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Toll-interacting protein participates in immunity and development of the lepidopteran insect *Antheraea pernyi*

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Research Paper

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Abstract

Toll-interacting protein (Tollip) participates in multiple biological processes. However, the biological functions of Tollip proteins in insects remain to be further explored. Here, the genomic sequence of tollip gene from *Antheraea pernyi* (named *Ap-Tollip*) was identified with a length of 15,060 bp, including eight exons and seven introns. The predicted *Ap-Tollip* protein contained conserved C2 and CUE domains and was highly homologous to those tollips from invertebrates. *Ap-Tollip* was highly expressed in fat body compared with other determined tissues. As far as the developmental stages were concerned, the highest expression level was found at the 14th day in eggs or the 3rd day of the 1st instar. *Ap-Tollip* was also obviously regulated by lipopolysaccharide, polycytidylic acid or 20E in different tissues. In addition, the interaction between Ap-Tollip and ubiquitin was confirmed by western blotting and pull-down assay. RNAi of *Ap-Tollip* significantly affected the expression levels of apoptosis and autophagy-related genes. These results indicated that *Ap-Tollip* was involved in immunity and development of *A. pernyi*.

Introduction

Toll-interacting protein (Tollip) localizes to early endosomes (Capelluto, 2012; Lu *et al.*, 2014), multivesicular bodies (Katoh *et al.*, 2004) and Golgi apparatus (Li *et al.*, 2004) and closely relates to IL-1β, IL-13, toll-like receptor, TGF-β and cGAS-STING signaling pathways (Burns *et al.*, 2000; Zhang and Ghosh, 2002; Zhu *et al.*, 2012; Ito *et al.*, 2018; Pokatayev *et al.*, 2020). Tollip from human contained four transcriptional forms (Lo *et al.*, 2009) with three typical domains, including the TBD (Tom1-binding domain), central C2 (conserved 2) domain and CUE (coupling of ubiquitin to endoplasmic reticulum degradation) domain (Yamakami *et al.*, 2003). As a lipid molecule binding domain (Ankem *et al.*, 2011), the C2 domain mediated the specific binding to PtdIns3P or PtdIns(3,4,5) (Li *et al.*, 2004) and resulted in membrane localization. The C-terminal domain triggered intramolecular ubiquitination (Kang *et al.*, 2003; Shih *et al.*, 2003) and protein-protein interactions (Zhang and Ghosh, 2002; Lim *et al.*, 2019).

In vertebrates, tollip inhibited TLR-mediated immune responses and apoptosis by binding to TLRs (Zhang and Ghosh, 2002; Mukherjee and Biswas, 2014) or IL-1R-mediated signaling pathway through regulation of phosphorylation (Burns *et al.*, 2000). It was reported that tollip activated the immune system by stabilizing STING proteins (Pokatayev *et al.*, 2020) and overexpression of tollip inhibited NF-κB activation. Some clues indicated the important role of tollip in human diseases like cancer (Ahmed *et al.*, 2018), inflammation (Maillard *et al.*, 2014; Katayanagi *et al.*, 2022) Alzheimer's disease (Cribbs *et al.*, 2012), Parkinson's disease (Ryan and Tumbarello, 2021), Huntington's disease (Doi *et al.*, 2004). Although some functions of tollip in few non-mammals have been partially documented (Rebl *et al.*, 2008, 2011; Zhang *et al.*, 2015; Shan *et al.*, 2016; Feng *et al.*, 2019; Jiang *et al.*, 2022), the roles of tollip in insects remained unclear.

The oak silkworm *Antheraea pernyi* is one of the important wild silk-producing insets in China and used for food, silk reeling, and medicinal purposes (Liu *et al.*, 2010; Li *et al.*, 2017). It was well known that diapause, molting, metamorphosis as well as immunity were very important for the growth of insects (Hillyer, 2016; Kang *et al.*, 2019; Truman, 2019; Numata and Shintani, 2023) and 20-hydroxyecdysone (20E) could promote the apoptosis and autophagy during these biological processes (Romanelli *et al.*, 2014; Dong *et al.*, 2015). In this study, a tollip homolog was first characterized from *A. pernyi* and its expression patterns were investigated in various tissues, developmental stages or under different challenges. The protein interaction between ubiquitin and *Ap-tollip* was determined. In addition, RNA interference of Ap-tollip was performed to explore its effect on the expression of target genes in apoptosis and autophagy signal pathways.

Materials and methods

Experimental animals

Healthy *A.pernyi* pupae were maintained at 4°C and released from stasis according to the method (Bao *et al.*, 2022), then the pupae were used for moth production. After copulation and laying eggs, some fertilized eggs were collected on days 2, 4, 6, 8, 10, 12, and 14. The other fertilized eggs were kept at 22°C with 70% humidity and 12 h light:12 h dark for ants hatching. The larvae were fed at 22°C with fresh oak leaves until 4th instar and used for experiments.

Sequence analysis of Ap-Tollip

Total RNA or DNA was extracted from fat body and used for PCR. Specific primers (shown in Table 1) were designed for the identification of genomic sequence of Tollip. The PCR procedure was 94°C 5 min pre-denaturation, followed by 35 cycles of 95°C 30s denaturation, 54–56°C 40s annealing and 72°C 1 min extension, and a final extension at 72°C for 5 min. PCR products were purified and sequenced by BGI Genomics Co., Ltd (Shenzhen, China).

The sequence of *Ap-Tollip* was analyzed by BLAST (https://blast.ncbi.nlm.nih.gov). The amino acid sequence and conserved domains of *Ap-Tollip* proteins was predicted using AAT Bioquest (Https://www.aatbio.com/) and SMART (https://www.smart.embl.de/) software, respectively. The Prabi (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and Phyre2 tool (Kelley *et al.*, 2015) were used for prediction of Secondary structures and tertiary structures. Multiple sequence comparisons were performed using DNAMAN with its default parameters. A neighbor-joining phylogenetic tree was constructed using MEGA software (Kumar *et al.*, 2018) based on Tollip sequences from different species.

Protein expression and purification

To investigate the interaction between Ap-Tollip and ubiquitin, fragments of *Ap-Tollip* and *Ap-ubiquitin* were amplified by PCR using specific primers (Tollip-F and Tollip-R, Ubi-F and Ubi-R, Table 2) and then ligated to pET-28a (+) or PGEX-4 T-1 vector after digestion with restriction enzymes. The recombinant plasmids were transformed into *E. coli* BL21(DE3) cells for protein expression induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The recombinant proteins were purified using Proteinlso® Ni-NTA Resin or Proteinlso® GST Resin (TransGen Biotech, Beijing), then were visualized by 12% SDS-PAGE and the concentration of purified proteins was quantified by BCA method (Walker, 1994). In addition, the antiserum against Ap-Tollip was prepared. Briefly, the purified Ap-Tollip proteins (100 μg) were incubated with adjuvant and immunized New Zealand white rabbits at two-week intervals, the antibodies were collected after the fourth immunization and used for western blotting.

Western blotting

Protein samples were separated by 12% SDS-PAGE electrophoresis, and then transferred to PVDF (polyvinylidene difluoride) membrane (Millipore Sigma, USA) and blocked with PBST (phosphate buffered saline containing 0.1% Tween-20) containing 5% non-fat milk powder for 1 h. Then, 5 mg of purified proteins were incubated with the PVDF membrane for 4 h at room temperature.

Table 1. Primers for the PCR amplification of Ap-Tollip genomic sequence

Name	Primer sequences (5'-3')
F1	CTGACCTCTTAGAATCGTTTGTT
R1	TCTGCGTTGAATACTTACTTTGG
F2	CCCAGACAAACAATGGACACTTCA
R2	CCACCTCTTCTACTTCCTGCTCAA
F3	AATGACGAATTTGAGCAGGAAGTA
R3	TATCCAGAAGCCTAAACAAGAAGA
F4	GTAGCCCACGGAAGAGTCATA
R4	GTCCCTCCCAGAAATAAATAG
F5	TGTCAGTAGTTCCGTCAAGTTGTGTT
R5	GCAAGGGTGGATTCTTATTTTTTTTT
F6	GCAGCCAGCATAAAGTAAAGAAAC
R6	AATAGGGGGCAAAAAATGACAGAT
F7	ACTATCTGTCATTTTTTGCCCCCT
R7	TATCTGTTGTTCCATCTGCG
F8	TGCTACTGGGTCCCCTCCCTAAT
R8	AAGATGACGCAGCAATAATA
F9	TATTATTGCTGCGTCATCTT
R9	TGTATTGTCATCGACCTTTG
F10	CAAAGGTCGATGACAATACA
R10	CCAGAACCCGAATAATAAAA
F11	TCTTTACTGGTATGTCTTCTTT
R11	CACTATATTTTTGTTGTTTGAT
F12	TAATAACACTGATAAAGTCAAAAA
R12	AACACTAATAAACAACAAAAAGAA
F13	TATCGTTCCCATACTCT
R13	TGTCTCGCTTTGGTTAC
F14	CTCGGTCTTCTACCCTATG
R14	TATTTCGTATCGGGCAACT
F15	AAGTTGCCCGATACGAAAT
R15	CACTTCCTTGTCAATGCTC
F16	GAGTGAGAAAGTCGCACGGAAAT
R16	CCTCGGCTTCAATGAATCCTAAT

After washing with PBST, membranes were incubated with primary antibodies for 2 h and then incubated with the secondary antibodies (TransGen Biotech, Beijing) for 50 min at room temperature. Finally, the membranes were stained with the Enhanced HRP-DAB Fifth Color Development Kit (Tiangen, Beijing).

Pull-down assay

To further confirm the interaction between Ap-Tollip and Ap-ubiquitin, Ap-Tollip protein was used as pery protein and Ap-Ubi as bait protein. Pull-down assay was performed using Pierce™ His Protein Interaction Pull-down Kit (Thermo Scientific, USA) according to the instructions. The

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Table 2. Primers used for real-time PCR, protein expression and RNAi

Name	Primer sequences (5'-3')
For protein expression	
Tollip-F	GGAATTCATGACTTCTGCAATACCTACTGATA
Tollip-R	CCGCTCGAGTTATTCTGACATCTGTAATAGAGAG
Ubi-F	CGGGATCCATGCAGATATTCGTTAAGACCCT
Ubi-R	CCGCTCGAGCTAATTGCCACCTCTAAGACGTA
For Tollip dsRNA synthesis	
Tollip-RNAi-F	GGATCCTAATACGACTCACTATAGGGTGCTGATTATCAAGCCGCAC
Tollip-RNAi-R	GGATCCTAATACGACTCACTATAGGGGACCCACACGCAATCTAACA
EGFP-RNAi-F	GGATCCTAATACGACTCACTATAGGCAGTGCTTCAGCCGCTACCC
EGFP-RNAi-R	GGATCCTAATACGACTCACTATAGGACTCCAGCAGGACCATGTGAT
For qRT-PCR	
Tollip-F1	AGGCATGATCAACCTTGTGC
Tollip-R1	CCTTGTCAATGCTCGGGAAC
18S-F	CGATCCGCCGACGTTACTACA
18S-R	GTCCGGGCCTGGTGAGATTT
Dronc-F	AGGTGGACTGCGAGAATCTC
Dronc-R	GAGCCCTTCCTCTCGTATCC
ATF-F	AACAAGAAGCCACGGAACAC
ATF-R	TGGCCTCCTTCAGTTGTTGA
Dredd-F	TGCTGATGGGATCTCGGATT
Dredd-R	GGTACTTGTGCACGCTTCAA
Daxx-F	TACGGCAGCAAATCATCACG
Daxx-R	CATCAGCTCGGGATCCTCAT
Fadd-F	TTTTGAGACAATTACGGAGGAAC
Fadd-R	CTCTGAGGATCCGCTCTCTG
Beclin-F	GCTACTATGGTCCCTGGCAA
Beclin-R	CAACCTGCTCCTTGAACTGC
ATG5-F	CCGGTTTGTTTCCAACTTGC
ATG5-R	AACCACATTTCACTGTCGGC
ATG7-F	GAGACCAGGAGTAGCTGCAA
ATG7-R	GCACACGTCGGTAAAACAGT
Pdk1-F	TCCTTTTAACGACGGGTCCA
Pdk1-R	CGATCACACGTTGCCATTCT
Rheb-F	AGCTCTTGGATATGGTCGGA
Rheb-R	ACACATCCATTCTTGTCAGGT
BNIP3-F	CTCCAGCGTAAAAGGAAGCC
BNIP3-R	ATAGACGAGGTGGCTGAAGG

Note: Restriction sites are underlined, T7 promoters are double underlined.

purified recombinant Ap-Tollip protein was immobilized on His agarose beads and then the purified recombinant Ap-ubiquitin proteins were incubated with Ap-Tollip proteins. The eluate was collected for SDS-PAGE electrophoresis and subsequently validated by western blotting using anti-GST antibodies. Non-treated agarose was used as a negative control.

Real-time PCR

The cDNA was reverse transcribed using PrimeScript™ RT Master Mix (Takara, China) with RNA templates from different tissues and the concentration of cDNA was adjusted to 150 ng/μL with ddH_2O. The primers of candidated genes for PCR were listed in Table 2 and the PCR program was: 95°C for 5 min,

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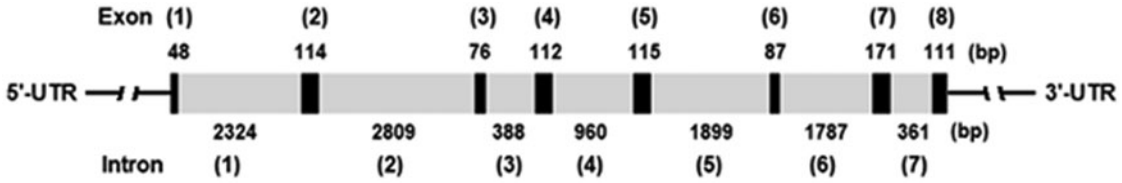

Figure 1. Genomic sequence analysis of *Ap-Tollip*. The exons and introns were marked by black and gray, respectively. UTR, untranslated region.

followed by 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. The relative expression of genes was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) and 18S rRNA gene (GenBank accession no. DQ347469) was selected as an internal reference. Each experiment was performed in three replicates. The data were analyzed by Student's *t*-test or one-way ANOVA analysis using SPSS software and difference was considered as significant when P-value was less than 0.05.

Expression pattern analysis of Ap-Tollip

The tissues epidermis, fat body, midgut, hemocytes, silk gland and malpighian tubule were collected for tissue distribution analysis. Total RNA was extracted using Trizol method and total protein from each tissue was extracted using the Total Animal Protein Extraction Kit (Coolaber, Beijing) according to the instructions. The expression level of *Ap-Tollip* was detected by real-time PCR or western blotting using anti-Tollip and anti-β-tublin antibodies. For different developmental stages, the fertilized eggs at 2, 4, 6, 8, 10, 12 or 14 day after oviposition as well as the larvae from 1st to 4th instars were collected for real-time PCR analysis. Thirty eggs or three larvae were concluded in each group and all the experiments were done in tripilcate. For various challenges, 10 μL of lipopolysaccharide (LPS, 1 μg/μL) or polyinosinic: polycytidylic acid (poly I: C, 1 μg/μL) or 20-hydroxyecdysone (20E, 1 μg/μL) was injected into each *A. pernyi* pupa, respectively, and hemocytes or fat bodies were collected at 3 h, 6 h, 9 h, 12 h and 24 h post-injection. Three pupae were injected in each group and biological

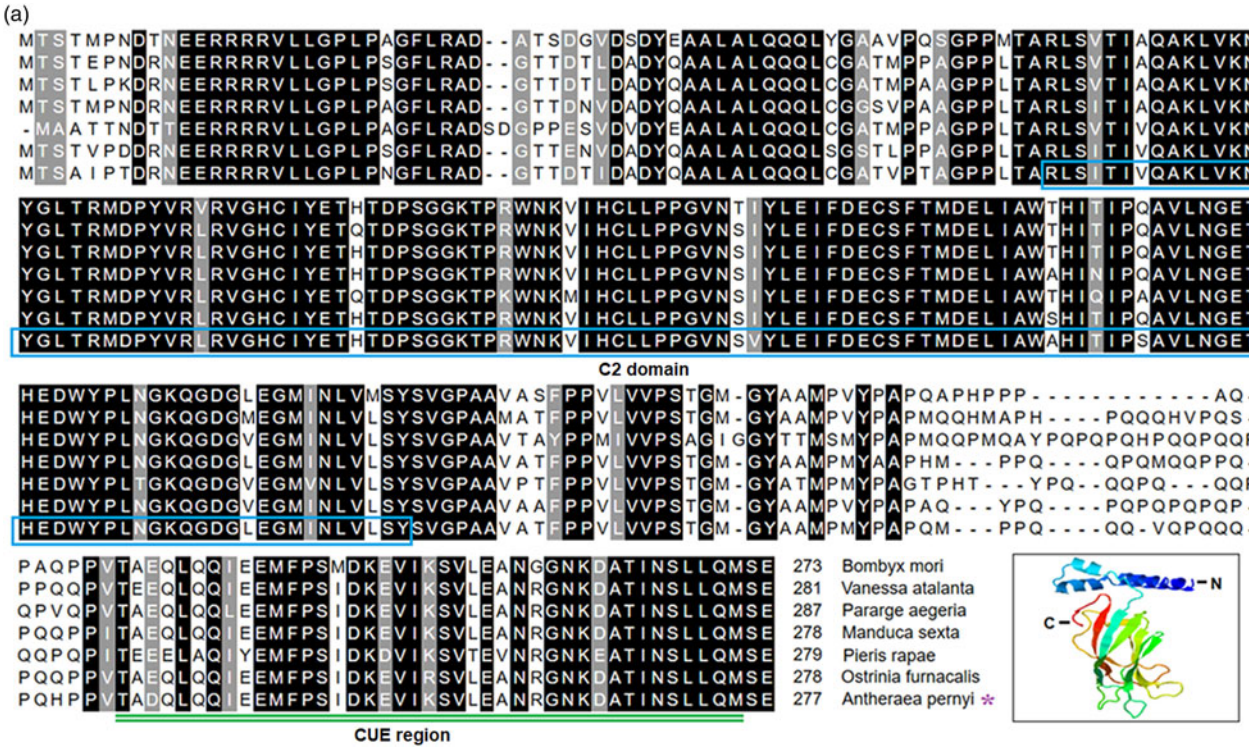

Figure 2. (a) Sequence alignment of Ap-Tollip with other insect Tollips. The conserved C2 domain was boxed and the CUE domain was double underlined. The predicted three-dimensional structure of Ap-Tollip was shown at the bottom right-hand corner. (b) Phylogenetic trees were constructed using the neighbor-joining method based on the amino acid sequences of Tollip proteins from different species. Numbers close to each branching point indicate the percentage of 1000 bootstrap iterations supporting the branch. The GenBank numbers for these tollip sequences are : *Bombyx mori* (XP_037871305), *Vanessa atalanta* (XP_047538853), *Pararge aegeria* (XP_039747777), *Manduca sexta* (XP_030035003), *Pieris rapae* (XP_022120383), *Ostrinia furnacalis* (XP_028157188), *Mus musculus* (CAB58121), *Gallus gallus* (NP_001006471), *Bos taurus* (NP_001035050), *Homo sapiens* (CAB58118), *Macaca mulatta* (NP_001248200), *Salmo salar* (ACI33751), *Ctenopharyngodon idella* (AFM09715),*Larimichthys crocea* (XP_027137215), *Penaeus vannamei* (AET79206), *Penaeus japonicus* (BAK19511), *Mizuhopecten yessoensis* (AKO62848), *Mytilus galloprovincialis* (AHI17285), *Apis mellifera* (XP_624417), *Anopheles gambiae* (XP_562476), *Anoplophora glabripennis* (XP_018572445), *Tribolium madens* (XP_044255367), *Halyomorpha halys* (XP_014283422), *Nilaparvata lugens* (XP_022189393), *Locusta migratoria* (AWJ58418), *Blattella germanica* (PSN31565), *Zootermopsis nevadensis* (XP_021925455), *Plutella xylostella* (XP_037976358), *Papilio machaon* (XP_045539518), *Eumeta japonica* (GBP04589), *Leguminivora glycinivorella* (AXS59135), *Bombyx mandarina* (XP_028042731), *Danaus plexippus plexippus* (XP_032522997), *Spodoptera frugiperda* (XP_050552012), *Galleria mellonella* (XP_026751574).

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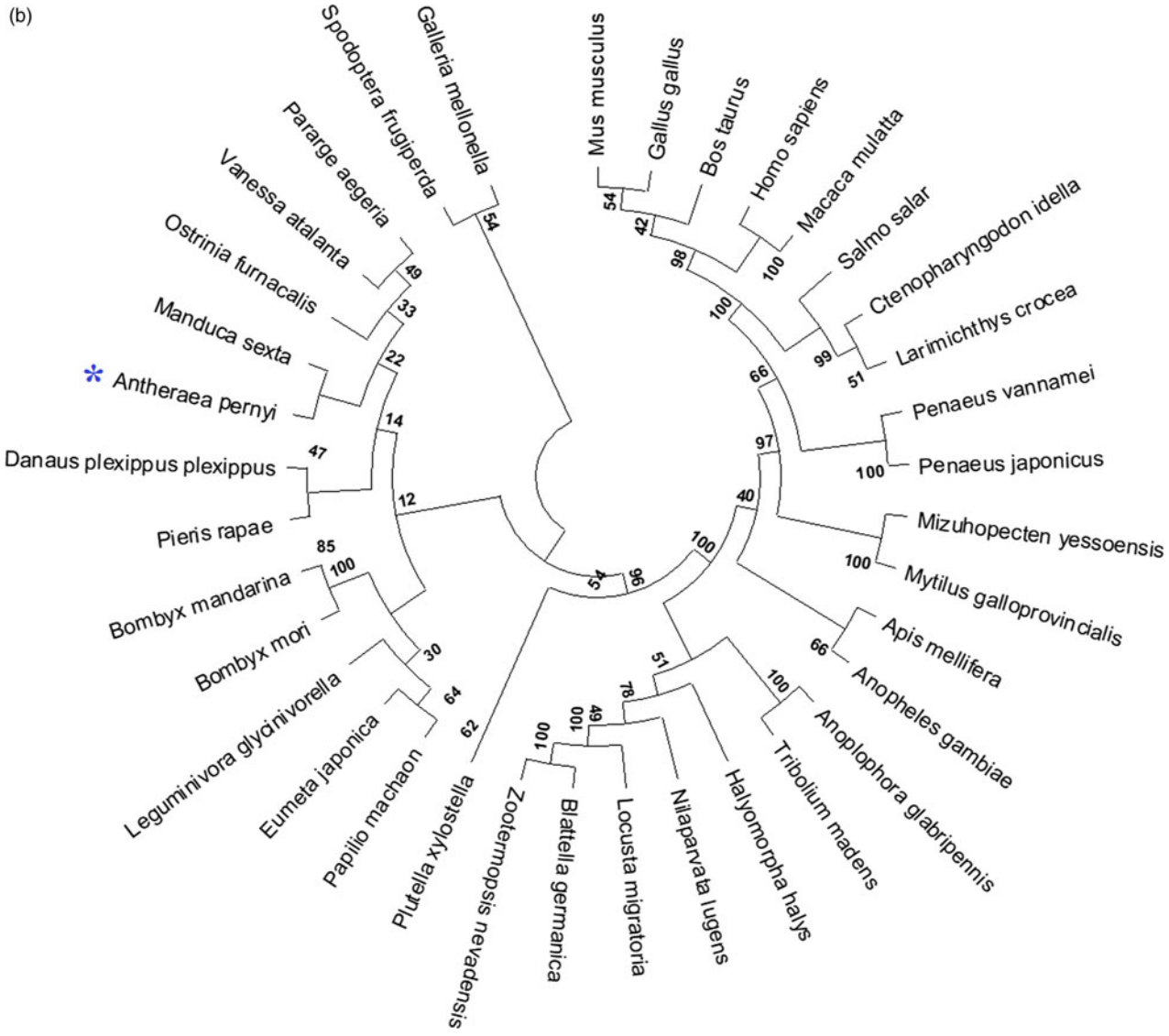

Figure 2. Fig 2. continue

sampling was repeated three times. The PBS injection group was used as a negative control,

RNAi of Ap-Tollip

The dsRNA sequences of *Ap-Tollip* and EGFP were synthesized in vitro using ScriptMAX^{TM} Thermo T7 Transcription Kit (TOYOBO, Japan)according to the instructions. dsRNA concentration was measured using an Ultra-micro nucleic acid concentration meter and adjusted to 1 μg/μL. 10 μl dsRNA was separately injected into each pupa and fat bodies were collected at 24 h and 48 h after injection. The changes in the expression levels of candidated genes involved in autophagy and apoptosis pathways were detected by real-time PCR after RNAi. The EGFP dsRNA injection group was used as a control.

Results

Sequence analysis of Ap-Tollip

The genomic sequence of *Ap-Tollip* was 15,060 bp long with eight exons and seven introns (shown in fig. 1 and supplementary file). Several possible promotor sites (1656–1706 bp and 2221–2271 bp) and binding sites for transcription factors CREB and ATF were predicted. The open reading frame of *Ap-Tollip* (GenBank accession no. OQ447887) was 834 bp long and encoded a protein including a conserved C2 domain (65–183 aa) and a CUE region (235–275 aa). The predicted Ap-Tollip protein included 31.77% alpha helix, 6.86% beta turn and 44.77% random coil (fig. 2A). It was highly homologous to those Tollip proteins from Lepidopteran insects in terms of conserved structural domains (fig. 2B).

Protein interaction between Ap-Tollip and Ap-ubiquitin

Recombinant Ap-Tollip (fig. 3) and Ap-ubiquitin (fig. 4) proteins corresponding to the predicted molecular weights (about 36 kDa and 34 kDa, respectively) were successfully expressed and purified from in *E. coli* cells, which was confirmed by SDS-PAGE and western blotting. After protein purification, the interaction between Ap-Tollip and ubiquitin was further verified by His pull-down method. In His pull-down assay, non-treated agarose was set as a negative control and immobilized bait was used as

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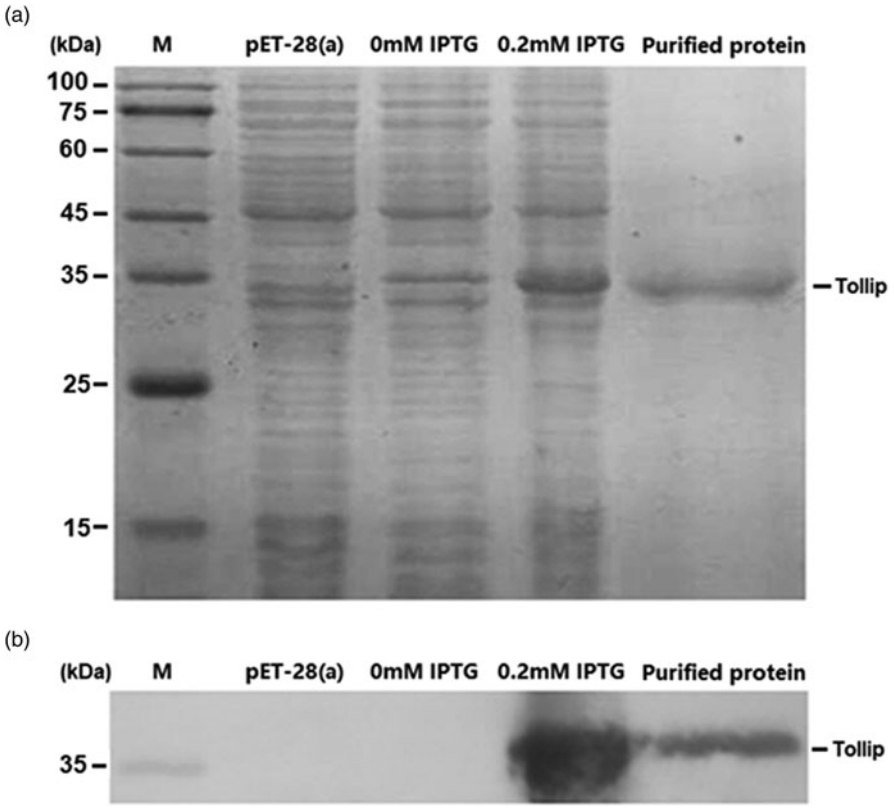

Figure 3. Protein expression, purification (a) and western blotting of Ap-Tollip (b). The protein expression of Ap-Tollip was induced by IPTG. Crude extracts of *E. coli* transformed with pET-28 (a+) vector or pET-28 (a+)-Tollip recombinant plasmids were subjected to SDS-PAGE and stained with Coomassie brilliant blue R250. The western blot was performed using anti-His tag antibodies. M, protein marker.

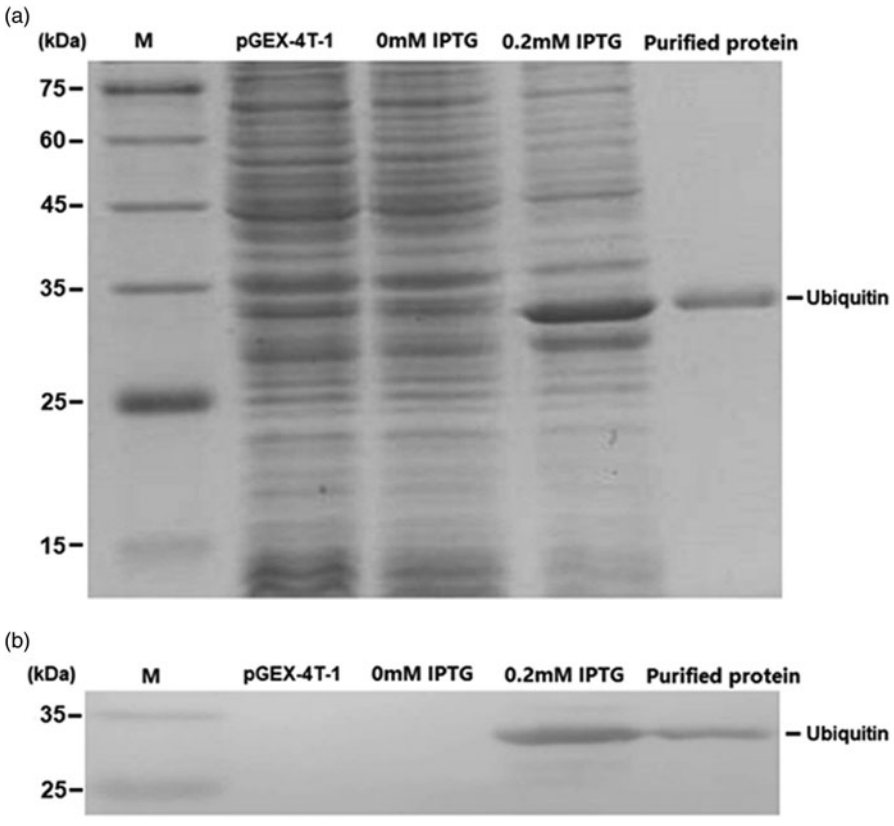

Figure 4. Protein expression, purification (a) and western blotting of Ap-Ubiquitin (b). Crude extracts of *E. coli* transformed with pGEX-4 T-1 vector or pGEX-4 T-Ubiquitin recombinant plasmids were used for SDS-PAGE analysis. The western blot was carried out using anti-GST tag antibodies. M, protein marker.

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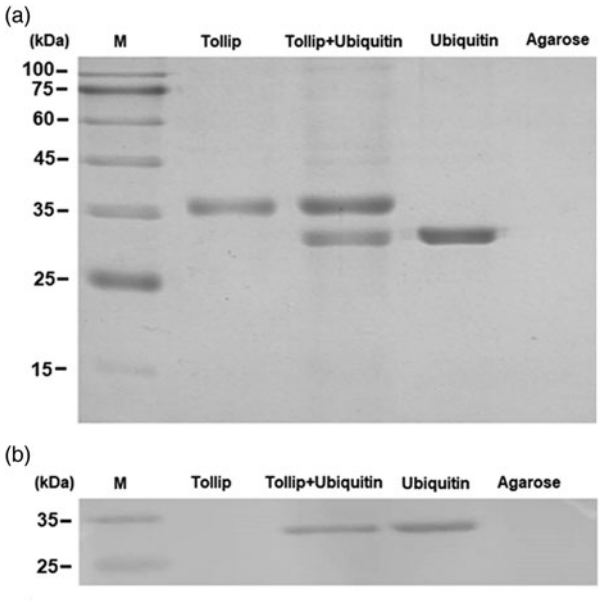

Figure 5. Pull-down assay of Ap-Tollip and Ap-Ubiquitin. (a) SDS-PAGE of collected eluate. (b) Western blotting of Ap-Ubiquitin. Tollip, purified Ap-Tollip protein; Tollip + Ubiquitin, protein eluate; Ubiquitin, purified Ap-Ubiquitin protein; Agarose, Agarose Gel control; M, protein Marker.

a positive control. Two specific protein bands were found after pull-down assay and were consistent with the sizes of Ap-Tollip and Ap-ubiquitin (fig. 5A), and further western-blotting also confirmed it (fig. 5B). His pull-down assay indicated that Ap-Tollip could bind ubiquitin in vitro.

Expression of Ap-Tollip *in different tissues and developmental stages*

Six tissues of *A.pernyi* larvae at the 3rd day of 4th instar were collected and total RNA and proteins was extracted for real-time PCR (fig. 6A) and western blotting (fig. 6B). The results showed that *Ap-Tollip* was widely expressed in examined tissues with the highest expression level in the fat body, while the lowest expression was found in the malpighian tubule. In addition, the result of real-time PCR was in agreement with that of western blotting. As far as the developmental stages were concerned, *Ap-Tollip* was obviously upregulated in eggs from the 10th day to the 14th day (fig. 7A). In larvae, higher expression levels were found at the 3rd day of the 1st or 2nd instar as well as the 1st day of the 4th instar (fig. 7B).

Expression patterns of Ap-Tollip *under various challenges*

Real-time PCR was carried out to investigate the role of *Ap-Tollip* in immunity and development of *A. pernyi*. In fat body, the expression of *Ap-Tollip* was significantly triggered by LPS or poly I: C from 3 to 9 h post-induction and more than 15-fold changes were detected at the peaks (fig. 8A, D). Although the *Ap-Tollip* expression was induced by poly I: C in hemocytes from 9 to 24 h post-induction, it was downregulated by LPS at 3, 12 and 24 h (fig. 8B, C). For 20E challenge, the transcript levels

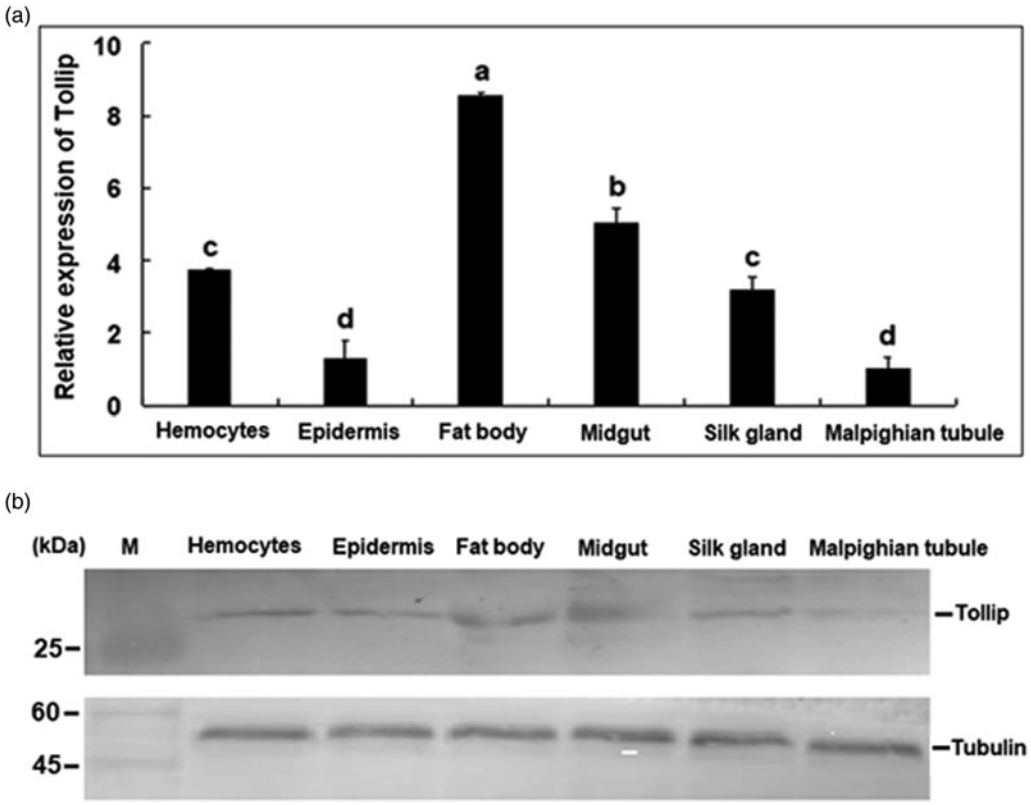

Figure 6. Tissue distribution of *Ap-Tollip*. The expression levels of *Ap-Tollip* were determined by real-time PCR and western blotting. The data were analyzed by one-way ANOVA and presented as the mean ± SE of independent experiments performed in triplicate. Bars labeled with different letters indicated significant differences ($P < 0.05$). Western blot of tubulin was used as a control.

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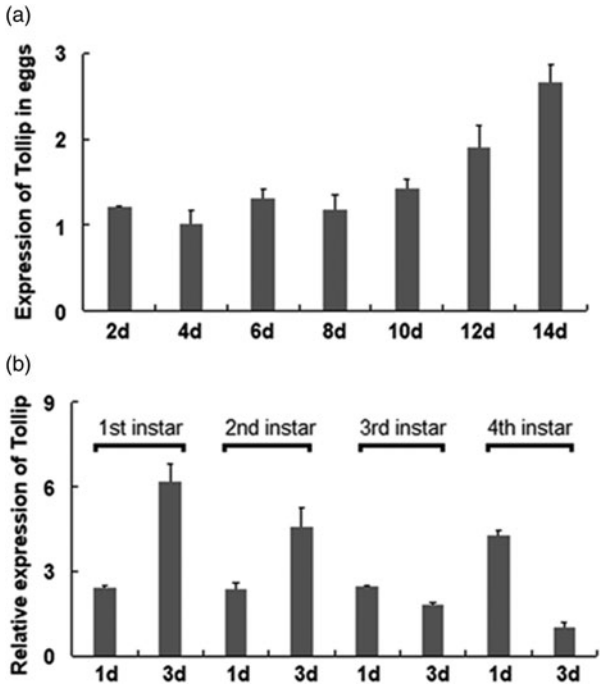

Figure 7. Expression of *Ap-Tollip* in egg (a) and larvae (b). Real-time PCR was used to determine the expression levels of *Ap-Tollip*. The relative expression of *Ap-Tollip* was calculated using the $2^{-\Delta\Delta ct}$ method and 18S rRNA gene was used as an internal reference. The data were presented as the mean ± SE and each experiment was performed in triplicate.

of *Ap-Tollip* showed a decreasing trend from 3 to 9 h and obvious up-regulation at 24 h was found (fig. 9).

Effects of Ap-Tollip *RNAi on the expression of apoptosis and autophagy-related genes*

RNAi was performed to explore the effects of *Ap-Tollip* on the candidate genes related with the immunity and development. Five apoptosis-related genes (*Dronc*, *ATF-2*, *Dredd*, *Daxx*, *Fadd*) and six autophagy-related genes (*Beclin-1*, *ATG5*, *ATG7*, *Pdk1*, *Rheb*, *BNIP3*) were selected for real-time PCR assays. As shown in Fig. S1, the expression level of *Ap-Tollip* decreased significantly at 48 h after RNA interference. Except for *Daxx* (fig. 10A) and *ATG7* (fig. 10B) genes, these candidate genes were significantly downregulated after RNAi.

Discussion

Tollip was reported to involve in multiple signal pathways (Yamakami and Yokosawa, 2004; Lang and Mansell, 2007; Toruń *et al.*, 2015). Although many tollip sequences from various insect species have been deposited in public database, their roles were less reported. Here, the genomic sequence of *Ap*-Tollip was identified with eight exons and seven introns as well as several binding sites of transcription factors (ATF and CREB) in the 5'-UTR, which was similar to that of *Bombyx mori* (GenBank accession no. BHWX01000014.1). Previous reports shown that ATF participated in apoptosis not only as a substrate for JNK and p38 (Maekawa *et al.*, 2008), but also bound to c-Jun to form a dimer to mediate apoptosis (Yuan *et al.*, 2009). CREB

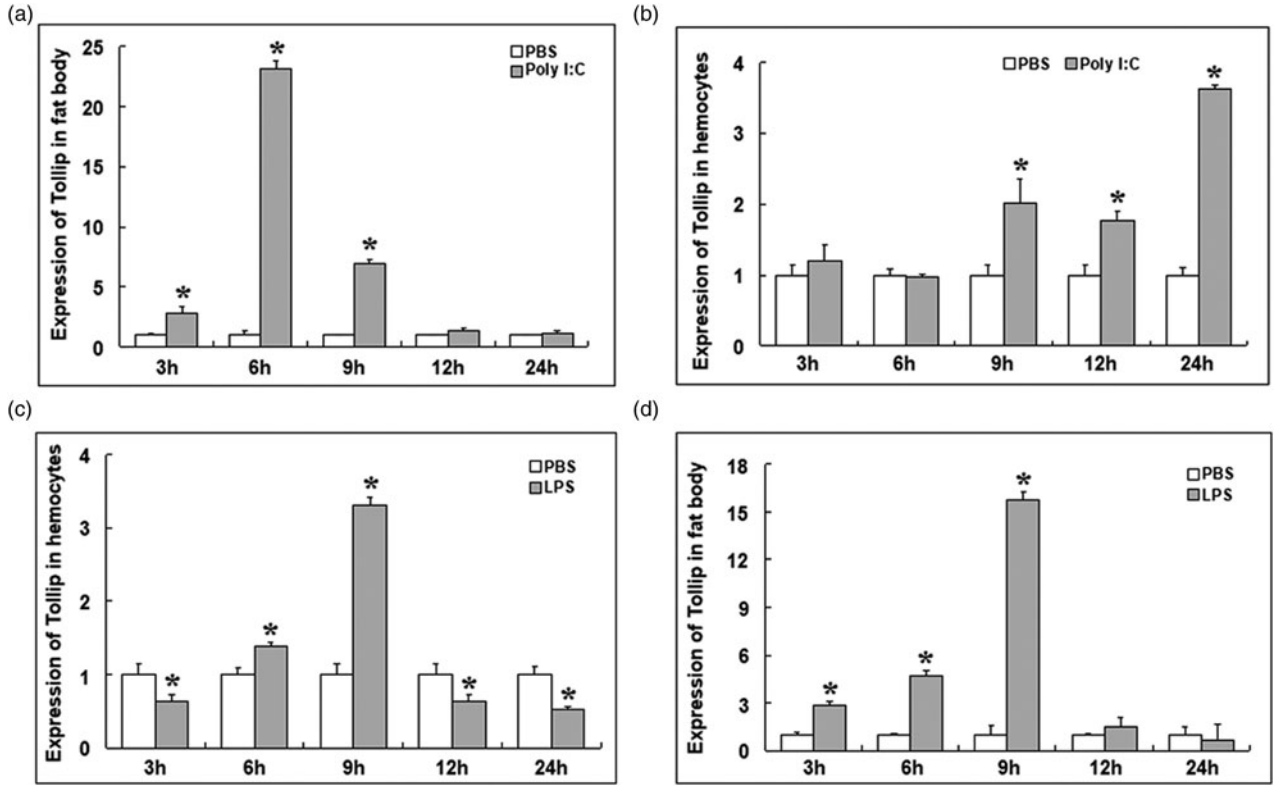

Figure 8. Expression profiles of *Ap-Tollip* in fat bodies and hemocytes under Poly (I: C) (a and b) or LPS (c and d) challenge. These tissues were collected 3, 6, 9, 12, or 24 h post-injection and the PBS-injected groups were used as the calibrators. Bars represent mean ± S.E. ($n = 3$) and asterisks represented significant differences ($P < 0.05$).

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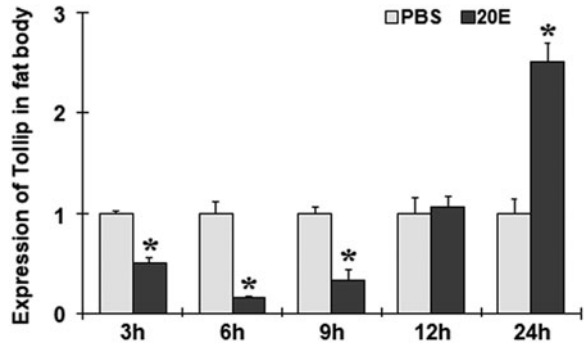

Figure 9. Expression levels of *Ap-Tollip* in fat bodies after 20-hydroxyecdysone induction. The *Ap-Tollip* mRNA level in the PBS-injected group was designated as a control. Significant differences between control and treatment groups were indicated by asterisks.

could induce the autophagy through multiple signal pathways (Seok *et al.*, 2014; Liu *et al.*, 2020; Wang *et al.*, 2021) enhance gene transcription (Jing *et al.*, 2016). We also found that the expression of CREB was upregulated in the fat body of *A. pernyi* upon fungi and virus challenges (Gao *et al.*, 2018). In addition, the predicted *Ap*-Tollip protein shared conserved C2 and CUE domains with other known tollips from invertebrates and vertebrates. The interaction between Ap-Tollip and Ap-Ubiquitin was also verified in vitro by His Pull-Down assay (fig. 5). All these suggested that *Ap-Tollip* had similar functions like tollips from other animals and was involved in various biological processes such as immunity and development in *A. pernyi*.

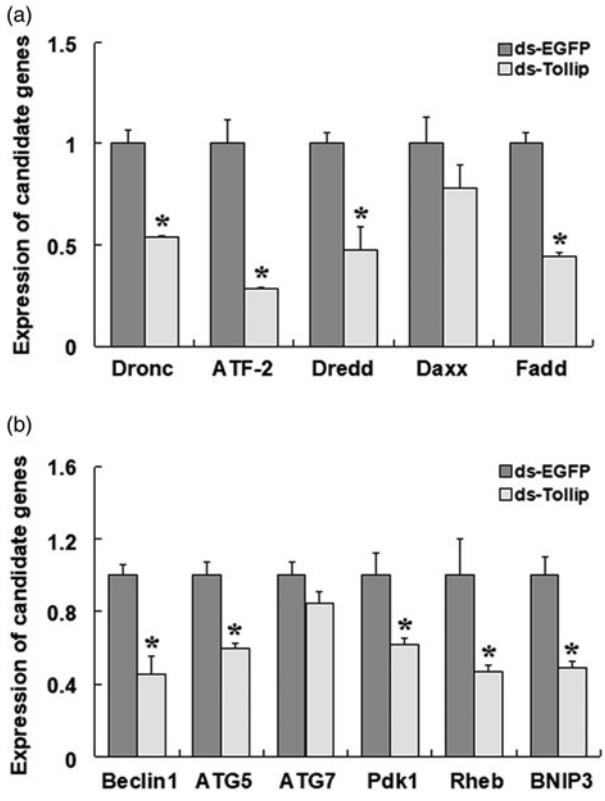

Figure 10. Effect of *Ap-Tollip* RNAi on the expression of candidate genes. (a) Expression of apoptosis-related genes at 48 h after RNAi. (b) Expression of autophagy-related genes at 48 h after RNAi. The dsEGFP-injected group was used as a control.

According to the results, *Ap-Tollip* was widely distributed in various tissues with higher expression levels in the immune-related tissues (fig. 6). Considering that LPS or poly (I:C) was extensively used as bacterial endotoxin and viral dsRNA to investigate the immune response in animals (Yamaguchi *et al.*, 2005; Bai *et al.*, 2021), the expression pattern of *Ap-Tollip* in the hemocytes and fat bodies induced by LPS or poly (I:C) was investigated (fig. 8). Both of LPS and poly (I:C) could regulate the expression of *Ap-Tollip* in these two tissues and the fat bodies were more sensitive to immune challenges compared with hemocytes. Some reports also confirmed the roles of tollip in the anti-virus response and triggering the NF-kapaB pathway in other animals (Lu *et al.*, 2013; Wei *et al.*, 2015; Jiang *et al.*, 2022; Wang *et al.*, 2022). It was considered that *Ap-Tollip* was involved in the regulation of innate immunity in *A. pernyi*.

To further investigate the function of *Ap-Tollip* in the developmental process of *A. pernyi*, the expression patterns of *Ap-Tollip* in eggs and larvae were examined. It was found that *Ap-Tollip* was highly expressed in late stages of eggs and the expression level of *Ap-Tollip* varied in different instars (fig. 7). Meanwhile, the expression levels of *Ap-Tollip* were detected in fat body of pupa induced by 20-hydroxyecdysone. It was known that 20-hydroxyecdysone played a key role in controlling molting and larval-pupal metamorphosis of insects (Liu *et al.*, 2015; Niwa and Niwa, 2016) as well as apoptosis (Li *et al.*, 2016*a*, 2016*b*). Recently, 20-hydroxyecdysone was found to take part in the pupal diapause termination of *A.pernyi* (Li *et al.*, 2020; Du *et al.*, 2022). In this experiment, 20E obviously induced the expression of *Ap-Tollip* in the fat body of pupa (fig. 9) and RNAi of *Ap-Tollip* influenced the expression of candidate genes involved in apoptosis pathway (*Fadd*, *Dronc*, *Dredd*, *ATF2*) and autophagy pathway (*ATG5*, *Beclin1*, *Rheb*, *Pdk1* and *BNIP3*) (fig. 10). These clues shown *Ap-Tollip* was correlated with the apoptosis and autophagy in the fat body of *A. pernyi* pupa.

In summary, a Tollip gene was identified from *A. perni* and it participated in the regulation of immunity and development. Further work should be focused on the *Ap-Tollip*-mediated signal pathways and its partners.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485323000184.

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Conflict of interest. The authors have no conflict of interest to declare.

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