ORIGINAL ARTICLE

The receptor tyrosine kinase torso regulates ecdysone homeostasis to control developmental timing in *Bombyx mori*

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Abstract Insect growth and development are precisely controlled by hormone homeostasis. The prothoracicotropic hormone (PTTH) receptor, Torso, is a member of the receptor tyrosine kinase family in insects. Activation of Torso by PTTH triggers biosynthesis and release of the steroid hormone in the prothoracic gland (PG). Although numbers of genes functioning in steroid hormone synthesis and metabolism have been identified in insects, the PTTH transduction pathway via its receptor Torso is poorly understood. In the current study, we describe a loss-of-function analysis of Torso in the silkworm, *Bombyx mori*, by targeted gene disruption using the transgenic CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/RNA-guided Cas9 nucleases) system. Depletion of *B. mori* Torso (*BmTorso*) did not eventually affect larval ecdysis and metamorphosis processes. Instead, *BmTorso* deficiency resulted in significant extension of developing time during larval and pupal stages with increased pupa and cocoon sizes. The ecdysteroid titers in the hemolymph of *BmTorso* mutants sharply declined. Transcriptional levels of genes involved in ecdysone biosynthesis and ecdysteroid signaling pathways were significantly reduced in *BmTorso*-deficient animals. Additionally, RNA-Seq analysis revealed that genes involved in the longevity pathway and protein processing in the endoplasmic reticulum pathway were affected after *BmTorso* deletion. These results indicate that Torso is critical for maintaining steroid hormone homeostasis in insects.

Key words *Bombyx mori*; CRISPR/Cas9; developmental timing; Torso

Introduction

During the development of multicellular organisms, tissue growth must be timed appropriately to ensure the emergence of adults with correct body size (Nijhout, 2015). Body size cannot be changed once the adult stage has been reached, which indicates that adult size is completely fixed by the growth that takes place during the juvenile stages (Nijhout et al., 2014). In holometabolous insects, the growth of larva is clearly punctuated by several times of molting process before metamorphosis and the transition to reproductively mature adults (Yamanaka et al., 2013). A variety of neuropeptides and hormones play distinct roles in the control of developmental transition (Roller et al., 2008; Nassel & Winther, 2010). In addition, the precise regulation of hormone titers is also critical in the process of molting and metamorphosis (Warren et al., 2006; Riddiford, 2012).

Steroid hormones are the primary endocrine molecules that regulate the developmental transition (Gilbert et al., 2002). In insects, the timing of hatching, molting and metamorphosis are modulated by a steroid hormone 20-hydroxyecdysone (20E). The precursor of 20E, ecdysone, is synthesized primarily in the prothoracic gland (PG) and secreted into the hemolymph (Yamanaka et al., 2015).
Production and release of ecdysone begins with the secretion of prothoracicotropic hormone (PTTH), a neuropeptide produced by two pairs of dorsolateral neurosecretory cells in the brain (McBrayer et al., 2007). Subsequently, PTTH signaling promotes ecdysone biosynthesis by stimulating the transcription of several Halloween genes including neverland (ndl), spookier (spok), phantom (phm), shadow (sad) and disembodied (dib) (Gilbert, 2004). Then, ecdysone is converted to the active form, 20E, in peripheral tissues (Petryk et al., 2003). In the model insect Drosophila, genetic ablation of PTTH-producing neurons strongly delayed larval development because of low ecdysone titers (McBrayer et al., 2007).

Recently, a receptor tyrosine kinase (RTK) called Torso, which serves as a master control of terminal pattern during early embryogenesis (Schoppmeier & Schroder, 2005), was identified as the receptor of PTTH in Drosophila (Rewitz et al., 2009). After early embryogenesis, Torso is expressed specifically in the PG. During the larval stage, PTTH binds to Torso and activates ecdysone synthesis via the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway, resulting in the activation of ERK (Lin & Gu, 2007; Rewitz et al., 2009). In Drosophila, loss of Torso in PG not only delayed the onset of metamorphosis, but also resulted in larger fly owing to excessive growth during the prolonged duration of the larval stage (Rewitz et al., 2009).

In the lepidopteran model insect Bombyx mori, Torso has been identified as a PTTH receptor in vitro (Rewitz et al., 2009). As in Drosophila, BmTorso is predominantly expressed in PG during the larval stage. BmPTTH specifically stimulates ERK phosphorylation through BmTorso, rather than other receptors (Rewitz et al., 2009). The Torso activation by PTTH is maintained by unusual intermolecular disulfide in the transmembrane region (Konogami et al., 2016). Although the physiological roles and activation mechanism for Torso have been extensively studied in B. mori, the comprehensive function of Torso have yet to be revealed in vivo. Recent advance in genome editing tools, such as the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/RNA-guided Cas9 nucleases) system, have provided a promising approach for loss of functional analysis (Hsu et al., 2014; Xu et al., 2017; Zeng et al., 2017). In B. mori, these techniques have been established, including the transgenic CRISPR/Cas9 systems (Li et al., 2015). The transgenic CRISPR/Cas9 would be risky, because it could not knock out the target gene completely, with mild phenotype. However, this somatic mutagenesis system still provides an efficient platform to perform gene loss-of-function analysis in B. mori.

In the current study, we established BmTorso mutants by using the CRISPR/Cas9 system. The mutants show significant developmental delay during the larval and pupal stages. The prolonged development period especially in feeding larval stage results in larger pupae. The ecdysteroid titers at wandering stage and pupal stage were sharply reduced. Furthermore, we found that transcription levels of Halloween genes in ecdysone synthesis were reduced. RNA-Seq analyses revealed that longevity pathway and protein processing in the endoplasmic reticulum were affected by BmTorso deletion. Taken together, our results indicate that Torso regulates the developmental timing from larval to pupal stages and determines the final body size by controlling the ecdysteroid production in silkworm.

Materials and methods

Silkworm strains

A multivoltine and non-diapausing silkworm strain, Nistari, was used in all experiments. Larvae were fed on fresh mulberry leaves under standard conditions at 25°C and 75% relative humidity.

Plasmid construction

To target BmTorso gene, the plasmid pBac[IE1-DsRed2-U6-BmTorso-sgRNAs] (Torso-sgRNAs) expressing BmTorso-specific single guide RNA (sgRNA) was constructed as previously described (Zhang et al., 2017). The primer sequences used for plasmid construction are listed in Table S1.

Silkworm germline transformation

A mixture of Torso-sgRNAs plasmid and piggyBac helper plasmids was injected into preblastoderm embryos (G0). G0 adults were sib-mated or crossed with wild-type (WT) to obtain G1 progeny. G1 positive progeny expressing red fluorescent protein were screened under a fluorescence microscope (Nikon AZ100).

RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was extracted from PG and fat body at wandering stage using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen) to digest genomic DNA. cDNA was synthesized using the ReverAid First
Strand cDNA Synthesis Kit (Fermentas) in accordance with the manufacture’s protocol.

Quantitative real-time polymerase chain reaction (q-RT-PCR) analysis

q-RT-PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo). The thermal cycling conditions were as follows: initial incubation at 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Three independent biological replicates were performed in quantitative messenger RNA (mRNA) measurements, and data were normalized to Bmrp49. The primers used in q-RT-PCR are listed in Table S1.

Genomic DNA extraction and mutagenesis analysis

Genomic DNA of WT and BmTorso mutants at adult stage were extracted as previously described (Zhang et al., 2017). The DNA fragments including the sgRNA targeting site were amplified using KOD-Plus polymerase (Toyobo). The PCR conditions were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 60 s, followed by a final extension period of 68 °C for 5 min. The amplified products were sub-cloned into pJET1.2 vectors (Fermentas) and subsequently sequenced. The primers used for mutagenesis analysis are listed in Table S1.

Determination of ecdysteroid titers

Hemolymph of WT and BmTorso mutants at wandering and pupal stages respectively were collected. The samples were extracted with methanol as previously described (Warren et al., 2006). The extracts were evaporated and re-dissolved in enzyme immunoassay (EIA) buffer (Cayman), and EIA kit (Cayman Chemical, MI, USA) as described before (Zeng et al., 2017). Anti-20-hydroxyecdysone EIA antiserum, acetylcholinesterase (AchE)-conjugated 20E, and standard 20E (Sigma) were used in competitive assay to quantify 20E titers. The AchE activity was quantified by Ellman’s buffer and measured at 405 nm by using a Multiskan FC microplate photometer (Thermo).

RNA-Seq analysis

Total RNA were extracted from the PG dissected from WT and BmTorso mutants with the methods described above. The cDNA libraries were generated by using the Illumina TruSeq™ RNA Sample Preparation Kit (Illumina) following the manufacturer’s recommendations. The cDNA libraries were then sequenced using the Illumina HiSeq 2000 platform (BGI). The raw data were qualified, filtered and mapped to the silkworm genome database (http://www.silkdb.org/silkdb/). Differentially expressed genes (DEGs) between WT animals and BmTorso mutants were functionally annotated by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes.

Results

Generation of BmTorso mutant using the transgenic CRISPR/Cas9 system

To genetically assess BmTorso function, we employed the binary transgenic CRISPR/Cas9 system that has been described in our previous studies (Li et al., 2015). This system included two independent lines, which expressing Cas9 and gene-specific sgRNAs. The Cas9-expressed line was established as described (Xu
The developmental time of the 2nd instar from ∆Torso was not different from WT (Fig. 2A). However, during the last instar, the developmental time of ∆Torso larvae increased ∼5.5 d compared to WT (Fig. 2A). After pupation, the time to eclosion for ∆Torso increased ∼8.5 d compared to WT (Fig. 2A). These results suggest that the deletion of BmTorso induces extreme life cycle expansion from larval to pupal stages. Compared to WT, the pupae and cocoons of ∆Torso were both increased sharply (Fig. 2B). Male mutant whole cocoons were 167% heavier than WT (Fig. 2C). Female mutant whole cocoons were 162% heavier than WT (Fig. 2C). The male mutant cocoon ratio increased by 5.4% and female mutant cocoon ratio increased by 5.7% (Fig. 2D). As expected, the depletion of BmTorso resulted in larger adults (Fig. S2).

Ecdysone titers in BmTorso mutants

Since PTTH is the ligand of Torso, and PTTH is thought to be the primary factor regulating the production and release of ecdysone (Rewitz & O’Connor, 2011). Thus, we measured the ecdysteroid titers in the hemolymph of WT and ∆Torso larvae at the wandering stage. We found that the ecdysteroid titers in ∆Torso larvae decreased ∼60% at wandering stage (Fig. 3A). To investigate whether developmental delay of ∆Torso was the result of a defect in ecdysone biosynthesis, we fed 20E to ∆Torso larvae from day 4 of the 5th instar. As expected, feeding ∆Torso larvae with mulberry leaves smeared with 20E solution completely rescued the developmental delay (Fig. S1). These results indicate that reduced synthesis of ecdysone in the PG is responsible for an extensive delay in the onset of pupal metamorphosis in ∆Torso larvae, demonstrating that Torso modulates the timing of pupal metamorphosis in the last instar through the control of ecdysteroid titers.

The precise regulation of ecdysteroid is required for pupal–adult development (Yamanaka et al., 2013). The ecdysteroid titers reached the peak concentration 2 d after pupation in B. mori (Mizoguchi et al., 2001). Then the titers decreased to undetectable levels before eclosion. Thus, we hypothesized that the prolonged pupal stage of ∆Torso animals might be determined by abnormal ecdysteroid titers. Thus, we measured the ecdysteroid titers during the pupal stage and we found that ecdysteroid titers in the hemolymph collected from WT reached the peak concentration at P3 (Fig. 3B). However, the ecdysteriod titers of BmTorso mutants remained at a very low level from P1 to P9 (Fig. 3B). Taken together, these results demonstrate that deletion of BmTorso disrupts the
Developmental delay and increased body size in △Torso animals. (A) The stages of larval and pupal development in WT (wild-type) and △Torso (L1, 1st instar; L2, 2nd instar; L3, 3rd instar; L4, 4th instar; L5, 5th instar; P, pupae). (B) The upper graph shows male pupae and cocoons for the indicated genotype. The down graph showed female pupae and cocoons for the indicated genotype. (C) The cocoon shell weight of WT and △Torso. (D) The cocoon shell ratio of WT and △Torso. The data are shown as the mean ± SEM (n = 30). ***$p < 0.001$ according to two-tailed t-test.

We then investigated the mRNA expression of three 20E regulated genes: E75A, E75B, HR3. Significant down regulation of E75A, E75B and HR3 were detected in △Torso animals (Fig. 3D). To further explore the molecular mechanisms of how BmTorso regulates the developmental timing, we performed RNA-Seq analysis by using PG at wandering stage between WT and △Torso. A total of 1699 DEGs (543 up-regulated and 1153 down-regulated) were identified. GO functional classification showed that DEGs were mainly involved in cellular and metabolic processes, and binding and catalytic activities (Fig. 4A). KEGG enrichment analysis revealed that longevity regulating pathway, protein processing in endoplasmic reticulum pathways and ABC transporters were in the top five affected pathways. (Fig. 4B) We further investigated the expression levels of genes known to play critical roles in longevity regulating pathway by using qRT-PCR. Four positively regulated genes including PI3K, AKT and S6K were down-regulated to 80%, 87% and 60%, respectively (Fig. 4C). Two negatively regulated genes: FOXO and 4EBP were up-regulated by 1.4 and 2.2-fold when compared to the levels in WT (Fig. 4C). Two key genes, UGT and MANI, of protein processing in endoplasmic reticulum pathway, were down-regulated to 63% and 53%, respectively. (Fig. 4C). Since Atet

ecdysteroid titers, leading to an increase in the duration of larval and pupal stages. Moreover, Western blotting using p-ERK antibody revealed a decrease in protein level in the △Torso larvae (Fig. S3), indicating that BmTorso knockout disrupts the MAPK/ERK pathway in the PG. Overall, BmTorso knockout disrupted ecdysone synthesis and caused developmental delay.

**q-RT-PCR and RNA-seq analysis in BmTorso mutants**

To investigate the reason for the low ecdysteroid titers in BmTorso mutants, we measured the expression of the ecdysteroid biosynthesis genes in the PG. mRNA levels of nvd, spok, phm, sad, and dib were analyzed by qRT-PCR at the wandering stage. Compared to WT, the transcription levels of nvd, spok, phm, sad, and dib in △Torso animals were reduced to 34%, 10%, 16%, 10%, 16% and 16% respectively (Fig. 3C). We also investigated the transcription expression of shd in fat body, another member of the Halloween genes which converted ecdysone into 20E in the peripheral tissues. Interestingly, the relative mRNA level of shd up-regulated 1.6-fold (Fig. 3C).
has been identified as the ABC transporter required for ecdysone secretion in the PG (Yamanaka et al., 2015), we detected the expression levels of two homologous genes of Atet, Atet1 and Atet2 were both significantly down-regulated (Fig. S4).

Recent study showed that ecdysone dose-dependently affects PTTH transcription, promoting its expression at lower concentrations and inhibiting it at higher concentrations (Christensen et al., 2020). The ecdysteroid titers in △Torso animals were dramatically reduced (Fig. 3A). Thus, we hypothesized that the expression levels of PTTH could be affected. As expected, the relative mRNA level of PTTH up-regulated 1.4-fold in △Torso animals (Fig. S4).

**Discussion**

Torso is a RTK that plays distinct roles in different developmental process. During embryonic development, activation of Torso is required for correct terminal pattern (Schoppmeier & Schroder, 2005). After embryonic development, Torso is predominantly expressed in PG, which acts as the receptor of PTTH. Torso mediates PTTH signaling through the activation of the ERK pathway and regulates ecdysone biosynthesis (Rewitz et al., 2009). Our data presented here indicated that Torso is required for ecdysone synthesis, and it plays a vital role in regulating developmental timing and body size in *B. mori*.

Previous study in *B. mori* showed that PTTH mutants showed a developmental delay during the larval and pupal stages (Uchibori-Asano et al., 2017). The developing timing of each larval stage showed significant difference among PTTH mutant individuals (Uchibori-Asano et al., 2017). The majority of PTTH mutants stopped developing at the second larval stage. Some larvae undergo precocious metamorphosis and pupating at the end of the 4th instar. The rest of the larvae could complete the five larval instars. The diversity of phenotypes emerged in PTTH mutants was different from the phenotypes observed in BmTorso mutants. The depletion of BmTorso showed also developmental delay throughout the larval and pupal stages. However, all BmTorso mutants undergo the five larval instars without developmental arrest or precocious metamorphosis. The phenotypic difference between PTTH homozygous mutants (Uchibori-Asano et al., 2017) and BmTorso mutants could be explained by: (1) different silkworm strains were used; (2) transcription activator-like effector nuclease-mediated mutagenesis was complete knock out while CRISPR/Cas9-mediated mutagenesis was incomplete; and (3) PTTH and
Fig. 4 RNA-Seq analysis reveals the differentially expressed genes (DEGs) between wild-type (WT) and △Torso. (A) Gene Ontology functional classification of the DEGs. (B) The top five enriched Kyoto Encyclopedia of Genes and Genomes pathways. (C) Quantitative real-time polymerase chain reaction validation for the genes associated with the regulation of longevity and protein processing signaling pathway. The data are shown as mean ± SEM (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001 according to two-tailed t-test.

its receptor Torso function differently in the PTTH signaling pathway. In addition, the high levels of PTTH were detected in △Torso animals (Fig. S4), supporting the idea that PTTH exerts an additional role independent of Torso.

An intriguing finding in the present study is that the life cycle was extremely extended in BmTorso mutants. The larval growth period of BmTorso mutants was extended ~5.5 days. In addition, the mutants took ~8.5 days longer to complete the pupal stage. And final body size was also increased. Low ecdysone synthesis by PG can delay developmental timing and induce overgrowth in insects (Colombani et al., 2005). We hypothesized, therefore, that the low ecdysteroid titers due to BmTorso depletion is likely responsible for this phenotype. In order to assess if Torso can regulate developmental timing and body size by reducing the production of ecdysone in B. mori, we measured the ecdysteroid titers of hemolymph at the wandering stage and pupal stage. As expected, the ecdysteroid titers were declined sharply in BmTorso mutants. The mRNA levels of ecdysone biosynthetic genes were

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all down-regulated in $\Delta$Torso animals. However, p-ERK levels were only reduced slightly in $\Delta$Torso PG. This is in agreement with previous experiments in Drosophila (Re-witz et al., 2009), which demonstrate that other signals might be responsible of ERK phosphorylation. A recent study shown that the epidermal growth factor receptor (EGFR) signaling is the major signaling that induced the phosphorylation of ERK and regulated ecdysone biosynthesis (Cruz et al., 2020). Taken together, these results showed that Torso signaling might act synergistically only to increase the ERK pathway activity and regulate developmental timing in B. mori.

RNA-Seq and subsequent q-RT-PCR analysis revealed that expressions of genes in the longevity pathway were inactivated in $\Delta$Torso animals, suggesting that low ecdysteroid titers in $\Delta$Torso could negatively modulate the longevity pathway. As a result, the transcription level of positively and negatively regulated genes were down-regulated and up-regulated in PG, respectively. Previous studies showed that PG was not only a tissue producing ecdysone, but also a size-assessing tissue by using longevity dependent PG cell growth to determine the final body size in insects (Mirth et al., 2005). The impaired longevity pathway only in PG resulted in low ecdysone, but also a size-assessing tissue by using the transmembrane region. The present study thus provides the first genetic evidence that BmTorso affects the ecdysone synthesis to regulate growth and development in the silkworm.

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Disclosure

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** 20-hydroxyecdysone (20E)-feeding experiments.

**Fig. S2** Photos of adults of wild-type (WT) and △Torso. Scale bars: 5 mm.

**Fig. S3** Immunoblot analysis of phospho-extracellular signal-regulated kinase (p-ERK) protein.

**Fig. S4** Messenger RNA (mRNA) expressions of PTTH and genes involved in ecdysteroid transport pathway.

**Table S1** Oligonucleotide primers used in this study.