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α-1,6-fucosyltransferase plays a critical role during embryogenesis of the hemimetabolous insect *Nilaparvata lugens*

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Abstract

Protein glycosylation is one of the most important post-translational modifications, modulating the properties of proteins. In insects, α-1,6-fucosyltransferase (FucT6) is an important enzyme in the glycosylation pathway, modifying the core structure of N-glycans on glycoproteins with the addition of a fucose residue. In our previous study, RNAi-mediated silencing of *FucT6* in the third-instar nymphs of *Nilaparvata lugens* caused a failure of the ecdysis process during nymphal development, leading to high mortality. These results suggested the requirement of FucT6 during nymphal development in *N. lugens*. In this study, RNAi-mediated gene silencing of *FucT6* in adults did not cause lethality. However, parental RNAi of *FucT6* led to full failure in the hatching of eggs, and this effect was maternally mediated. Interestingly, gene expression levels of *FucT6* in the eggs peaked at the katatrepsis event, where the embryo rotates 180° resulting in the head pointing towards the anterior side of the egg. Proteome analysis showed significant differences in the abundance of proteins between different embryonal developmental stages, suggesting the crucial role of *FucT6* mediated core N-fucosylation in embryonal development. Therefore, correct α-1,6-fucosylation of glycoproteins is important for

N. lugens during embryonic development and this study provides new insights into the role of N-glycosylation in embryogenesis in insects.

Keywords: *Nilaparvata lugens*; fucosyltransferase; embryonic development; RNAi; fucosylated proteome

Abbreviation[1]

[1]BPH, brown planthopper; RNAi, RNA interference; Fuc, fucose; FucT, fucosyltransferase; Asn or N, asparagine; Ser, serine; Thr, threonine; Pro, proline; dsRNA, double-stranded RNA; GFP, green fluorescent protein; AEL, after egg laying; RIPA, radio-immunoprecipitation assay; LCA, *Lens culinaris* agglutinin; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; MS, mass spectrometry; AP-MS, affinity purification mass spectrometry; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LFQ, label-free quantification.

1. Introduction

Protein glycosylation is known to be essential in a plentitude of biological processes, including protein trafficking, cell adhesion, cellular localization, etc. (Moremen et al., 2012; Ohtsubo and Marth, 2006). As one of the most common post-translational modifications of proteins, the attached glycan structures are important for the quality control, correct folding and functioning of the proteins they decorate (Suzuki et al., 2022). Disorders of protein glycosylation have been reported to be associated with a variety of human diseases, including inflammation, tumors and cancers (Li et al., 2022; Loaeza-Reyes et al., 2021; Pinho and Reis, 2015; Reily et al., 2019). Recently, glycosylation was found not only to be important for human immunoglobulin G in the defense against the coronavirus 2019 (COVID-19) caused by SARS-CoV-2 virus, but also important for invasion of the virus into the host by glycosylated spike (S) protein of SARS-CoV-2 virus (Larsen et al., 2021; Watanabe et al., 2020).

In N-linked glycosylation, an oligosaccharide precursor is attached to the Asn residue of polypeptides in an Asn-X-Ser/Thr (where X is any amino acid but Pro) consensus sequence. Subsequently, these oligosaccharides are further processed by glycosyltransferases, which add sugar moieties, and glycosidases, which remove sugar moieties, to generate different glycan structures (Helenius and Aebi, 2001; Rini et al., 2022; Schjoldager et al., 2020). One of these enzymes, α-1,6-fucosyltransferase (FucT6), is involved in the modification of N-glycans by adding fucose residues in an α-1,6-linkage to the core N-glycan structure. While RNAi-mediated gene silencing of *FucT6* has mild effects in *Drosophila melanogaster*, *Tribolium castaneum* and *Leptinotarsa decemlineata* (Liu et al., 2022; Walski et al., 2016; Yamamoto-Hino et al., 2015), it resulted in almost complete mortality in the brown planthopper (BPH), *Nilaparvata lugens*, due to failure of the ecdysis event between two nymphal stages (Yang et al., 2022). This discrepancy between the observed phenotypes suggests that the role of FucT6 in the development of insects differs between the holometabolous flies and beetles, which undergo a complete metamorphosis in the development from larva to adult, and the hemimetabolous *N. lugens,* which gradually undergoes metamorphosis when developing to an adult through distinct nymphal stages. However, the molecular mechanisms by which FucT6 influences *N. lugens* development are still unknown.

N. lugens is a monophagous pest on rice and outbreaks of BPH cause enormous losses in rice yield (Heong and Hard, 2009). To minimize yield losses caused by BPH, an increasing number of studies on the genome, transcriptome and proteome were carried out in order to better understand the molecular composition of this destructive pest (Bao et al., 2013; Jing et

al., 2017; Xue et al., 2010; Xue et al., 2014; Zhai et al., 2013). Of interest, the N-glycome of *N. lugens* showed that fucosylated glycans account for 36% of the total glycan pool in the nymphal stages and significant differences in glycan types were observed between female and male planthoppers, suggesting fucosylation differs in different developmental stages and sexes of *N. lugens* (Scheys et al., 2019).

Extraembryonal membranes play an important role during the embryonic development of animals. While the well-known holometabolous model insect *D. melanogaster* has an extremely reduced extraembryonic component, the amnioserosa, most insects retain the ancestral component of two distinct extraembryonic membranes, amnion and serosa (Panfilio, 2008). In the embryonal development of hemimetabolous insects, these membranes are involved in anatrepsis and katatrepsis, referred to as blastokinesis. During anatrepsis, tissue invaginates into the yolk forming a sock-like germ rudiment (amnion and embryo). In katatrepsis, the embryo emerges from the serosa and rotates to the ventral surface, reversing its orientation in the egg (Panfilio, 2008). In holometabolous insects, these events are simplified and do not have distinct movements (Panfilio, 2008). Recently, Fan et al. (2020) described the morphology of the BPH embryo during its development and reported the embryonal transcriptome, paving the way for research into the embryonal development of BPH.

While α-1,3-fucosylation was shown to play an important role in the neural development of *Drosophila* embryos (Rendić et al., 2010), literature about the role of α-1,6-fucosylation in embryonic development is rare and limited. Analysis of the N-linked glycan profile in the *D. melanogaster* embryos showed that the abundance of fucosylated glycans increased in the late embryo (Aoki et al., 2007). These results suggest the involvement of fucosylation in embryonic development. In this study, the role of FucT6 in the embryonal development of BPH will be investigated. Studying the role of genes in embryogenesis can be challenging when abolishing a gene activity that leads to lethality. However, using transgenerational or parental RNAi, the gene silencing phenotype can be observed in the progeny of the treated parent organism (Khajuria et al., 2015). This ability to silence gene expression in the next generation makes parental RNAi a powerful tool to study the role of genes in embryonic development. After treatment of male or female planthoppers, fecundity and egg production will be analyzed, and embryonic development will be evaluated. In addition, in a pull-down assay with lectin-coated beads, the proteome during BPH embryogenesis will be characterized, aiming to decipher the underlying molecular information of α-1,6-fucosylation in BPH embryonic development.

2. Materials and methods

2.1 Insects

The BPH colony used for this study was reared at the laboratory on 8 to 10-week-old rice plants (*Oryza sativa* japonica, Nipponbare, USDA Agricultural Research Service, Beltsville, MD, USA) in an incubator under standard conditions of 27 ± 1 °C, 75% ± 5% relative humidity and a 16 h/8 h (light/dark) photoperiod. For synchronized BPH, an appropriate amount of pregnant female adults was transferred to fresh 8 to10-week-old rice plants. After laying eggs for 12 h, all females were removed from the plants and the eggs were kept on the rice plants until their fifth nymphal instar. Then, these synchronized fifth instar nymphs were transferred to fresh rice seedlings to rear until emergence.

2.2 Analysis of expression profiles for FucT6

Once emerged, the synchronized BPH colony was checked every two hours and the newly emerged females and males were separated and transferred to fresh rice seedlings to collect samples at different time points. For embryos, pregnant females from the BPH colony were transferred to fresh rice seedlings to lay eggs for 3 h. Subsequently, all females were removed from the plants, and the eggs were kept on the rice seedlings until the timepoint of collecting for expression profile analysis. All samples were immediately frozen with liquid nitrogen after collection, and total RNA was isolated using the RNeasy Mini Kit (QiaGen, Venlo, The Netherlands) according to the manufacturer's protocol. Synthesis of cDNA, quantification of mRNA expression levels by RT-qPCR, and analysis of the expression profile by qBase plus were carried out as described in our previous study (Yang et al., 2022). The genes coding for the ribosomal protein S15 (RPS15) and α-tubulin (TUB) were used as reference genes as previously described (Scheys et al., 2019; Yang et al., 2022). Visualization of the expression profiles and statistical analysis of the data were performed by GraphPad Prism 9.0.

2.3 RNAi and microinjection

Double-stranded RNA (dsRNA) targeting *FucT6* and *GFP* was synthesized and purified using the MEGAscript RNAi Kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. dsRNA concentration was measured with a DeNovix DS-C Spectrophotometer (DeNovix, Wilmington, USA) and its quality was checked by electrophoresis using an 1.5% agarose gel. The primers used for dsRNA synthesis were previously described (Yang et al., 2022). The dsRNA targeting the green fluorescent protein (GFP) gene is used as a control since no homologous gene is present in the *N. lugens genome.*

Gene silencing efficiency for the targets was checked at 72 h post-microinjection. The analysis was done in three biological replicates with for each replicate, six to eight BPH individuals.

Junior and senior BPH adults were injected with 100 ng (30 nL of 3.3 μg/μL) dsRNA targeting *FucT6* or *GFP* (*FucT6*^{RNAi} or *GFP*^{RNAi}, respectively) using a Nanoject III programmable nanoliter injector (Drummond Scientific, Broomall, USA). Before injection, all insects were anesthetized in a falcon tube containing a cotton plug soaked in 400 μL diethyl ether for 3 min and aligned on an agarose gel as previously described (Yang et al., 2022). All injected BPHs were allowed to recover for 2 h in an incubator under standard rearing conditions. The surviving BPHs were then selected and transferred to fresh rice seedlings for further analysis.

For the junior adults, newly emerged BPH females and males (≤ 12 h old) were collected from the synchronized colony and injected with dsRNA targeting *GFP* and *FucT6*, respectively. After recovering from microinjection, five pairs of *GFP*^{RNAi}-females and *GFP*^{RNAi}-males, and five pairs of *FucT6*^{RNAi}-females and *FucT6*^{RNAi}-males were pooled, respectively. Six pools for each treatment were obtained. All pooled insects were reared on fresh rice seedlings and followed for 10 days to observe the survival rate. For fecundity evaluation, five pairs of injected adults were pooled and transferred to fresh rice seedlings, allowing to mate for 48 h to increase mating rate. Four groups were included, *GFP*^{RNAi} ♀ × *GFP*^{RNAi} ♂, *GFP*^{RNAi} ♀ × *FucT6*^{RNAi} ♂, *FucT6*^{RNAi} ♀ × *GFP*^{RNAi} ♂, and *FucT6*^{RNAi} ♀ × *FucT6*^{RNAi} ♂. Three pools for each group were used. Subsequently, all males were removed, and the females were allowed to lay eggs for 72 h. Then, all females were removed and the eggs were kept on the rice seedlings until hatching. Hatchlings were counted and removed after daily checking. The eggs that were not hatched were dissected from rice seedlings after day 10 and counted.

For the senior adults, the pregnant females collected from the BPH colony were used for microinjection. After recovering, the females were reared on fresh rice seedlings and transferred to fresh rice seedlings daily. The eggs were kept on rice seedlings until analysis. As a control, pregnant females were collected from the BPH colony under the same rearing conditions but without any treatment.

2.4 Observation of embryo morphological features during embryonic development

Pregnant females from the BPH colony were transferred to fresh 8-week-old rice seedlings to lay eggs for 2 h. After removal of the females, the eggs were dissected from rice and kept on an 1.5% agarose gel in a clean plastic Petri dish for daily observation and for imaging by microscopy (Leica Microsystems DFC295). For the observation of parental RNAi treated eggs,

females were first injected with dsRNA targeting *FucT6* and allowed to recover for 2 h before being placed on the rice plants to lay eggs. After removal of females, the eggs were dissected from the rice plants, following the same procedures of observation and imaging as described above.

2.5 Preparation of BPH protein extracts

To better understand the role of fucosylation in the BPH embryo during embryonic development, especially in the katatrepsis related rotation, the embryonal proteomes at pre-katatrepsis (early embryo) and post-katatrepsis (late embryo) were investigated. Total protein extracts were isolated from early and late embryos and homogenized in 1 mL radio-immunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Illinois, USA) using stainless steel beads in a TissueLyser II (Qiagen, Hilden, Germany) at a frequency of 30 Hz/s for 3 min. Each sample was prepared in three biological replicates with each replicate consisting of 1000 eggs. Protease and Phosphatase Inhibitor Cocktail (100×) (Thermo Scientific, Illinois, USA) was added at 10 µL/mL to the RIPA lysis buffer before use. Subsequently, the homogenized samples were incubated and agitated on the shaker for 2 h on ice to allow efficient lysis. After that, the samples were centrifuged at 20,000× *g* for 5 min at 4 ℃ to remove debris and beads, and the resulting supernatant was transferred into a new 1.5 mL Eppendorf tube and centrifuged again at 20,000× *g* for 20 min at 4 ℃ to remove remaining debris. Finally, the supernatant was collected in a new 1.5 mL Eppendorf tube and stored at -80 ℃ until analysis. The protein concentration was determined with the RC DC protein assay kit (Bio-Rad, Bulletin, USA) according to the manufacturer's instructions, using bovine serum albumin (BSA) for setting up the calibration curve (Bio-Rad, Bulletin, USA). 10–12 µg of protein extracts per replicate were loaded onto a 4–20% Mini-PROTEAN TGX Protein Gel (Bio-Rad, Winninglaan, Belgium) to separate proteins at 200 V for 35 min and the Pierce Silver Stain Kit (Thermo Scientific, Illinois, USA) was used to visualize the distribution of separated proteins according to the manufacturer's protocol.

2.6 Pull-down of N-fucosylated glycoproteins

α-1,6-fucosylated N-glycoproteins were enriched for downstream analysis through a pull-down assay with *Lens culinaris* agglutinin (LCA) macrobeads (bioWORLD, Washington, USA). In brief, 150 µL of LCA macrobeads were transferred into a 1.5 mL Eppendorf tube and centrifuged at 500× *g* for 30 s to remove the storage buffer. Then, the LCA macrobeads were washed three times with a total of 5 volumes wash buffer (20 mM Tris-HCl pH 7.6, 50 mM NaCl, 2 mM $CaCl_2$ and 2 mM $MnCl_2$) to remove the remaining storage buffer and equilibrate

the LCA macrobeads to working conditions. Subsequently, 2 mg of protein extracts were applied onto the LCA macrobeads and incubated with an end-over-end rotation for 30 min at room temperature. After centrifugation at 500× *g* for 5 s and removal of the supernatant, the LCA macrobeads containing the interacting α-1,6-fucosylated proteins were washed once with 300 µL wash buffer and three times with 1 mL trypsin digestion buffer (20 mM Tris-HCl pH 8.0 and 2 mM $CaCl_2$). Finally, the LCA macrobeads were re-suspended in 150 µL trypsin digestion buffer and stored at -80 °C until further analysis. 15 µL of pull-down samples per replicate were loaded onto a 4–20% Mini-PROTEAN TGX Protein Gel (Bio-Rad, Winninglaan, Belgium) to separate the α-1,6-fucosylated proteins at 200 V for 35 min and the Pierce Silver Stain Kit (Thermo Scientific, Illinois, USA) was used to visualize the separated proteins according to the manufacturer's protocol.

2.7 Mass spectrometry (MS)

Before proceeding to MS, the pull-down samples were treated with trypsin to digest proteins into peptides and release them from the LCA macrobeads. Briefly, 1 µg trypsin was added and samples were incubated for 4 h at 37 °C to allow on-bead protein digestion. Next, another 1 µg trypsin was added for overnight digestion at 37 °C. Peptide samples were acidified to a final concentration of 1% Trifluoroacetic acid, purified on C18 omix tips (Agilent), transferred to MS vials and dried down.

Peptides were re-dissolved in 20 µL loading solvent A (0.1% trifluoroacetic acid in water/acetonitrile (ACN) (98:2, v/v)) of which 15 µL was injected for LC-MS/MS analysis on an Ultimate 3000 RSLCnano system in-line connected to a Q Exactive HF mass spectrometer (Thermo). Trapping was performed at 20 µL/min for 2 min in loading solvent A on a 5 mm PepMap trapping column (Thermo scientific, 300 µm internal diameter (I.D.), 5 µm beads). The peptides were separated on a 250 mm Waters nanoEase M/Z HSS T3 Column, 100Å, 1.8 µm, 75 µm inner diameter (Waters Corporation) kept at a constant temperature of 45 °C. Peptides were eluted by a non-linear gradient starting at 1% MS solvent B reaching 33% MS solvent B (0.1% FA in water/acetonitrile (2:8, v/v)) in 60 min, 55% MS solvent B (0.1% FA in water/acetonitrile (2:8, v/v)) in 75 min, 70% MS solvent B in 90 minutes followed by a 5-minute wash at 70% MS solvent B and re-equilibration with MS solvent A (0.1% FA in water). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the 12 most abundant ion peaks per MS spectrum. Full-scan MS spectra (375–1500 m/z) were acquired at a resolution of 60,000 in the Orbitrap analyzer after accumulation to a target value of 3,000,000. The 12 most intense ions above a threshold

value of 13,000 were isolated with a width of 1.5 m/z for fragmentation at a normalized collision energy of 30% after filling the trap at a target value of 100,000 for maximum 80 ms. MS/MS spectra (200–2000 m/z) were acquired at a resolution of 15,000 in the Orbitrap analyzer.

2.8 MS data analysis

All spectra obtained from the LC-MS/MS runs were searched using MaxQuant (version 2.0.3) with mainly default search settings, including a false discovery rate set at 1% on peptide-to-spectrum match (PSM), peptide and protein level. Identified spectra were searched against the *N. lugens* protein sequences in the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein; BioProject: PRJNA177647) (Xue et al., 2014), containing 27,571 sequences. The mass tolerance for precursor and fragment ions was set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to arginine and lysine, also allowing cleavage at proline bonds with a maximum of two missed cleavages. Variable modifications were set to oxidation of methionine residues and acetylation of protein N-termini. Proteins were quantified using the MaxLFQ algorithm integrated in the MaxQuant software. A minimum ratio count of two unique or razor peptides was required for quantification.

Further data analysis of the LC-MS/MS results was performed with an in-house R script, using the protein groups output table from MaxQuant. Reverse database hits were removed, LFQ intensities were $\log_2$ transformed and replicate samples were grouped. Proteins with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution centered around the detection limit using the DEP package (Zhang et al., 2018), leading to a list of quantified proteins used for further data analysis. To compare protein abundance following LCA-enrichment between pairs of sample groups, statistical testing for differences between two group means was performed using limma (Ritchie et al., 2015). Statistical significance for differential regulation was set at false discovery rate (FDR) < 0.05 and $|\log_2$ fold change ($\log_2$FC)$| = 2$. Proteomics data sets have been deposited to PRIDE. All identified proteins were analyzed for the presence of a signal peptide (https://services.healthtech.dtu.dk/service.php?SignalP) (Teufel et al., 2022) or a transmembrane domain (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) (Krogh et al., 2001).

2.9 Statistics analysis

Statistical analysis of the survival curves was performed in SPSS 28 using the Kaplan-Meier log-rank (Mantel-Cox) test. Graphs and survival curves were visualized using GraphPad Prism

9.0. Statistical differences were evaluated by student independent sample T-test and by one-way ANOVA when comparing more than two groups.

3. 3. Results

3.1 FucT6 expression profile during adult stage and embryonal development

The temporal expression pattern of *FucT6* in male and female adults revealed a similar pattern for both sexes. The mRNA abundance of *FucT6* increased in both sexes shortly after adult emergence, reaching a peak at 6 h after emergence (Fig. 1A–B). After this peak, the transcript abundance decreased to levels lower than those in the newly emerged adults. However, when comparing the transcript levels between the two sexes, a significant difference in abundance of *FucT6* transcripts can be observed. The transcript levels in male insects are, in average, about 5-fold higher than those in female insects (Fig. 1A–B).

In embryos, transcription levels were found to gradually increase after egg laying, reaching a peak at 120 h after egg laying (AEL) (Fig. 1C). Then, expression levels slightly decreased, nevertheless, they remain higher than the levels in early embryos (≤ 90 h AEL).

Observing the embryonic development in the egg allowed the identification of several key milestones in the development (Fig. 1D). As previously described by Fan et al. (2020), after oviposition, the content of the egg gradually became dense and turned from clear to opaque (≤ 12 h AEL). While during this period the diameter of the egg had not changed, around 24 h AEL, a clear increase in the diameter in the middle of the egg could be observed (Fig. 1D). While initially the cells were uniformly distributed through the whole egg, gradually a germ band, destined to give rise to the embryo, was formed and around 48 h AEL, enlarged germ bands were clearly visible. Around 56 h AEL, a 'sock-like' embryo was observed, which gradually grows and develops to form the head lobe, thorax and abdomen. At 72 h AEL, a white-to-yellow yolk-like mass gathers at the anterior of the egg, attaching to the abdomen of the embryo (Fig. 1D). At this stage, the embryo shape was quite evident, and the egg was larger and harder. In this period, organ systems and appendages were being formed. Subsequently, katatrepsis began and the embryo gradually moved along the ventral axis of the egg followed by an 180° rotation of the embryo. This movement of the embryo took place within hours and the majority of the eggs underwent this rotation movement between 90 h to 100 h AEL. At 96 h AEL, a red eyespot appeared at the anterior side of the egg, while the yolk mass can now be seen at the posterior of the egg (Fig. 1D). At 120 h AEL, the red eyespot of the embryo had become larger and more visible, and a clear segmentation of the embryo body was seen. The organ systems and

appendages continued to develop while the yolk was re-absorbed by the embryo. At 144 h AEL, the red eyespot was darker and larger. The complete appendages of the pre-nymph were evident at 168 h AEL (Fig. 1D). In this stage, the dark tarsus of the pre-nymph in the eggshell was clearly observed. Once the dark tarsus of the pre-nymph is observed, the pre-nymph will gradually move forward and will soon come out from the eggshell, normally within hours. When comparing the expression profile of *FucT6* in the embryo with the observed developmental milestones of the embryo, the increase in *FucT6* transcripts coincided with the development of the embryo (Fig. 1C–D). *FucT6* expression increased significantly (6.5 times) after embryo rotation (120 h AEL) compared to the levels before rotation (72 h AEL).

3.2 Silencing of FucT6 in adult affected female survival but not egg production

Newly emerged adults, within 12 h after eclosion, were injected with dsRNA targeting *FucT6* ($FucT6^{RNAi}$) or control-treated with dsRNA targeting *GFP* (GFP^{RNAi}). Different from the observations in nymphs, disruption of the expression of *FucT6* in adults did not cause complete mortality. Compared to the control treatment (GFP^{RNAi}), the survival rate of $FucT6^{RNAi}$-females showed a significant decrease of 24% (corrected mortality) at 10 days post microinjection (Fig. 2A) while no negative effects on the survival rate was observed in $FucT6^{RNAi}$-males (Fig. 2B). Gene silencing efficiency was checked at 72 h post microinjection (Fig. S1).

To evaluate egg production, GFP^{RNAi}- and $FucT6^{RNAi}$-females were allowed to mate with GFP^{RNAi}- or $FucT6^{RNAi}$-males (GFP^{RNAi} ♀ × GFP^{RNAi} ♂, GFP^{RNAi} ♀ × $FucT6^{RNAi}$ ♂, $FucT6^{RNAi}$ ♀ × GFP^{RNAi} ♂, and $FucT6^{RNAi}$ ♀ × $FucT6^{RNAi}$ ♂). According to the analysis of the accumulating total number of eggs at 10 days after treatment, no reduction in the total number of eggs was observed for the $FucT6^{RNAi}$-females neither after mating with GFP^{RNAi}-males or $FucT6^{RNAi}$-males (Fig. 2C). In contrast, a small significant increase in the total number of eggs was observed in the $FucT6^{RNAi}$ ♀ × $FucT6^{RNAi}$ ♂ group compared to the GFP^{RNAi} ♀ × GFP^{RNAi} control group.

3.3 Parental RNAi of FucT6 disturbed embryonal development

While disruption of *FucT6* in female or male adults did not affect egg production, the hatching rate of eggs laid by $FucT6^{RNAi}$-females, mated either with GFP^{RNAi}- or $FucT6^{RNAi}$-males, was reduced to 0% (Table 1). In the GFP^{RNAi}-female groups, 69% to 74% (GFP^{RNAi} ♀ × GFP^{RNAi} ♂, GFP^{RNAi} ♀ × $FucT6^{RNAi}$ ♂, respectively) of the eggs hatched within 8–10 days AEL, and 21% to 17% (GFP^{RNAi} ♀ × GFP^{RNAi} ♂, GFP^{RNAi} ♀ × $FucT6^{RNAi}$ ♂, respectively) of the

GFP^{RNAi}-female eggs failed to develop an embryo during embryonic development. In contrast, no hatchlings could be observed from the eggs laid by the *FucT6*^{RNAi}-females mated with either *GFP*^{RNAi}-males or *FucT6*^{RNAi}-males with > 98% of eggs having failed to develop an embryo (Table 1). These eggs appeared shrunken and dried, while the eggs without a developed embryo from the *GFP*^{RNAi}-female appeared full and hydrated (Fig. 3A).

At day 10 AEL, 5.3% to 14.1% of the eggs laid by *GFP*^{RNAi}-females were unhatched but contained a normal developed embryo with the red eyespot at the anterior of the egg (Fig. 3A and Table 1). These embryos showed a delay of development but would still hatch. In the eggs laid by *FucT6*^{RNAi}-females however, less than 2% of the eggs were found to contain an embryo with a red eyespot and this eyespot was seen at the posterior of the egg (Fig. 3A and Table 1). Eggs where the embryo had not rotated will not hatch.

To analyze in-depth the role of FucT6 in embryos, the development of embryos from *FucT6*^{RNAi}-females was compared to that of embryos from untreated females (Fig. 3B). As described above, most eggs did not develop an embryo. For those that did develop, no differences could be observed between the development of eggs from untreated or *FucT6*^{RNAi}-females up to 93 h AEL. In the eggs laid by the untreated females, a rotated embryo can be observed at 128 h AEL while in the eggs laid by *FucT6*^{RNAi}-females the embryo had not rotated. In those embryos however, the eyespot did appear (at the posterior of the egg), suggesting that development had not halted but that the katatrepsis event had failed (Fig. 3B). Analysis of the transcription levels in eggs just before and just after this rotation event supports the involvement of α-1,6-fucosylation in katatrepsis, as in the untreated eggs, a significant upregulation of *FucT6* levels was observed after katatrepsis (Fig. 3C).

Treatment of pregnant females with dsRNA targeting *FucT6* revealed that for the eggs already formed in the female before treatment (eggs laid at 1–2 days after treatment), the percentage of embryos that failed katatrepsis is 30–15%, respectively (Table S1). Analysis of the *FucT6* transcription levels confirmed the parental RNAi efficacy at the moment of katatrepsis (Fig. 3C). In the eggs laid ≥ 3 days after treatment (not yet formed in the female at the time of treatment), the percentage of embryos that failed katatrepsis decreased and the percentage of embryos that did not develop an embryo increased (Table S1).

3.4 Analysis of the N-fucosylated proteome in the BPH embryo

To gain insight into the mechanisms behind the katatrepsis phenotype, the N-fucosylated proteome of embryos was analyzed at the moment of katatrepsis. Two untreated samples were collected: early embryo, with a yolk mass at the anterior of the egg (pre-katatrepsis, 72–96 h AEL) and late embryo, with a yolk mass at the posterior and red eyespot at the anterior of the egg (post-katatrepsis, 96–120 h AEL). In addition, a sample of *FucT6*^{RNAi}-induced inversed embryos (failed-katatrepsis, 96–120 h AEL) was included in the analysis. After total protein isolation, the N-fucosylated proteins were enriched by pull-down with *Lens culinaris* agglutinin (LCA) macrobeads before LC-MS/MS analysis. Due to a low number of proteins identified in one biological repetition of the inverted embryo sample after processing the LC-MS/MS data, the quantified proteins were analyzed based on at least two valid label-free quantification intensity values in one of the experimental conditions and were used for downstream analyses. In total, we identified 328 protein groups containing 993 proteins over all the embryo samples, of which 265 protein groups were from early embryo, 179 protein groups from late embryo and 131 protein groups from inversed embryo (Fig. 4A and Table S2). These proteins were further analyzed for the presence of a signal peptide or a transmembrane (TM) domain to confirm their processing through the secretion pathway. Of the 993 proteins, 230 (23%) were predicted to contain a TM domain and 294 (30%) were predicted to have a signal peptide. Thus, together, 377 proteins (38%) carry either a TM domain, a signal peptide or both, which suggests a rather low enrichment in membrane-bound and/or secreted proteins and possible contamination of non-fucosylated proteins. In addition, a scan for the presence of predicted signal peptides or TM domains in the full proteome (https://www.ncbi.nlm.nih.gov/protein/, released version: 2023_Jan.) of BPH, containing 35,818 proteins, was performed for comparison. In this proteome, 8426 proteins (24%) have at least one TM domain and 4711 proteins (13%) carry a signal peptide; thus, together, 13,063 proteins (36%) carry either a TM domain and/or a signal

peptide. When comparing the proportion of proteins with a signal peptide in the embryo samples to those in the full proteome, the enrichment of proteins carrying a signal peptide is apparent. However, based on the proportion of proteins with a TM domain, the enrichment is not evident.

3.5 Analysis of protein levels reveals early and late embryonal groups

Pairwise analysis of protein levels following LCA-enrichment identified a total of 79 protein groups as with different levels over the different samples (Fig. 4B and Table S2) ($|\log_2 FC| \geq 2$, FDR < 0.05). Following hierarchical clustering analysis, these proteins were grouped into six clusters (Fig. 4B). Of interest is the proteins of cluster 2 (including sixteen protein groups) and cluster 6 (including five protein groups) (Table S2), which are respectively upregulated and downregulated in the late embryo compared to the early embryo and the inversed embryo samples, as these are potentially involved in katatrepsis. Compared with the late embryo, 24 protein groups were found significantly upregulated and 44 protein groups significantly downregulated in the early embryo (Fig. S2A). When compared with the inversed embryo, 17 protein groups were significantly upregulated and 34 protein groups significantly downregulated in the early embryo (supplementary Fig. S2B). There were only two protein groups significantly upregulated and no proteins significantly downregulated in the late embryo when compared with the inversed embryo (supplementary Fig. S2C).

4. Discussion

Glycosyltransferases and glycosidases are important enzymes involved in protein glycosylation. Glycan structures are sequentially modified by a series of these enzymes that add or remove oligosaccharide moieties, giving rise to different types of glycans. During the formation of paucimannose-, complex- and hybrid-type N-glycans, α-1,3-fucosyltransferase and α-1,6-fucosyltransferase catalyze the core modification of N-glycans by adding fucose moieties to the proximal GlcNAc-residues (García-García et al., 2021; Li et al., 2018).

Alteration of protein fucosylation in humans has been reported to be associated with many diseases (Loaeza-Reyes et al., 2021; Miyoshi et al., 2008; Reily et al., 2019). For example, in many cancer patients the expression of Fut8, an α-1,6-fucosyltransferase, was found to be upregulated (Bastian et al., 2021; Liao et al., 2021; Tu et al., 2017). In mice, disruption of the Fut8 ortholog by gene knock-out leads to severe growth retardation, which indicates that Fut8 activity is needed for mice growth (Wang et al., 2006). Similarly, RNAi-mediated disruption

of FucT6 activity in the hemimetabolous insect *N. lugens* blocked nymphal development and resulted in complete lethality (Yang et al., 2022). On the other hand, gene silencing of *FucT6* in the holometabolous model insect *D. melanogaster* only caused mild effects such as abdominal depigmentation in adults (Yamamoto-Hino et al., 2015). In other holometabolous insects, such as *T. castaneum* and *L. decemlineata*, RNAi-mediated gene silencing of *FucT6* did not cause any observed negative effects (Liu et al., 2022; Walski et al., 2016).

In *N. lugens*, expression of *FucT6* increased shortly after adult eclosion, to subsequently decrease below initial levels. Interestingly, the transcript levels of *FucT6* in females remain significantly lower than those in males during the whole adult lifespan. This can be correlated with the abundance of fucosylated glycans in the male and female N-glycome. In BPH females, fucosylated glycans comprise 8% of the total N-glycome, while in BPH males they represent 43% (Scheys et al., 2019). However, given this higher abundance of fucosylated N-glycans and higher expression levels of *FucT6* in male insects, RNAi-mediated gene silencing had no observed negative effects on the survival of males. In BPH females, however, $FucT6^{RNAi}$ had a slight impact on their survival.

Despite the mild effects on female planthoppers, silencing of *FucT6* did not reduce their egg production. However, parental RNAi of *FucT6* was found to impair embryonic development, leading to a loss of egg hatching. While in the majority of the eggs laid by $FucT6^{RNAi}$-females, embryonal development was aborted, leading to an absence of an embryo, a considerable proportion of the eggs (up to 30%) did develop an embryo. However, while in normal embryonic development, during the process of katatrepsis, the embryo rotates 180°, the $FucT6^{RNAi}$-embryos failed to undergo katatrepsis and displayed an eyespot at the posterior of the egg and a yolk mass at the anterior of the egg. These inverted embryos were not able to hatch. This phenomenon was only seen in the eggs of $FucT6^{RNAi}$-female groups, suggesting it is maternally mediated. The importance of FucT6 activity in embryonic development and especially at the timing of katatrepsis was supported by the significant increase in *FucT6* transcript levels in the (untreated) late embryos (post-katatrepsis) compared to the early embryos (pre-katatrepsis).

To elucidate the potential glycoproteins involved in the process of katatrepsis, a proteomics study was performed after enriching the fucosylated proteins through affinity purification using *Lens culinaris* agglutinin (LCA), which has a strong specificity to α-1,6-fucosylated proteins (Bojar et al., 2022; Do Nascimento et al., 2015; Rubén et al., 2021). However, contamination with non-specifically bound proteins is inherent in all enrichment techniques. Despite washes to reduce potential contaminants, the enrichment of proteins with a signal peptide and/or

transmembrane domains, and therefore potentially subjected to core-fucosylation in the secretion pathway, appears rather limited. Indeed, based on the number of proteins predicted to have at least one TM domain, the enrichment of such proteins is not evident in our study. However, an apparent enrichment is observed in proteins carrying a signal peptide compared to the full BPH proteome. Nevertheless, our data provide some level of insight into the (N-fucosylated) proteome of BPH during the process of katatrepsis. In total, we identified 328 protein groups over all samples, of which 79 were identified at differential levels over the different samples, revealing clusters of proteins upregulated in embryos pre- or post-katatrepsis. At current, the knowledge of glycoproteins is still limited in insects, thus further studies will be needed to confirm these identified proteins and their corresponding glycans.

Correct glycosylation is essential for recognition by and interactions with glycoprotein-dependent lectins. Disruption of these interactions, involved in many essential biological processes, can lead to problematic cellular homeostasis and regulation (Brown et al., 2018; Varki, 2017). For example, recently, the RNAi-mediated gene silencing of *Nllet1*, a C-type lectin in BPH was found to disrupt the structure of the serosal cuticle, causing complete embryonal mortality (Lu et al., 2022). Here, we noticed that $FucT6^{RNAi}$-eggs were easily broken by touching after 72 h AEL, were prone to fungal infection and that many $FucT6^{RNAi}$-eggs were gradually shrunken (desiccation) at the late stage of embryonic development. These observations suggest that the silencing of *FucT6* might have an influence on the formation or structure of the extraembryonic membranes. Proteomics revealed a few potential targets, e.g. chitin- and cuticle-related proteins (LOC111062103, LOC120353154, LOC111048624 and LOC120354132), that might play a role in the formation of the extraembryonal membranes, the serosa and amnion. Absence of these protective membranes has been shown to impair embryonal development and lead to desiccation during embryonic development (Jacobs et al., 2013; Schmidt-Ott and Kwan, 2016). While our data have brought some insight into the role of α-1,6-fucosylation in embryonal development, the regulatory mechanisms behind these processes are still unclear and more research is needed to better understand and interpret the impact of α-1,6-fucosylation in the katatrepsis events, formation of extraembryonal membranes and embryogenesis.

Author contributions

QY, GS and KDS conceptualized this study; QY conducted the experiment, performed the data analysis, and drafted the manuscript; AS and KG performed the LC-MS/MS analysis; QY

and KDS performed the data curation; GS and KDS supervised the experiments. KDS revised the manuscript. All authors approved the submission of the manuscript.

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Conflict of interest

Authors have declared no conflict of interest.

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Table 1. Summary of the effects on BPH egg by parental RNAi of *FucT6*

Treatment	Total number of eggs	Hatched	Unhatched		
			Normal embryo	Inversed embryo	Failed to further develop
GFP^{RNAi} ♀× GFP^{RNAi} ♂	321	237 (73.8%)	17 (5.3%)	1 (0.3%)	66 (20.6%)
GFP^{RNAi} ♀ × $FucT6^{RNAi}$ ♂	404	278 (68.8%)	57 (14.1%)	0	69 (17.1%)
$FucT6^{RNAi}$ ♀ × GFP^{RNAi} ♂	466	0	3 (0.6%)	6 (1.3%)	457 (98.1%)
$FucT6^{RNAi}$ ♀ × $FucT6^{RNAi}$ ♂	503	0	2 (0.4%)	4 (0.8%)	497 (98.8%)

Supplementary tables are listed separately:

Table S1. Summary of morphological features of eggs deposited from $FucT6^{RNAi}$-females at different time after microinjection (.docx).

Table S2. Summary of α-1,6-fucosylated glycoproteins in BPH embryos (.xlsx).

633 **Figure legends**

634 **Fig. 1. Expression profiles of *FucT6* and embryo morphology**. (A–C) Temporal expression
635 profiles of *FucT6* in female, male and embryo. Three independent biological repetitions were
636 prepared for RNA isolation for each sample at different time points (n = 8–10 for females, 15–
637 20 for males, and 120–150 for eggs for each biological repetition, respectively). Two technical
638 replicates were prepared for each biological repetition when performing the qPCR. Bar values
639 indicate the means ± SEM. Different lowercase letters above the bar indicate statistical
640 differences (one-way ANOVA at $P < 0.05$). (D) Morphological features of BPH embryo during
641 embryonic development.

642 **Fig. 2. Effects on adult survival and egg production after RNAi of *FucT6*.** (A–B) Dynamic
643 survival rate of females and males after RNAi. Analysis was assessed on 60 individuals for
644 each treatment. Statistical differences are analyzed using Kaplan-Meier log-rank (Mantel-Cox)
645 test, "*" indicates a significant difference at $P < 0.05$. (C) Effects on adult egg production after
646 RNAi. Analysis is based on three biological replicates with each includes five pairs of females
647 and males as a pool. Bar values indicate the means ± SEM. Different lowercase letters above
648 the bar indicate statistical differences (one-way ANOVA at $P < 0.05$).

649 **Fig. 3. Effects on eggs after parental RNAi of *FucT6***. (A) Effects on eggs by parental RNAi
650 in newly emerged adults. (B) Effects on eggs by parental RNAi in pregnant females. The scale
651 bar indicates 200 μm. AEL, after egg laying; A, the anterior of the egg; P, the posterior of the
652 egg. (C) Expression profiles of *FucT6* in early embryo and late embryo. Three independent
653 biological repetitions were prepared for RNA isolation and two technical replicates for each
654 biological repetition when performing the qPCR. Different lowercase letters above the bar
655 indicate statistical differences (one-way ANOVA at $P < 0.05$).

656 **Fig. 4. Distribution of the quantified proteins and hierarchical clustering heatmap.**

657 **Fig. S1. Relative expression level of *FucT6* after microinjection.** Gene silencing efficiency
658 was checked at 72 h post-RNAi. FM, female; M, male. Three independent biological repetitions
659 were prepared and of each with six to eight individuals. Two technical replicates were prepared
660 for each biological repetition when performing qPCR. Bar values indicate the means ± SEM.
661 Statistical significances indicate with "*" at $P < 0.05$ (Independent Samples T Test).

662 **Fig. S2. Volcano plot of proteins obtained from LC-MS/MS.** EE, early embryo; LE, late
663 embryo; IE, inversed embryo. Upregulation is indicated by red and downregulation is indicated
664 by blue.

665