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The UDP-Glycosyltransferase Gene Family in *Achelura yunnanensis* (Lepidoptera: Zygaenidae): Identification, Phylogeny, and Diverse Expression Patterns

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Abstract: The caterpillars of the Lepidoptera are important herbivores as most of them belong to serious agricultural and forestry pests. To adapt to their habitats and feeding host plants, the larvae utilize uridine diphosphate (UDP)-glycosyltransferases (UGTs) to metabolize plant defensive compounds and insecticides. However, information on the UGT gene family in *Achelura yunnanensis* remains scarce. Here, we characterized the UGT genes through gene identification, phylogenic analyses, and comprehensive expression profiles regarding sexes, tissues, and stages. Transcriptome analyses led to the yields of 50 transcripts encoding UGTs in *A. yunnanensis*, representing a comparable gene number compared to those in other lepidopteran species. Sequence and phylogenetic analyses revealed a low amino acid identity of 28.23% among 31 full-length AyunUGTs, but some members shared relatively high conservation (>50% identities) with a phylogenetically clustered distribution. In addition, the majority of AyunUGTs possessed conserved residues involved in the catalysis and sugar-donor binding. Combining RNA sequencing and PCR approaches, a number of AyunUGTs were found to have the expression in chemosensory or detoxification tissues, possibly associated with the sensing of odorant molecules and the metabolism of toxic chemicals. More importantly, at least 27 AyunUGTs displayed detectable expression in reproductive tissues of both sexes. This study identifies candidate *A. yunnanensis* UGTs responsible for detoxification, olfaction, and reproduction, allowing us to address putative roles of UGTs in the adaptation of larvae to the habitats and feeding hosts.

Keywords: *Achelura yunnanensis*; UDP-glycosyltransferase; detoxification; olfaction; reproduction

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1. Introduction

The evolutionary adaptation of insects to habitats is associated with various biotransformation processes, especially for the detoxification pathway that is indispensable for herbivorous insects to detoxify the toxic chemicals derived from their food host plants or the external environment. Usually, these detoxification-related enzymes mainly refer to the phase I (cytochrome P450 monooxygenases, P450s, and carboxylesterases, COEs) and II (glutathione S-transferases, GSTs, and uridine diphosphate (UDP)-glycosyltransferases, UGTs), which are of utmost importance for the survival and reproduction of insects. The phytophagous insects utilize these enzymes to metabolize xenobiotics and endobiotics, which are often toxic to herbivores [1,2]. In comparison to the three superfamilies of P450s, GSTs, and COEs, UGTs in insects have received relatively little attention. As one of the most important detoxification enzymes, insect UGTs have been demonstrated to be mainly responsible for the metabolism of plant allelochemicals and insecticides [3–5].

Moths constitute the majority of the Lepidoptera, and their larvae are all herbivores [6,7]. To adapt to the constantly changing habitats and feeding host plants, they must cope with plant-produced or man-made chemicals via a xenobiotic-metabolizing enzyme system.

Moth UGTs are capable of catalyzing the conjugation of small lipophilic compounds with sugars, including plant defensive compounds, pesticides, hormones, odorants, and other xenobiotics [4,8–10]. The biotransformation process makes the harmful substrates become more water-soluble and readily excretable, helping the caterpillars avoid plant toxins and insecticides. In *Bombyx mori*, a genome-wide analysis resulted in the yields of 42 UGT genes, of which they were mainly transcribed in reproductive and/or detoxification-related tissues [11]. Furthermore, some BmorUGTs responded to flavonoids, coumarins, and terpenoids, serving as key enzymes in the detoxification of plant toxic compounds and the degradation of odorant molecules [12,13]. In addition, the polyphagous noctuid species also employ the UGT enzymes to metabolize plant-derived defensive substrates. For example, a cotton-produced defensive compound of gossypol was glycosylated by HarmUGT40D1 and HarmUGT41B3 in *Helicoverpa armigera*, as an adaptation of the cotton bollworm larvae to a major feeding host plant [14]. In *Spodoptera frugiperda*, SfruUGT33F28 was responsible for the glycosylation of benzoxazinoids, the main defensive compounds derived from maize plants [15]. Similarly, three *Helicoverpa* species could overcome the defense of capsaicin, especially for the specialist *H. assulta* [16]. Beyond the detoxification of plant secondary metabolites, moth UGTs are involved in olfaction and insecticide resistance [8,9,17–19].

The zygaenid moth, *Achelura yunnanensis*, is an oligophagous insect herbivore with the larvae feeding solely on the Rosaceae family, particularly its favorable hosts of *Cerasus yunnanensis* and *Photinia glomerata*. Therefore, it is considered to be a serious pest of garden plants that generally eats the leaves of host plants and then moves to other hosts for continuing damage [20–22]. Like other moths, *A. yunnanensis* caterpillars are able to metabolize a variety of toxic exogenous and endogenous compounds by detoxification-related tissues, including midguts, fat bodies, and Malpighian tubules. However, no information is available about the detoxification mechanism in this species. Here, we identified and characterized the UGT gene family from *A. yunnanensis*, one of the most crucial detoxification enzymes. Through sequence and phylogenetic analyses, and expression profiling characteristics, this work identifies molecular candidates involved in detoxification, olfaction, or reproduction, and provides insights into the adaptation of the *A. yunnanensis* larvae to the feeding host plants.

2. Materials and Methods

2.1. Insects and Tissues

A. yunnanensis larvae rearing was described in detail by Li et al. (2021). Briefly, newly emerged adults were separated by sex, and males and females were maintained in individual cages, supplying with 10% sugar solution. To examine the expression profiles of UGT genes, the following tissues were collected: the 3rd day eggs after virgin females laid eggs; antennae, heads (removing antennae), maxillary palps, midguts, fat bodies, and Malpighian tubules from 4th instar larvae; the 3rd day pupae of both sexes after pupation; adult tissues from 3-day-old moths, including antennae, heads (removing antennae), thoraxes, abdomens, legs, and wings of both sexes, and female pheromone glands; and reproductive tissues from 3-day-old adults, comprising accessory glands (MAG), testes (Te), seminal vesicles (SV), and ejaculatory ducts (ED) of males, as well as FAG, bursa copulatrix (BC), ovaries (Ov), and spermathecae with spermathecal glands (SS). The reproductive tissues were isolated and related fat bodies were cleaned in phosphate-buffered saline (PBS, pH 7.4). The collected tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA Extraction and Synthesis of First-Strand cDNA

TRIzol Regent (Ambion, Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from each tissue of *A. yunnanensis*, along with the protocols. RNA concentration and quality were checked on an agarose gel (1%, *w*/*v*) and a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA). Genomic DNA from purified RNA was removed using gDNA Eraser at 42 °C for 2 min. Then, reverse transcription was

carried out using a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) at 37 °C for 15 min, and stopped at 85 °C for 5 s. For reverse transcription (RT)–PCR analyses, 5-fold dilutions of cDNA templates were used, and 15-fold for quantitative real-time PCR (qPCR).

2.3. Identification of Candidate UGTs in A. yunnanensis

Initially, UGT protein sequences of four lepidopteran species, including *B. mori*, *H. armigera*, *Manduca sexta*, and *Athetis lepigone*, were pooled as a local sequence database. Using these sequences as queries, we identified the UGT genes in *A. yunnanensis* by TBLASTN against the transcriptomes previously published [20,21]. To confirm whether newly identified AyunUGTs were members of the Glycosyltransferase_GTB-type superfamily, BLAST searches were repeated with each new AyunUGT against the National Center for Biotechnology Information (NCBI) Non-redundant (Nr) protein sequences database.

2.4. Sequence and Phylogenetic Analysis of UGTs

Open reading frames (ORFs) of genes were predicted using an online tool ORF Finder in the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/ (accessed on 4 March 2022)). Isoelectric point (*p*I) and molecular weight (Mw) of full-length UGTs were computed by the Compute *p*I/Mw tool (https://web.expasy.org/compute_pi/ (accessed on 4 March 2022)). The prediction of signal peptides and *N*-glycosylation sites (NPS) were conducted using the SignalP version 6.0 (Technical University of Denmark, Kongens Lyngby, Denmark) [23] and NetNGlyc version 1.0 (Technical University of Denmark, Kongens Lyngby, Denmark) servers (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0 (accessed on 4 March 2022)), respectively. A multiple alignment program MAFFT V7.450 was employed to align the protein sequences and to calculate amino acid identities [24]. If necessary, the alignments were manually adjusted and edited using Jalview version 2.8 (University of Dundee, Scotland, UK) [25].

The maximum-likelihood tree was built using FastTree v2.1.12 (Lawrence Berkeley National Lab, Berkeley, CA, USA), under the Whelan-And-Goldman (WAG) 2001 model with the Gamma20 likelihood and 1000 replications [26]. To determine the distribution of UGT genes typically clustering into monophylogenetic clades on chromosomes, the genes in *B. mori*, *M. sexta*, and *H. armigera* were mapped onto their respective reference genomes by TBLASTN, implemented in Geneious R10.1.3 (https://www.geneious.com/ (accessed on 10 March 2022)). The genomic sequences of three species were retrieved from the NCBI Genome database, referring to *B. mori* (Bmori_2016v1.0, accession number: GCF_014905235.1) [27], *M. sexta* (JHU_Msex_v1.0, accession number: GCA_000262585.1) [28], and *H. armigera* (Harm_1.0, accession number: GCA_002156985.1) [29].

2.5. Expression Profiling Analysis of UGT Genes in A. yunnanensis

Based on the sequenced transcriptomes of 13 tissues [20,21], we first mapped clean reads onto the unigene transcriptomes using Bowtie2 [30]. The expression values of genes were calculated by RSEM v1.2.15 with default parameters [31], as normalized by FPKM (fragments per kilobase of transcript per million mapped reads) [32]. Next, RT–PCR was employed to determine the expression profiles of 31 full-length UGTs in different tissues of *A. yunnanensis*. The quality and quantity of cDNA templates were measured using a reference gene, ribosomal protein L8 (AyunRPL8) [20]. The primers were designed by Primer Premier 5 (PREMIER Biosoft International, Palo Alto, CA, USA), with the expected amplification sizes of 400–500 bp (Table S1). The reaction was performed with a total volume of 25 µL, following a Taq DNA Polymerase Kit's instruction (TaKaRa, Dalian, China). The conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 5 min. PCR amplification products were analyzed on 1.5% (*w*/*v*) agarose gels.

In addition, we chose six candidate UGT genes that showed high expression in main chemosensory (larval antennae, larval maxillary palps, antennae, and legs of both sexes) or detoxification (larval midguts, fat bodies, and Malpighian tubules) tissues to examine

their relative expression levels. Gene-specific primers were designed by Beacon Designer 8.14 (PREMIER Biosoft International, Palo Alto, CA, USA) (Table S1). qPCR was performed with qTOWER 2.2, according to Bestar® SybrGreen qPCR Mastermix's suggestions (DBI® Bioscience, Ludwigshafen, Germany). In brief, a reaction mixture of 20 µL was prepared and run under the procedures: 95 °C for 2 min and 40 cycles at 95 °C for 10 s, 58 °C for 31 s, and 72 °C for 30 s. Three biological pools were run for each tissue. Two reference genes, AyunRPL8 and RPS4 [20], were used to normalize the relative expression of target genes using a Q-Gene method [33,34]. Statistical analyses in the data were conducted by one-way ANOVA followed by Fisher's least significant difference (LSD) test. A significant level was set as p value < 0.05.

3. Results

3.1. Identification of A. yunnanensis UGT Genes

Combining Li et al. (2021) and Nuo et al. (2022) transcriptomic data, a BLAST-based homology search led to the yields of 50 transcripts encoding UGTs in *A. yunnanensis*. Out of the identified AyunUGTs, 31 relatives harbored complete ORFs, ranging from 1512 to 1641 bp. The remaining 19 genes were partial sequences, encoding 117 to 480 amino acids. Among the full-length AyunUGTs, most of them exhibited extremely low identities to each other at the amino acid level (<30%), with an average value of 28.23%. Exceptionally, four pairs of the proteins shared a high degree of conservation (>80% identities), i.e., AyunUGT8 and UGT12 (93.46%), AyunUGT15 and UGT31 (87.96%), AyunUGT9 and UGT28 (82.66%), as well as AyunUGT19 and UGT30 (82.43%). Like other insect UGTs, AyunUGTs had signal peptides (15–24 amino acids) and similar *p*I (6.37–9.50) and Mw (57.08–62.57 kDa). The identification of *N*-glycosylation sites revealed that all 31 full-length UGTs contained at least one site, particularly as many as six for AyunUGT6/17/21/29 (Table 1 and Supplementary Material Additional File S1).

Table 1. Information for 31 full-length UGT genes in *A. yunnanensis*.

Gene	ORF (bp)	SP (aa)	*p*I	Mw (kDa)	NPS
UGT1	1560	18	7.73	59.90	114, 273, 419
UGT2	1557	17	7.32	59.53	63, 330
UGT3	1593	17	8.89	60.03	63, 184, 247, 294, 343
UGT4	1575	18	7.65	60.28	67, 427, 474
UGT5	1542	18	8.78	58.27	49, 169, 318, 447, 501
UGT6	1602	19	7.68	61.39	68, 138, 199, 308, 479, 523
UGT7	1575	21	6.52	61.29	52, 244, 326, 452, 516
UGT8	1563	20	9.11	58.33	237, 302
UGT9	1554	20	9.18	59.29	82, 175, 245, 511
UGT10	1641	21	8.97	62.57	52, 239, 333
UGT11	1545	17	9.22	59.21	71, 89, 102, 237, 285
UGT12	1563	20	9.06	58.27	103, 237, 302
UGT13	1557	20	8.95	59.75	128
UGT14	1569	22	7.71	60.69	71, 192, 289, 472
UGT15	1545	18	8.98	59.13	120, 240
UGT16	1581	20	6.37	60.23	147, 236
UGT17	1566	20	8.97	60.88	51, 93, 240, 274, 426, 449
UGT18	1593	21	8.83	60.56	252, 436
UGT19	1539	19	8.84	59.41	60, 65, 102
UGT20	1563	18	7.29	59.64	117, 251
UGT21	1512	15	7.34	57.08	61, 72, 279, 315, 416, 417
UGT22	1575	24	7.25	60.83	73, 291
UGT23	1584	20	6.55	60.81	69, 429, 477
UGT24	1536	21	8.99	58.60	74, 238, 289

Table 1. *Cont.*

Gene	ORF (bp)	SP (aa)	*p*I	Mw (kDa)	NPS
UGT25	1554	17	8.10	59.96	66, 147, 463
UGT26	1578	22	7.59	60.24	71, 477
UGT27	1557	17	6.69	59.51	66, 189, 474, 515
UGT28	1578	20	9.13	60.10	82, 175, 245
UGT29	1623	16	9.50	61.29	78, 119, 205, 231, 327, 406
UGT30	1539	19	8.76	59.34	65
UGT31	1548	18	8.16	58.91	120, 240, 415, 507

ORF, open reading frame; SP, signal peptide; aa, amino acid; *p*I, isoelectric point; Mw, molecular weight; and NPS, *N*-glycosylation predicted site.

3.2. Sequence and Phylogenetic Characteristics of A. yunnanensis UGTs

To identify conserved residues involved in the catalysis and sugar-donor binding, 31 full-length sequences were aligned. As expected, two catalytic residues were highly conserved, of which the majority of AyunUGTs had identical amino acids at two positions (the first: histidine, H, and the second: aspartic acid, D), with the exception of AyunUGT8/12/16/20/24. In human UGT2B7, eight residues interacting with sugars were as follows: serine–tryptophan–glutamine–glutamic acid (S–W–Q–E, nucleotide binding), threonine–H (T–H, phosphate binding), and D–Q (glucuronic acid binding) [35]. For the nucleotide binding, 30 AyunUGTs possessed the conserved pattern at all the four sites, except for AyunUGT16 (asparagine (N) instead of S at the first position). Nearly all the AyunUGTs had the same "D–Q" pattern with human UGT2B7, though not AyunUGT10 (D–H). In comparison, the residues involved in the binding of phosphates were less conserved, with 16 AyunUGTs presenting the "T–H" pattern. In addition, two donor binding regions (DBRs) containing a signature motif in DBR–2 exhibited high conservation. On the contrary, the transmembrane domain and cytoplasmic tail sequences were more divergent (Figure 1).

With the aligned protein sequence of 184 UGTs from four moths, the phylogenetic tree was generated. In the tree, moth UGTs could be classified into 14 different clades, 12 of which possessed at least one member from *A. yunnanensis*. None of the AyunUGTs were found in the UGT43 and UGT48 subfamilies. Remarkably, two large clusters were formed in *A. yunnanensis*, representing AyunUGT6/14/22/32/33/38/39/47 in UGT33 and AyunUGT9/11/15/24/28/31/34/35/36/40/43/45/46/48/49 in UGT40. Such species-specific expansions were also observed in the UGT33 subfamily from three other species, as each representative was presented in one species. To determine the distribution of the clustered genes on chromosomes, they were aligned to the corresponding genomes using BLAST. As a result, the UGT genes were situated on one or two scaffolds in close proximity. In *B. mori*, eight UGT members were located on chromosome 28 and shared a mean identity of 67.69%. *H. armigera* UGTs presented a major distribution on scaffolds 395 and 562, of which the latter harbored 14 relatives varying from 56.59% to 91.41% identities. In the case of 11 *M. sexta* UGTs, they were distributed on scaffolds 00405 and 00641. Moreover, it was noticed that MsexUGT33C1–C6 were six alternatively spliced variants that shared a common 3'–terminal region and more than 75% identities with each other. Among four moth species, the high conservation was obtained in UGT44, UGT47, and UGT50, with a single gene copy in each species. In particular, members within each subfamily shared over 60% identities (Figure 2).

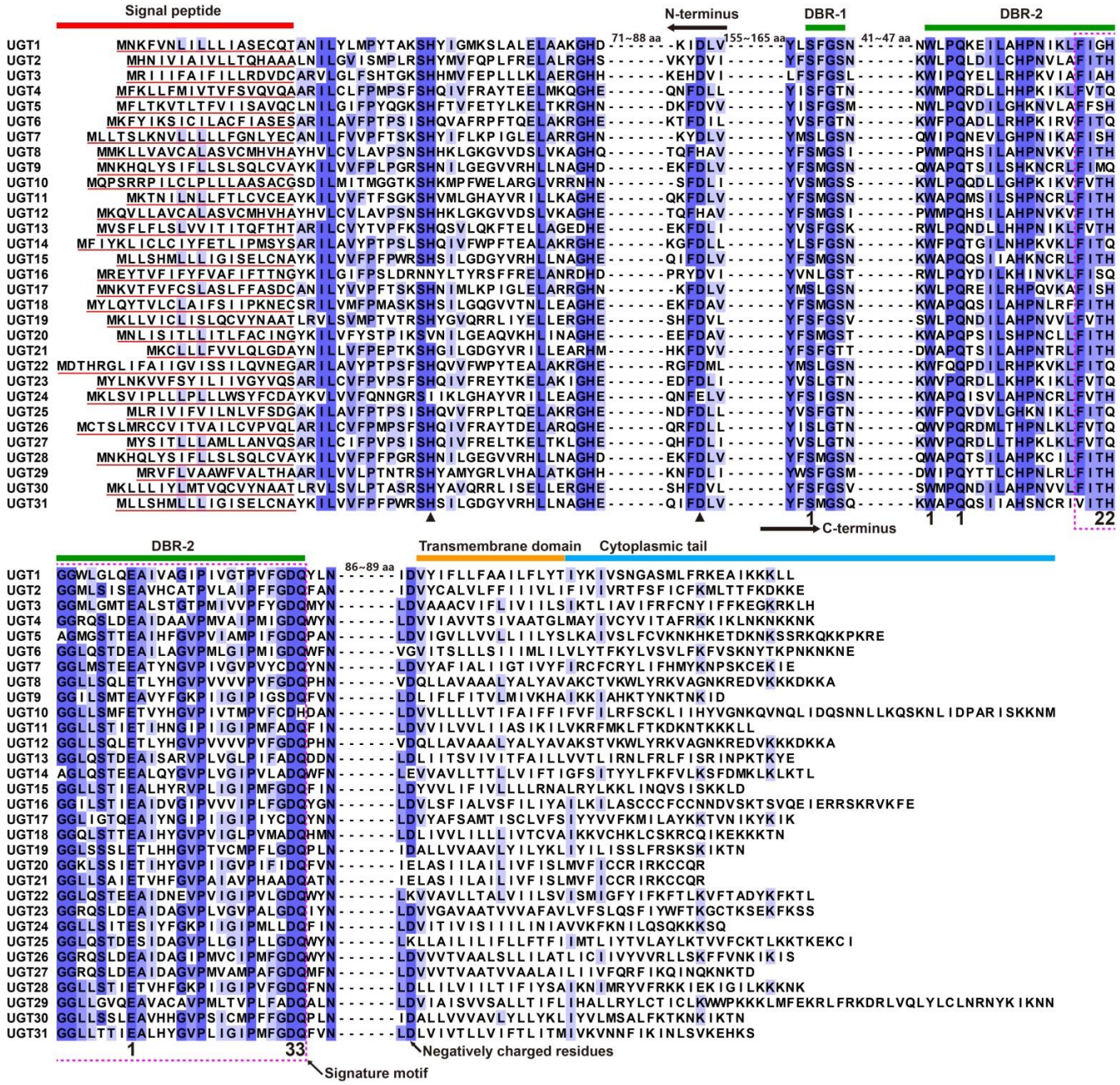


Figure 1. Multiple alignment of amino acid sequences of 31 full-length UGTs in *A. yunnanensis*. The signal peptide, N–terminus, C–terminus, donor binding regions (DBR–1 and DBR–2), a transmembrane domain, and a cytoplasmic tail are marked on the top of the alignment. Triangles represent two catalytic residues. The signature motif is indicated by a magenta dotted box. Numbers 1, 2, and 3 represent amino acids interacting with nucleotides, phosphates and glucosides, respectively.

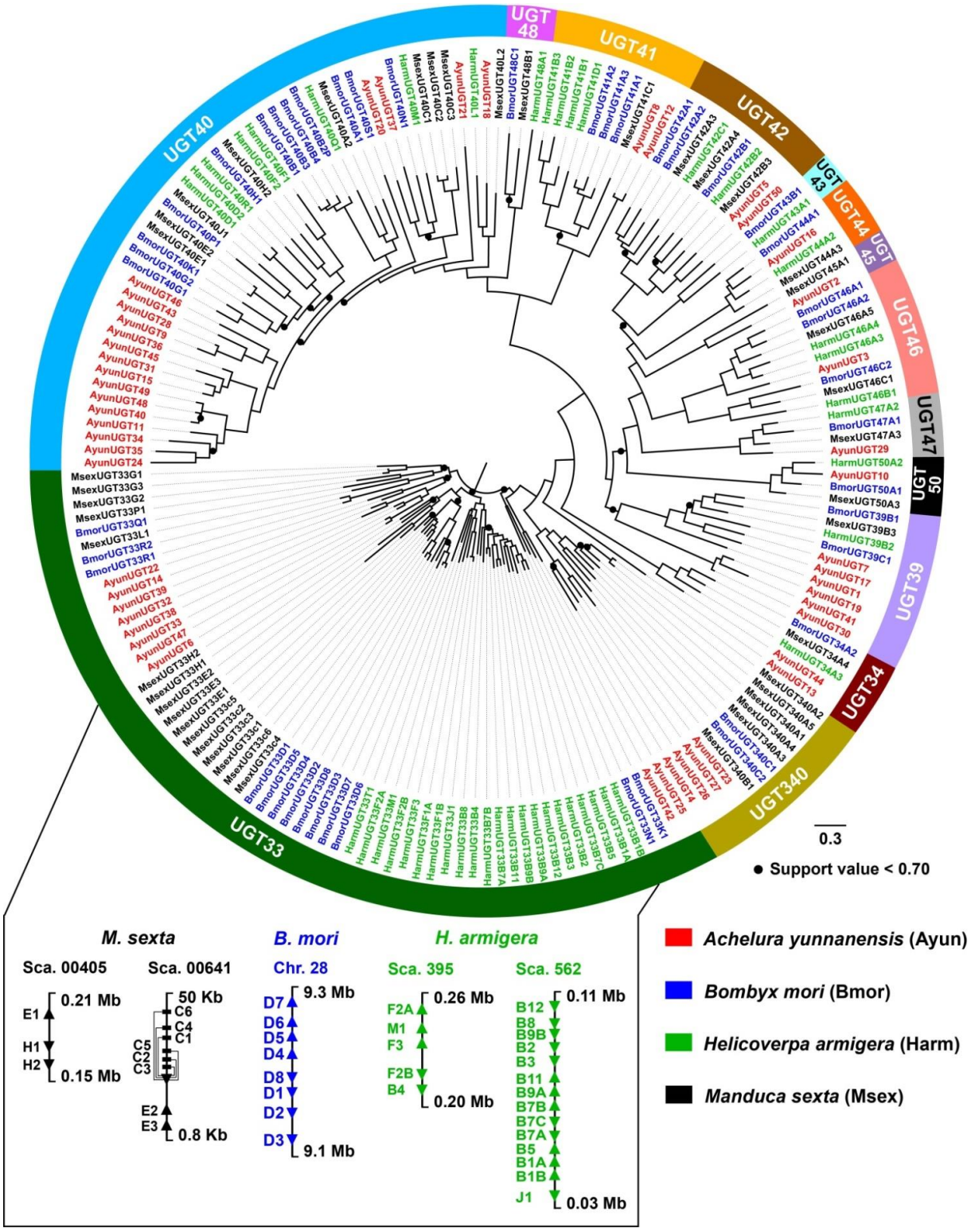


Figure 2. Phylogenetic tree of UGTs in four moths. Based on the classification of UGT subfamilies in moth species, AyunUGTs clustered into 12 subfamilies. Newly identified AyunUGTs are shown in red. In the UGT33 subfamily, three typical UGT clusters in three species with available genomes are presented on scaffolds (Sca.) or chromosomes (Chr.). Arrows indicate the transcriptional orientation of genes. In *M. sexta*, six alternative splicing variants (UGT33C1–UGT33C6) sharing a common 3′-terminus are indicated by solid lines.

3.3. Stage- and Sex-Specific Expression Profile of A. yunnanensis UGT Genes

As 10 transcripts encoding UGTs were identified exclusively from the initially assembled transcripts which were not clustered by Corset v1.05 [36], their FPKM values were unable to be calculated in various tissues. Thus, we constructed an expression profiling map of 40 AyunUGTs in 13 tissues. The majority of the genes were widely transcribed in tissues, such as AyunUGT1–3/5/7/16–22/25. Focusing on the genes specifically or highly expressed in tissues, it was found that six AyunUGTs had an antenna-enriched expression, including AyunUGT8/9/18/25/30/37/42/46 where AyunUGT8 appeared to be a male-biased relative in the antennae with a 5.12-fold difference compared to females. In contrast, AyunUGT22 expression was female-antenna-predominant. In addition, AyunUGT5, UGT41, and UGT48 were abundantly expressed in female abdomens, legs of both sexes, and male abdomens, respectively (Figure 3A).

To reconstruct and investigate the expression of 31 AyunUGTs with full-length sequences, nine additional tissues from different developmental stages were selected, including three detoxification-related tissues of larvae (midguts, fat bodies, and Malpighian tubules). Consistent with the results by RNA sequencing (RNA-Seq), most of the genes displayed a broad tissue expression. Intriguingly, the transcription of two genes was restricted to larval heads (AyunUGT11) and larval midguts (AyunUGT15). Some genes appeared to have a dominant expression in tissues, for example, AyunUGT4 and UGT31 expressed in larval midguts, AyunUGT6 expressed in larval midguts and fat bodies, and AyunUGT23 expressed in male abdomens. Out of the 31 AyunUGTs, 24 and 22 relatives were detected in antennae and maxillary palps of the larval stage, respectively, while 20 and 18 genes were presented separately in male and female antennae. In three main detoxification tissues, a number of AyunUGTs were found to have the expression in larval midguts, fat bodies, and Malpighian tubules with 26, 22, and 19 relatives, respectively (Figure 3B).

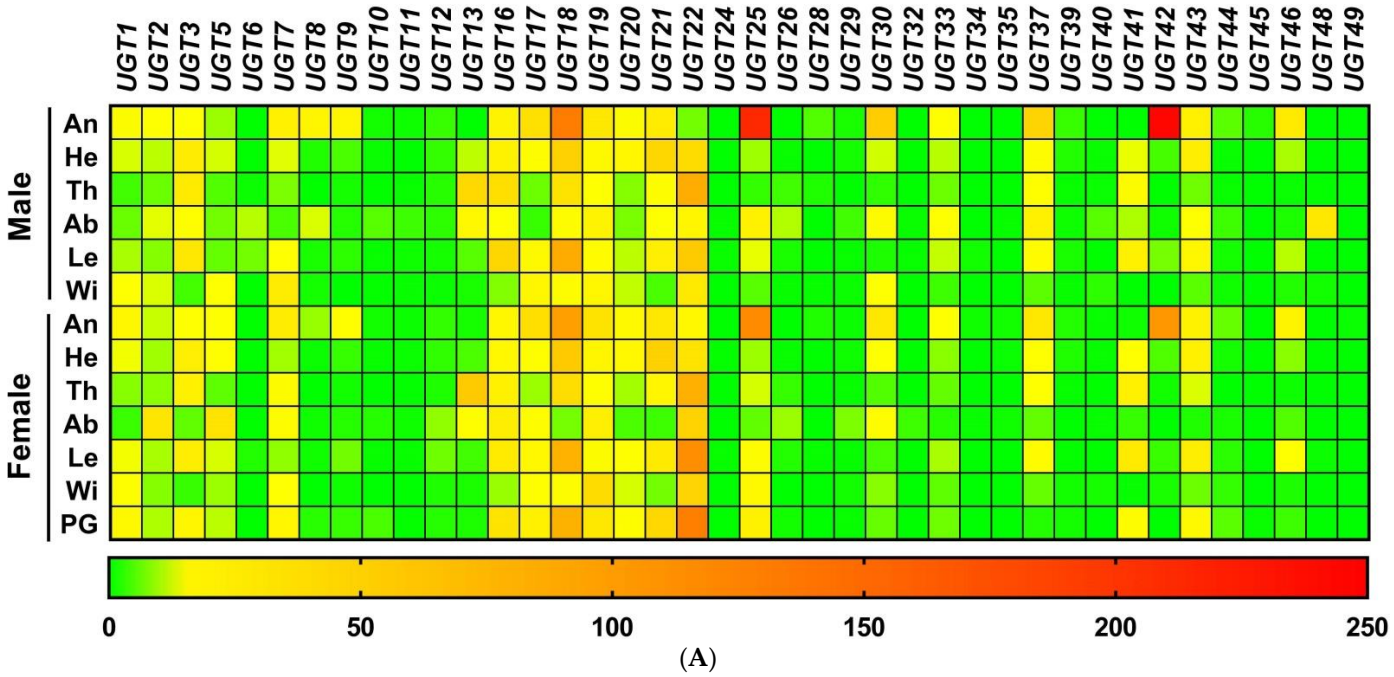
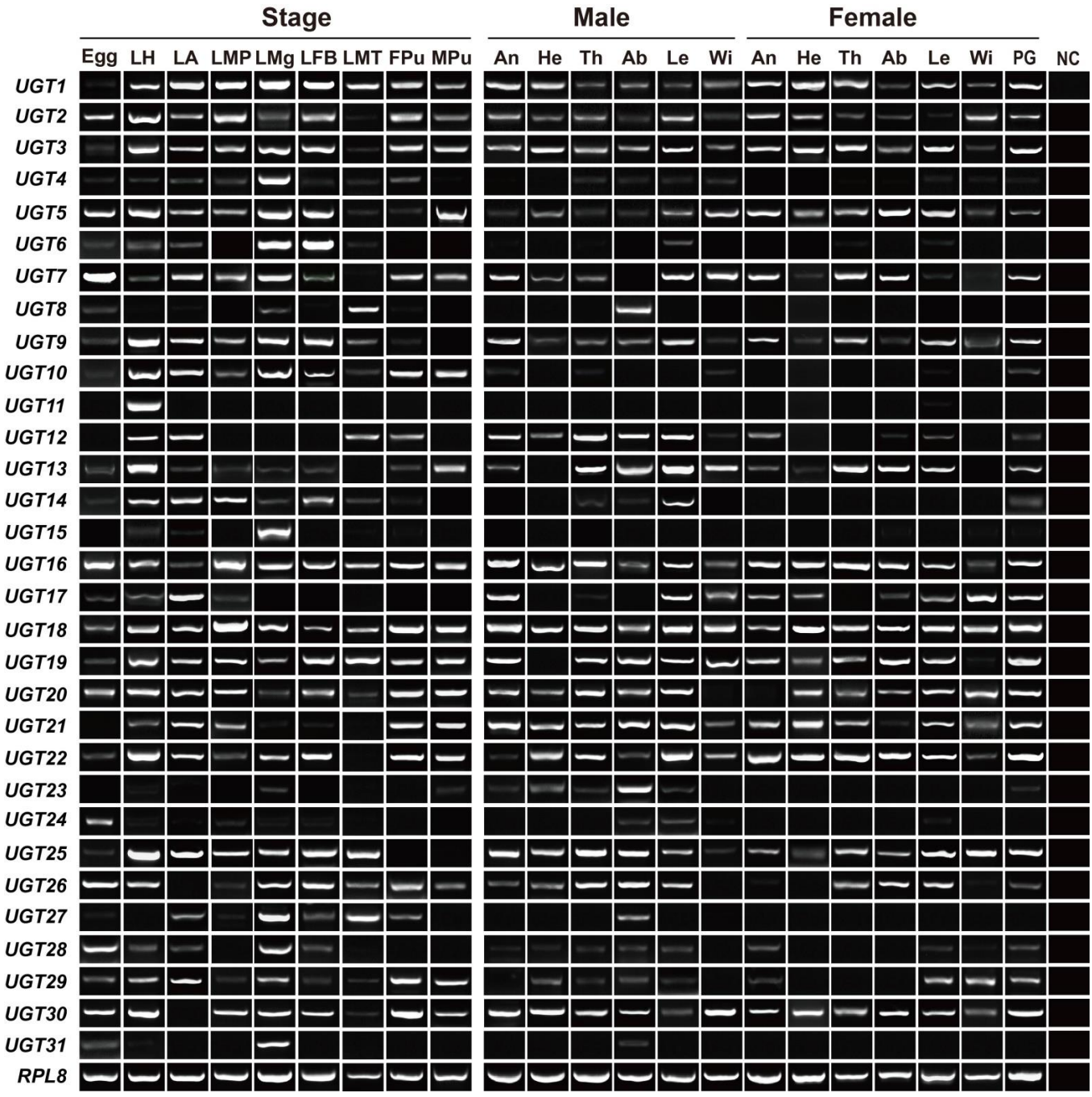


Figure 3. *Cont.*



(**B**)

Figure 3. Expression profile of 31 full-length UGT genes in different tissues of *A. yunnanensis*. (**A**) Expression of UGT genes as determined by FPKM values. (**B**) Expression of UGT genes as determined by RT–PCR. LA, larval antennae; LH, larval heads without antennae; LMP, larval maxillary palps; LMg, larval midguts; LFB, larval fat bodies; LMT, larval Malpighian tubules; FPu, female pupae; Mpu, male pupae; An, antennae; He, heads without antennae; Th, thoraxes; Ab, abdomens; Le, legs; Wi, wings; and PG, pheromone glands.

Based on the FPKM and RT–PCR results, we further selected six candidate UGT genes abundantly expressed in chemosensory and/or detoxification tissues to determine their relative levels. qPCR revealed that three genes (AyunUGT9, UGT12, and UGT18) were significantly enriched in chemosensory tissues and slightly presented in some detoxification tissues. AyunUGT9 had a significantly higher expression in larval antennae and

maxillary palps in comparison to other tissues. In contrast, the dominant expression of AyunUGT18 was presented in the antennae of the adult stage. AyunUGT12 had the highest expression level in male legs among tested tissues. Notably, AyunUGT8 exhibited particularly high transcription in larval Malpighian tubules, with a 1751.21-fold difference relative to male antennae. The remaining two genes, AyunUGT25 and UGT30, had comparable transcription levels in adult antennae and larval Malpighian tubules (Figure 4).

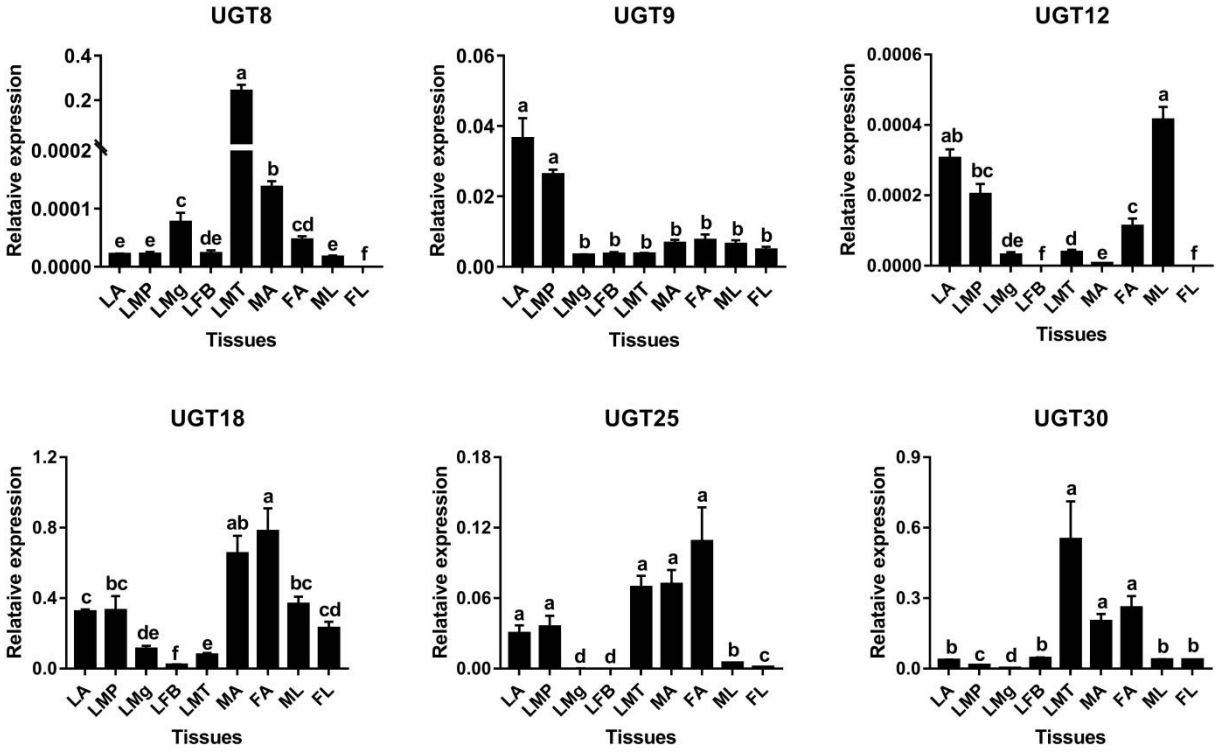


Figure 4. qPCR analysis of six candidate UGT genes in *A. yunnanensis*. Data denote mean ± standard errors. Different lowercases above bars represent significant differences among tissues ($p < 0.05$, ANOVA, LSD). LA, larval antennae; LFB, larval fat bodies; LMP, larval maxillary palps; LMg, larval midguts; LMT, larval Malpighian tubules; MA, male antennae; ML, male legs; FA, female antennae; FL, female legs.

3.4. Candidate UGT Genes in A. yunnanensis Involving Reproduction

Like most lepidopteran species, *A. yunnanensis* produces their offspring via sexual reproduction. As indicated in previous studies, reproductive-related genes in insects are rapidly evolving, greatly extending the gene repertoires associated with reproduction, including the UGT gene family [37–39]. Here, we surveyed the expression of 31 UGTs with full-length sequences in reproductive tissues. As shown in Figure 5A, the internal reproductive system of female *A. yunnanensis* was composed of a pair of accessory glands, four pairs of oviducts (ovary), one bursa copulatrix, and one spermatheca with a spermathecal gland. The reproductive system of male moths was made up of four portions, including a pair of accessory glands, one testis, one ejaculatory duct, and a pair of seminal vesicles. Out of the 31 genes, four AyunUGTs had no detectable transcription in eight reproductive tissues, i.e., AyunUGT11/14/29/31. The other 27 AyunUGTs were transcribed in at least one tissue, but eight of them only displayed weak bands such as AyunUGT8/12/15/17/21/23/27/28. Two genes, AyunUGT4 and UGT19, were presented in all tissues. Some genes appeared to be sex-specific transcripts in reproductive tissues, for example, AyunUGT2, UGT6, and UGT24 expressed in males, as well as AyunUGT9 and UGT13 expressed in females (Figure 5B).

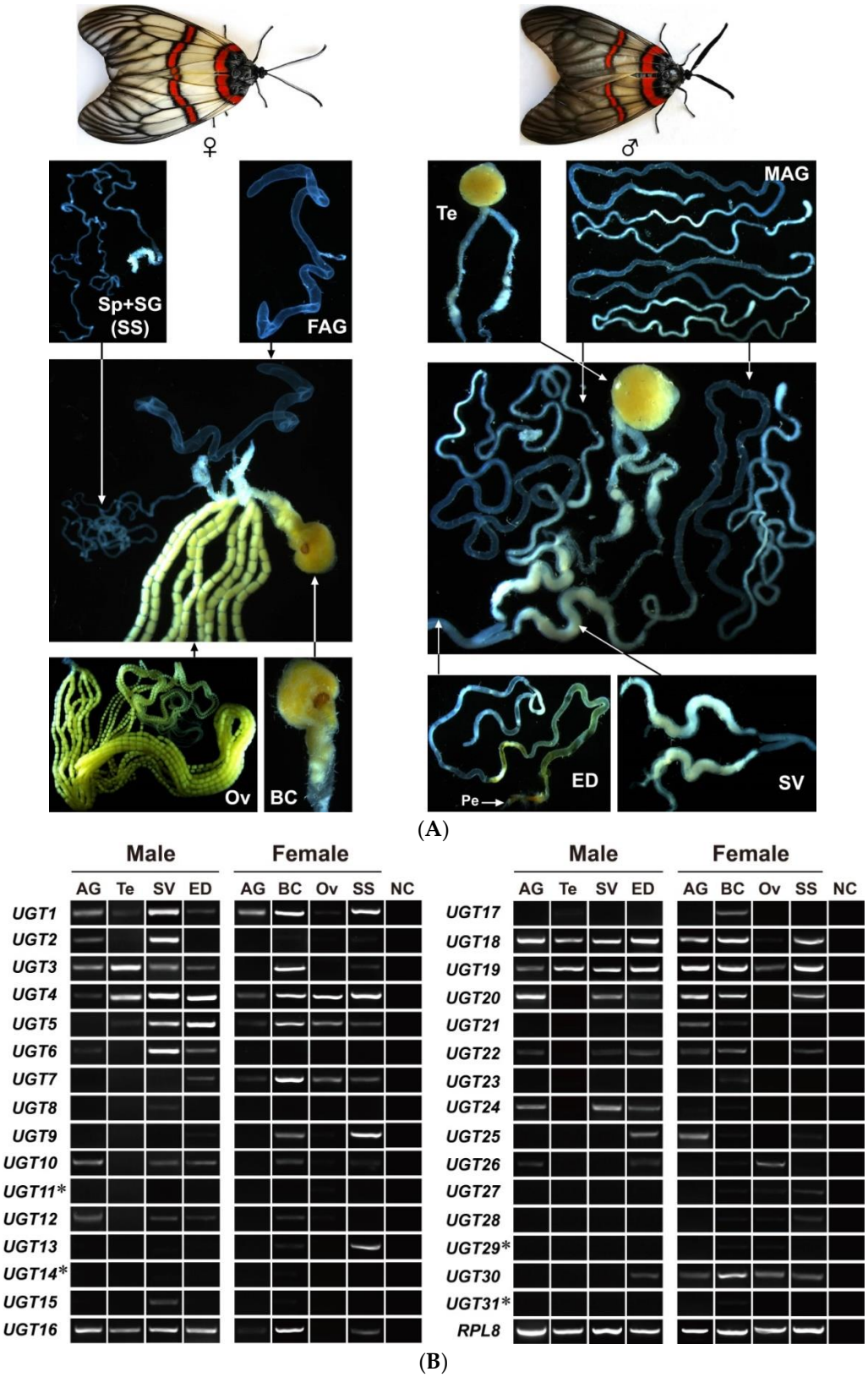


Figure 5. Expression profile of 31 full-length UGT genes in reproductive tissues of *A. yunnanensis*. (**A**) Reproductive systems of female and male moths. (**B**) Expression of UGT genes in eight reproductive tissues. Asterisks mean that the transcription of the genes was not detected in tissues. MAG, male accessory glands; Te, testes; SV, seminal vesicles; ED, ejaculatory ducts; FAG, female accessory glands; BC, bursa copulatrix; Ov, ovaries; and SS, spermathecae with spermathecal glands.

4. Discussion

In Lepidoptera, the caterpillars feed on diverse plant species. As a positive feedback, the plants will synthesize and release the secondary metabolites to cope with the herbivores. With the coevolution of moths and plants, the larvae also develop a variety of strategies to overcome the plant toxins, such as metabolic resistance. After encountering plant allelochemicals, the expression of detoxification enzyme genes in larvae is regulated as an adaptation of the larvae to xenobiotics [1,2]. Like the herbivorous insects, the larvae of *A. yunnanensis* have evolved to recognize and eat specialized host plants. During the process, the UGT gene family associated with the detoxification and insecticide resistance is indispensable for the glycosylation of xenobiotics, including plant secondary metabolites and insecticides [12,14,15,40,41]. Our current study identified and characterized the UGT enzymes in *A. yunnanensis*. This work has constituted a crucial step underlying the detoxification mechanism and allows the exploration of the specialized feeding adaptation of the *A. yunnanensis* larvae to the Rosaceae plants.

Insect UGTs have several typical characteristics, including signal peptides and conserved catalytic and sugar-donor binding residues, as implied in gene identification and functions [4]. *A. yunnanensis* UGTs possessed most, if not all, of these features. Moreover, not only did the majority of key sites have identical residues across *A. yunnanensis* and other insects, but this conservation also existed in *A. yunnanensis* and humans [4,17,35,42,43]. Outside the key residues at the conserved positions, most of AyunUGTs shared an average of only 28.23% identities to each other, especially for the diverse N-terminal binding domain. The low identities were generally presented in other insects, such as *B. mori* (30.44%) [4,11], *H. armigera* (35.30%) [4,29], *M. sexta* (31.99%) [28], *Tribolium castaneum* (35.51%) [4], *Anoplophora glabripennis* (33.10%) [44], *Xylotrechus quadripes* (35.03%) [42], and *Rhaphuma horsfieldi* (34.90%) [43]. Nevertheless, some clustered UGTs possibly derived from gene duplications retained high conservation (>50% identities) as presented in the tree, typically regarding the UGT33 and UGT40 subfamilies as discussed below.

In comparison to phase I metabolizing enzyme genes such as P450s and COEs, moth UGTs harbor a relatively stable gene number, ranging from 42 in *Helicoverpa zea*, 44 in *M. sexta*, 45 in *B. mori*, and 46 in *H. armigera* to 48 in *S. frugiperda* [4,11,28,29,45]. Our current study identified a comparable UGT number through the transcriptome analyses (50 relatives), very close to those of the above species with available genomes, and slightly more than 41 conreads in a zygaenid moth *Zygaena filipendulae* derived from the sequenced transcriptome [46]. In phylogeny, *A. yunnanensis* UGTs were expanded mainly in the UGT33 and UGT40 subfamilies, accounting for approximately half of all identified genes. Such large-scale expansions of UGTs in the two subfamilies were also reported in *S. frugiperda* [45], *H. armigera* [29], *B. mori* [4], and *Epiphyas postvittana* [47]. Notably, most members of the UGT33 and UGT40 subfamilies in *Plutella xylostella* [48] and *Spodoptera exigua* [19] have been found to have inducible expression by multi-insecticides. Hence, it is reasonably inferred that the lineage-specific expansions in UGT33 and UGT40 of *A. yunnanensis* may be associated with insecticide resistance, as an adaptation of insects to man-made chemical pesticides.

Concerning these UGT clusters in UGT33, we detected their physical locations in the chromosomes. The tandem arrays of UGTs on scaffolds or chromosomes were probably attributed to gene duplication events, coupled with their high identities. The clusters and high conservation of UGTs were also found in UGT33 and UGT40 of *A. yunnanensis*, reflecting a common phenomenon that species-specific expansions have occurred in moths. Of the 14 proposed clades, three orthologous groups (UGT44, UGT47, and UGT50) still retained a strict single gene copy in each species, suggesting the genes in each group may bear similar functional roles in moths, together with conserved catalytic and sugar-donor binding residues. Notably, the singleton in UGT50 was also presented in other insects [4,42]. Moreover, insect UGT50 members generally possessed conserved gene structure, for example, UGT50s in *B. mori*, *M. sexta*, and *H. armigera* comprised four introns

as characterized in the present study, in agreeable with those of putative orthologs in some coleopteran species and human [42].

In *Z. filipendulae*, ZfilUGT33A1 could catalyze the synthesis of two cyanogenic glucosides by glycosylation, i.e., linamarin and lotaustralin secreted by *Zygaena* larvae [49]. Intriguingly, the two toxic cyanogenic glucosides were also isolated and identified from the *A. yunnanensis* larvae [50]. To identify putative UGTs in *A. yunnanensis* in response to the two chemicals, we screened putative ortholog(s) of ZfilUGT33A1 by a combination of the identities and phylogeny. As a result, AyunUGT14 and UGT22 showed the highest identities of 62.94% and 63.13% with ZfilUGT33A1, respectively. Further, the three UGT members phylogenetically formed an independent small clade with a high support value. Hence, it is suggested that AyunUGT14 and UGT22 belonging to the UGT33 subfamily probably play a critical role in the glycosylation of the two cyanogenic glucosides.

At the larval stage, detoxification-related tissues, referring to midguts, fat bodies, and Malpighian tubules, are of utmost importance for larvae to metabolize various harmful chemicals. In the expression profiling analyses, a number of AyunUGTs were transcribed in the three tissues. In other lepidopteran species, UGTs enriched in larval midguts, far bodies, or Malpighian tubules were common, and moreover, responded to plant defensive compounds and insecticides [4,11,12,15,19,48]. Given the fact that the larvae of *A. yunnanensis* feed on the plants of the Rosaceae family [20–22], AyunUGTs presented in larval detoxification tissues (especially AyunUGT4/8/15/27/31) are key molecular targets for detoxifying defensive compounds of host plants. Beyond putative roles of AyunUGTs in detoxification, it was found that a comparable number of genes had the expression in main chemosensory tissues, including antennae and maxillary palps. In moths, several studies have suggested olfactory roles of UGTs, such as *M. sexta* [9], *B. mori* [4,11], *S. littoralis* [8], *A. lepigone* [17], and *P. xylostella* [18]. In addition, this olfactory association was also indicated in non-lepidopteran species, such as *D. melanogaster* [51–53], *Holotrichia parallela* [54], *X. quadripes* [42], and *R. horsfieldi* [43]. Accordingly, it is inferred that the antenna-enriched AyunUGTs in *A. yunnanensis* (particularly AyunUGT9, UGT12, and UGT18) may participate in the sensing of odorant molecules, and are regarded as odorant degrading enzymes. Notably, the majority of AyunUGTs had detectable expression in reproductive tissues, suggesting their reproductive roles. In *S. litura*, transcriptome and proteome analyses revealed the presence of UGTs in reproductive tissues [37]. A similar reproductive expression feature of UGTs was also found in *B. mori* [4,11], *H. armigera* [29], and *D. melanogaster* [4,55], but their functions remain to be identified.

5. Conclusions

Our current study has characterized the UGT gene repertoire in *A. yunnanensis*, with a combination of transcriptomics, bioinformatics, phylogenetics, and PCR approaches. From the transcriptomes, a total of 50 UGTs were identified, of which some shared high conservation and phylogenetically clustered together. At the key sites, most of AyunUGTs harbored conserved residues with the UGTs from other insects and human. In the expression profiling analyses, a number of UGTs were found to have the expression in chemosensory tissues as supported by PCR and RNA–Seq (FPKM > 1), such as 24 and 22 relatives separately in larval antennae and maxillary palps, 27 in male antennae, 24 in female antennae, 31 in male legs, and 25 in female legs. Moreover, a comparable number of genes were observed in detoxification tissues, including 26, 22, and 19 relatives in larval midguts, fat bodies, and Malpighian tubules, respectively. The expression of at least 27 UGTs was detected in reproductive tissues of female and male moths. Together, this study reports, for the first time, the detoxification-related UGT gene family, and allows for exploring the adaptation of the *A. yunnanensis* larvae to feeding hosts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14050407/s1. Table S1: Primers used for the expression profiling analyses of UGT genes in *A. yunnanensis*; Additional File S1: Amino acid sequences of UGTs in *A. yunnanensis*.

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