (A) and bovine β4GalT1 (PDB: 1YRO, *cyan*) (B). The stick model of UDP-GalNAc was superimposed from bovine β4GalT1 complexed with UDP-GalNAc (PDB: 1OQM). van der Waals radii of the acetamide group of GalNAc, 2-hydroxy group of Gal, and residues interacting with these groups are shown in dotted spheres. The residues mutated in this study and the corresponding residues in bovine β4GalT1 are labeled in *red*. **(C)** Sequence alignment of GT7 β-1,4-galactosyltransferases (human β4GalT1, β4GalT2, β4GalT3 and bovine β4GalT1) and β4GalNAcT from *B. mori*, *T. ni*, *D. melanogaster*, and *C. elegans*. The numbers of the first amino acid residues of each sequence are labeled. The residues that interacted with the 2-acetamide or 2-hydroxy groups of the donor substrate in β4GalNAcT and β4GalT are highlighted in *green* and *cyan*, respectively. The figure was generated using ESPript 3.0 (Robert and Gouet, 2014). **(D)** Relative glycosyltransferase activity of rBmGalNAcT mutants toward the UDP-GalNAc donor and GlcNAc-pNP acceptor under the conditions described in the Materials and Methods. N.D. means 'not detected'. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

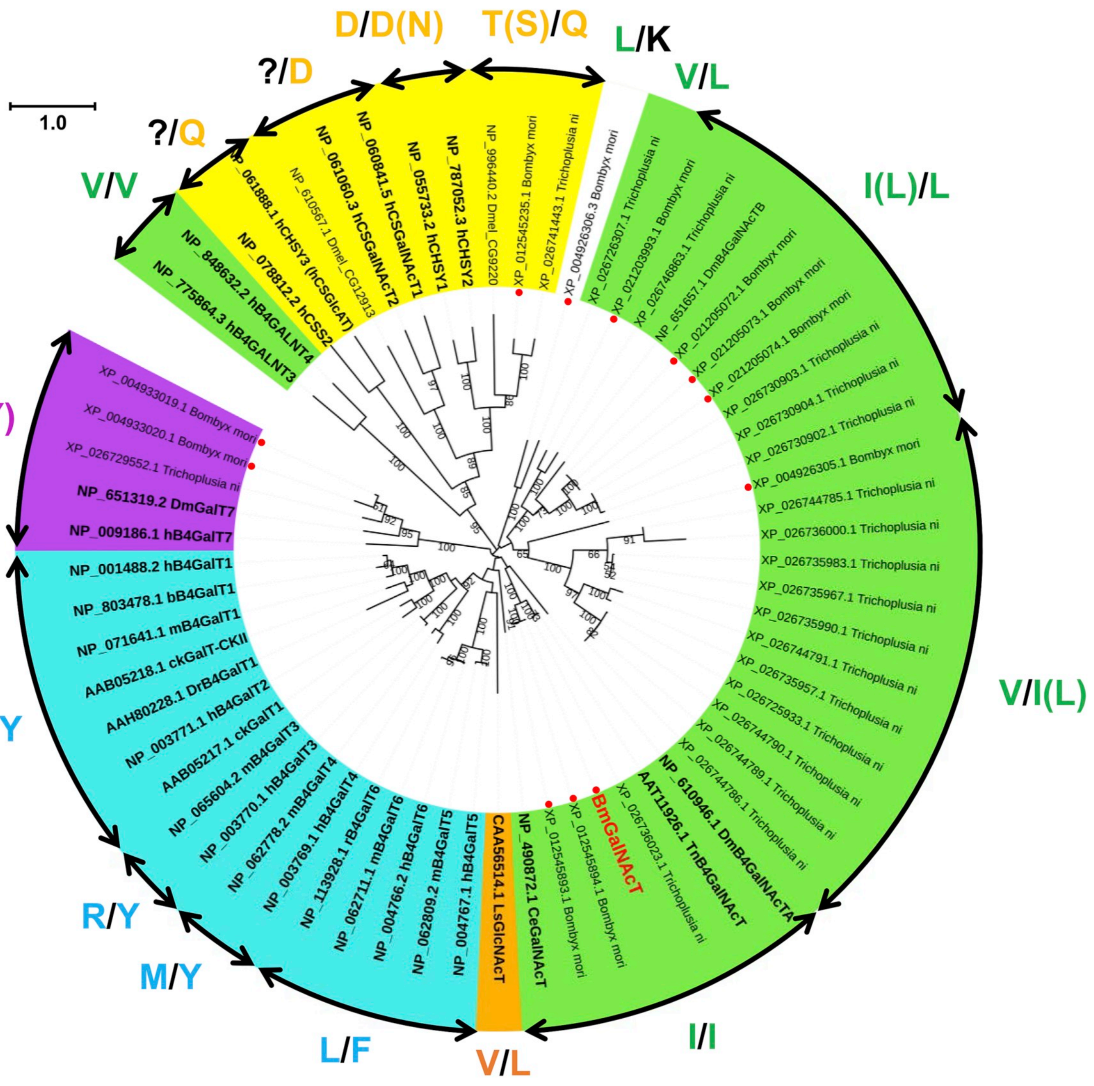

Fig. 6. Phylogenetic tree and distribution of substrate specificity in the GT7 family. The amino acid sequences were aligned using the MUSCLE program (Edgar, 2004), and the phylogenetic tree was constructed by the maximum likelihood method and visualized using iTOL v4 (Letunic et al., 2018). The amino acid sequences used were the characterized GT7 enzymes (*bold*) listed in the CAZy database and uncharacterized proteins of *B. mori*, *T. ni*, and *D. melanogaster* that are homologous to the GT7 enzymes. The multiple sequence alignment is shown in Supplementary Fig. S1. The amino acid motifs involved in the donor substrate specificity, that is, the residues corresponding to Ile298 and Ile310 in BmGalNAcT, are shown around the tree. The specificity based on the motif is distinguished with the following colors: *green* (β4GalNAcT), *cyan* (β4GalT), *purple* (xylosylprotein 4-β-galactosyltransferase), *orange* (β4GlcNAcT), and *yellow* (β4GalNAcT related to chondroitin sulfate biosynthesis). Proteins from *B. mori* are indicated with *red* circles. The scale bar indicates the number of inferred amino acid substitutions per site; the bootstrap values are displayed at branch points. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

corresponding to Ile298 and Ile310 in BmGalNAcT enabled us to predict the substrate specificity of uncharacterized GT7 enzymes (Supplementary Fig. S1). *B. mori* possesses seven genes for GT7 proteins as described above, four of which are suggested to prefer GalNAc as a donor substrate (BmGalNAcT, XP_021205072.1, XP_021203993.1, and XP_004926305.1). BmGalNAcT has higher homology with Dmβ4GalNAcTA than with Dmβ4GalNAcTB, which was reportedly involved in LacdiNAc (GalNAcβ-1,4-GlcNAc) structure formation in glycosphingolipids (Stolz et al., 2008). Two other proteins (XP_004933019.1 and XP_012545235.1) are predicted to be xylosylprotein β-4-galactosyltransferase GalT7 and chondroitin sulfate synthase, both of which are involved in the formation of glycosaminoglycan (Ramakrishnan and Qasba, 2010; Uyama et al., 2002). The other one (XP_004926306.3) belongs to the clades of β4GalNAcT, but the corresponding residue of Ile310 is substituted by a bulky lysine residue, suggesting that the protein might have different substrate specificity from BmGalNAcT. Further studies including analysis of *in vivo* expression profiles and enzymatic characterization of these gene products are necessary to identify glycosyltransferase genes related to β-1,4-galactosylation in *B. mori N*-glycans.

In conclusion, we cloned BmGalNAcT, a member of the GT7 family, for the first time, and the enzyme exhibited a strict preference of a donor substrate UDP-GalNAc compared with the preference of other insect β4GalNAcT enzymes. The mutational analysis identified two amino acid residues located in the active site as one of the determinants of GT7 substrate specificity, and *B. mori* has possible GT7 glycosyltransferases with different specificities and functions. This study would help us to identify novel enzymes related to the glycoconjugates of insects and to reveal the structure-function relationships of GT7 glycosyltransferases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103254.

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