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ORIGINAL ARTICLE

Functional analysis of *Ultrabithorax* in the silkworm, *Bombyx mori*, using RNAi

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Abstract The formation of abdominal appendages in insects is suppressed by the *Hox* genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), but mechanisms of the suppression can differ among species. As the function of *Ubx* and *abd-A* has been described in only a few species, more data from various insects are necessary to elucidate the evolutionary transition of regulation on abdominal appendages. We examined the function of *Ubx* in the silkworm *Bombyx mori* (*Bm-Ubx*) by embryonic RNA interference (RNAi). This is the first case in which functional analysis for *Ubx* is performed in lepidopteran insects. Larvae treated with *Bm-Ubx* dsRNA displayed an additional pair of thoracic leg-like protuberances in A1, whereas the other abdominal segments had no transformation. Our results suggest that *Bm-Ubx* is a suppressor of leg development in A1.

Keywords *Bombyx mori* · *Ultrabithorax* · Embryonic RNAi · Abdominal appendages

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Introduction

The homeotic complex (*Hox*) genes are required to assign segmental identity along the anterior-posterior body axis of an embryo. In insects, the abdominal appendages are suppressed by the *Hox* genes *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*; reviewed by Hughes and Kaufman 2002), but mechanisms of the suppression could differ among species. In *Drosophila melanogaster*, both *Ubx* and *abd-A* act to suppress the development of abdominal appendages by repressing the expression of *Dll* (Vachon et al. 1992). Although *Ubx/abd-A* also suppresses appendage development in the butterfly *Junonia coenia* and the moth *Manduca sexta*, the downregulation of *Ubx/abd-A* expression as circular holes in abdominal segments A3–A6 allow *Dll* to be derepressed, and, thus, prolegs are developed in these abdominal segments (Suzuki and Palopoli 2001; Warren et al. 1994). In the sawfly, prolegs did not express *Dll* at any time, and expressed *Ubx/abd-A* throughout development, so it is suggested that larval prolegs have evolved independently in Lepidoptera and Hymenoptera (Suzuki and Palopoli 2001). In the A1 appendage (pleuropodia) of the beetle *Tribolium castaneum* and grasshopper *Schistocerca americana*, and in the A1 (ventral tube), A3 (retinaculum), and A4 (furca) appendages of the springtail *Folsomia candida*, *Dll* is expressed despite high levels of *Ubx/abd-A* (Palopoli and Patel 1998). It is suggested that *Ubx/abd-A* does not repress *Dll* expression in certain segments in these species. In the milkweed bug *Oncopeltus fasciatus*, *Ubx* and *abd-A* act independently and repress *Dll* expression in the abdominal segments, but are redundant in *D. melanogaster* (Angelini et al. 2005; Castelli-Gair and Akam 1995; Vachon et al. 1992). *Ubx* and *abd-A* have not yet been identified outside the onychophora/arthropod clade, and in the onychophora

and the arthropod groups except for insects, the regulatory interaction between *Ubx/abd-A* and *Dll* has not been reported (e.g., Grenier et al. 1997). The Ubx proteins of the onychophoran *Akanthokara kaputensis* and the crustacean *Artemia fransicana* that were expressed in transgenic *D. melanogaster* were unable to repress *Dll* expression or prevent the initiation of appendage development (Galant and Carroll 2002; Ronshaugen et al. 2002; reviewed by Pavlopoulos and Averof 2002). These results indicate that the regulatory interaction between *Ubx/abd-A* and *Dll* has evolved and diverged among insects (e.g., Palopoli and Patel 1998; Suzuki and Palopoli 2001; reviewed by Angelini and Kaufman 2005).

In *Bombyx mori*, a number of homeotic mutants with extra crescent markings and extra legs in the abdominal segments have been reported, and these mutant genes belong to the *E* group (reviewed by Tazima 1964). The *E* loci are located at position 21.1 on the sixth-linkage group (Banno et al. 1997) and include homeotic genes specifying the identities of the larval abdominal segments, the *Ubx*, *abd-A* and, possibly, *Abd-B* genes (Ueno et al. 1992; Yasukochi et al. 2004). For instance, larvae homozygous for E^N express thoracic-type legs in A1–A7 segments and intermediate thoracic/abdominal-type legs in A8 segment (Ueno et al. 1992). As a further example related to our work, E^{Cw} heterozygous larvae show extra crescents in A1, and E^{Cw} homozygous larvae show not only the extra crescents but also extra thoracic legs in A1 and rudimentary thoracic leg-like protuberances in A2 (Hirokawa 1998). However, the relationships between defects of each *Hox* gene and mutant phenotypes are still unclear. Intriguingly, unlike *D. melanogaster*, *E* mutants of *B. mori* have a different transformation between the dorsal and ventral sides (Itikawa 1943; Tazima 1964).

Recently, it was reported that *abd-A* expression is required for proleg development in *B. mori* by RNAi experiments (Tomita and Kikuchi 2009; Pan et al. 2009). Although *Ubx* expression has been examined in various insect species (reviewed by Hughes and Kaufman 2002), functional analysis of *Ubx* by RNA interference (RNAi) has been limited to *Tribolium* (Lewis et al. 2000), *Acheta* (Mahfooz et al. 2007), and *Oncopeltus* (Angelini et al. 2005; Mahfooz et al. 2007). For evolutionary comparisons of regulation in abdominal appendages, more data from various insects groups are necessary. Here, we applied *Ubx* RNAi to *B. mori* in order to analyze the function of *Ubx* in a lepidopteran insect.

In the present study, we cloned full-length cDNA for the *Ubx* homolog from *B. mori* (*Bm-Ubx*) and analyzed the expression pattern in embryos of *B. mori* by whole-mount in situ hybridization. The expression of *Bm-Ubx* in the embryonic stages is very similar to that in the moth *M. sexta* (Zheng et al. 1999) and butterfly *J. coenia* (Warren et al. 1994). We also analyzed the function of *Bm-Ubx* with an embryonic RNAi experiment. Consequently, the larvae treated with *Bm-Ubx* dsRNA displayed an additional pair of thoracic leg-like protuberances in A1. This phenotype is very similar to that of E^{Cw} homozygous larvae (Hirokawa 1998). Our results suggest that *Bm-Ubx* is a suppressor of leg development in A1. Furthermore, we show that the embryonic RNAi method is efficient in *B. mori*.

Materials and methods

Insects

Strain No. 459 (sex-linked black eggs) and a polyvoltine strain (N_4) of the silkworm, *B. mori*, were used. Eggs were incubated at 25°C and the embryos were staged according to morphological markers as described by Ohtsuki (1979) and Morita et al. (2003).

Cloning

Total RNA was extracted from 48-h-old female *B. mori* embryos (No. 459) with TRIzol (Gibco BRL) according to the manufacturer's instructions. The first-stranded cDNA was synthesized with a SMART polymerase chain reaction (PCR) cDNA Amplification kit (Clontech) using 1 μg total RNA. *Bm-Ubx* cDNA fragments were amplified by PCR with the following pair of degenerate primers corresponding to the highly conserved amino acid sequences found in the sequences of *Ubx* from several arthropods and an onychophora.

The degenerate primer set for *Ultrabithorax* (*Ubx*):

Ubx-1: 5′-CARACITAYACIMGITAYCARAC-3′ (23 mer)
Ubx-3: 5′-TGIGCYTGYTTYTCYTGYTCRTT-3′ (23 mer)
(R=A+G, M=A+C, Y=T+C, I=inosine)

PCRs were performed using 2.5 μl of the tenfold diluted first-stranded cDNA, the above pair of primers, and AmpliTaq Gold (Perkin Elmer).

To obtain full-length cDNA, 5′ RACE and 3′ RACE were performed with the following gene-specific primers and the SMART PCR cDNA Amplification kit (Clontech) according to the manufacturer's instructions.

Gene-specific primers for 5′ RACE:

Bm-Ubx-3: 5′-GCGTCTCCTTCGCGTAAGGTAGTGGTTC-3′ (28 mer)
Bm-Ubx-4: 5′-TGATTTGCCTCTCCGTGAGGCACAACGC-3′ (28 mer)

Gene-specific primers for 3′ RACE

Bm-Ubx-1: 5′-GAACCACTACCTTACGCGAAGGA GACGC-3′ (28 mer)
Bm-Ubx-2: 5′-GCGTTGTGCCTCACGGAGAGG CAAATCA-3′ (28 mer)

The 5′ RACE and 3′ RACE were performed using 2.5 μl of the tenfold diluted first-stranded cDNA from 32-h-old male embryos, 10× Universal Primer Mix, Bm-Ubx-4 for 5′ RACE or Bm-Ubx-1 for 3′ RACE, and Advantage 2 Polymerase Mix. The nested PCRs for 5′ RACE and 3′ RACE were performed using 0.2 μl of the primary PCR product, Nested Universal Primer, Bm-Ubx-3 for 5′ RACE or Bm-Ubx-2 for 3′ RACE, and an Advantage 2 Polymerase Mix.

Sequencing and sequence analysis

The PCR product was subcloned into the *Eco*R V site of the pBluescript KS vector (Stratagene). Nucleotide sequence determination was performed by the dideoxy chain-termination method using an automatic DNA sequencer CEQ 2000XL (Beckman Coulter). Sequence analysis was performed using the DNASIS system (Hitachi Software Engineering). Deduced amino acid sequences were aligned with ClustalW to determine amino acid sequence identities. The DDBJ/EMBL/GenBank accession number for *Bm-Ubx* is AB505052.

Whole-mount in situ hybridization

Embryos (N_4) between stage 16 (neural groove formation) and stage 22 (complete embryonic reversal) were dissected from their eggs in saline solution (0.75% NaCl) and fixed overnight with a chilled PLP fixative (4% paraformaldehyde, 30 mM $NaPO_4$, 10 mM $NaIO_4$, and 75 mM lysine, pH 6.8). After fixation, embryos were washed several times in 100% methanol and stored in 100% methanol at −20°C until use.

The in situ hybridizations were performed using standard procedures (Tomoyasu et al. 2005). Embryos were exposed to Proteinase K (10 μg/ml) for 20 min. Digoxigenin-labeled RNA probes were synthesized based on the *Bm-Ubx* ORF sequence (765 bp) using a DIG RNA Labeling kit (Boehringer). Embryos were subjected to hybridization with the probes in hybridization buffer at 55°C for 18 h, washed at 55°C for several hours, and treated with blocking buffer (Boehringer blocking reagent, Triton, and NGS) at room temperature for 1 h. Embryos were then stained with the alkaline phosphatase-conjugated anti-digoxigenin antibody at 4°C overnight.

Preparation of dsRNA

Two regions of *Bm-Ubx* cDNA were subcloned into the *Eco*R V site of the pBluescript KS vector, a 765 bp fragment (the ORF of *Bm-Ubx* cDNA), obtained using Bm-Ubx-5 (5′-ATGAACTCTTACTTCGAGCA-3′) and Bm-Ubx-8 (5′-TTAATGTTCGGGGTGTCCCT-3′), and a 445-bp fragment (encoding the N-terminus sequences to the conserved YPWM motif) was obtained using Bm-Ubx-5 and Bm-Ubx-6 (5′-ATGATTAGTAGGCTGTTGGT-3′) as primers (Fig. S1; Electronic Supplementary Material). For the production of a template for in vitro transcription, PCR was performed using plasmids containing either *Bm-Ubx*-ORF (the 765 bp PCR product) or *Bm-Ubx*-HDless (the 445-bp PCR product, which does not contain homeobox; Fig. S1; Electronic Supplementary Material), two universal primers (T7-KS, 5′-TAATACGACTCACTATAGGGAGACCACTC GAGGTCGACGGTATC-3′; T7-SK, 5′-TAATACGACT CACTATAGGGAGACCACCGCTCTAGAACTAGTG GATC-3′) containing the T7 polymerase promoter sequence at their 5′ ends, and AmpliTaq Gold (Perkin Elmer). As a negative control, *DsRed* ORF (678 bp) was amplified using two primers with a T7 promoter sequence at the 5′ end (T7-DsRed5, 5′-TAATACGACTCACTATAGGGAGACCA CATGGTGCGCTCCTCCAAG-3′; T7-DsRed3, 5′-TAA TACGACTCACTATAGGGAGACCACCTACAGGAA CAGGTGGTG-3′). Sense and antisense transcripts were simultaneously synthesized using 1 μg PCR product and a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. After DNase I treatment, RNA precipitated with LiCl was dissolved in RNase-free water. The RNA solution was heated at 65°C for 30 min and cooled slowly to room temperature for annealing of the dsRNA. The concentrations of dsRNA for *Bm-Ubx*-ORF, *Bm-Ubx*-HDless, and *DsRed* were 1.2 μg/μl. The quality of the dsRNA was examined by agarose gel electrophoresis and small aliquots of the dsRNA were stored at −80°C until use.

Injection of dsRNA into *B. mori* embryos

Injections were performed under a dissection microscope (Stemi 2000, Carl Zeiss). *B. mori* embryos (N_4) were collected within 5 h of oviposition to perform dsRNA injection during the syncytial blastoderm stage. The dsRNA was injected ventrally into an egg using a micromanipulator (Narishige) and FemtoJet (Eppendorf). The injection into an embryo was performed with a special glass needle (uMPm-02, Natsume Optical Corporation).

Scanning electron microscopy

Dissected embryos were fixed with FAA solution (37% formaldehyde: acetic acid anhydride: 50% ethanol=1:1:18), dehydrated in a graded ethyl alcohol series and then transferred to acetone. The embryos were dried in a critical-point dryer, coated with platinum and observed under a scanning electron microscope (S-3000N, Hitachi).

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Bm-Ubx   MNSYFEQG-GFYGAHGVHQ---GGGGGDQ----------YRGFPLGL---TYAQP----H
Jc-Ubx   MNSYFEQG-GFYGAHGVHQ---GGGGGDQ----------YRGFPLGL---TYAQP----H
Tc-Ubx   MNSYFEQS-GFYGSHHHQS---GSVAGHHHEQSAAAAAAYRSFPLSLGMSPYASSQHHHH
Dm-Ubx   MNSYFEQASGFYGHPHQATGMAMGSGGHHDQTASAAAAAYRGFPLSLGMSPYANH-----
         *******. ****          . .*.:          **.***.*   .**.

Bm-Ubx   ALHQPRPQDSPYDASVAAACKLYAGEQQ-------YPKADCSKPGGEQQNGYG-------
Jc-Ubx   ALHQPRPQDSPYDASVAAACKLYAGEQQ-------YAKADCSKAGGEQQNGYG-------
Tc-Ubx   HLQARPPQDSPYDASVAAACKLYSSEGQQNSNYSSNSKPDCSK-GNADQNGYASVVAAAA
Dm-Ubx   -HLQRTTQDSPYDASITAACNKIYGDGAG------AYKQDCLNIKADAVNGYKDIWNTGG
               .********::***:   .:           * ** :      ***

Bm-Ubx   GKEAWGSG--------------------------------LGALVRPAACTPEARYSE--
Jc-Ubx   GKEAWGSG--------------------------------LGALVRPAACTPEARYSE--
Tc-Ubx   VKDVWQSATSGGGANL---------------------TNSLTGPVRPAACTPDSRVGYGS
Dm-Ubx   SNGGGGGGGGGGGGGAGGTGGAGNANGGNAANANGQNNPAGGMPVRPSACTPDSRVGG-Y
          :    ..                                    ***:****::* .

Bm-Ubx   --------SSSP---------------------------GRALPWGNQCALPGSAASAAQ
Jc-Ubx   --------SSSP---------------------------GRALPWGNQCALPG-AAASAQ
Tc-Ubx   VGLVGGDPASSPGAAAG--------------------RTGNSLSWNNPCSINSTSSQPVG
Dm-Ubx   LDTSGGSPVSHRGGSAGGNVSVSGGNGNAGGVQSGVGVAGAGTAWNANCTISGAAAQTAA
                  *                              * . .*.  *:: . :: ..
                  YPWM
Bm-Ubx   P-VHQQPTNHTFYPWMAIA-----------------------------------------
Jc-Ubx   P-VQHQPTNHTFYPWMAIA-----------------------------------------
Tc-Ubx   T-QIHQQTNHTFYPWMAIADSMT-------------------------------------
Dm-Ubx   ASSLHQASNHTFYPWMAIAGECPEDPTKSKIRSDLTQYGGISTDMGKRYSESLAGSLLPD
         .   :* :***********
                              homeodomain
Bm-Ubx   --GANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRR
Jc-Ubx   --GANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRR
Tc-Ubx   -FGANGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRR
Dm-Ubx   WLGTNGLRRRGRQTYTRYQTLELEKEFHTNHYLTRRRRIEMAHALCLTERQIKIWFQNRR
           *:********************************************************

                 Ubd-A peptide  QAQAQK  Poly-Ala
Bm-Ubx   MKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAAAAQGHPEH    :254
Jc-Ubx   MKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAAAAQGHPEH    :253
Tc-Ubx   MKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAVAAQVDPN       :314
Dm-Ubx   MKLKKEIQAIKELNEQEKQAQAQKAAAAAAAAAAAVQGGHLDQ    :389
         *******************************.**
```

Fig. 1 Comparison of amino acid sequence of *B. mori* Ubx with those of *Ubx* orthologs from *J. coenia*, *D. melanogaster*, and *T. castaneum*. *Asterisks* indicate identical amino acids among the four species. The amino acids with conservative substitutions and semi-conservative substitutions based on the physiochemical criteria are marked with *double dots* (:) *and single dots* (.), respectively. *Dm D. melanogaster* (P83949), *Jc J. coenia* (AY074760), *Tc T. castaneum* (AY074761). Accession numbers are in *parentheses*

Results and discussion

Cloning of the *B. mori Ubx* homolog

Partial *Bm-Ubx* cDNA was PCR-amplified using the first-strand cDNA prepared from *B. mori* embryos with a pair of degenerate primers based on the highly conserved amino acid sequence. The size of the PCR product for *Bm-Ubx* was 160 bp. Following this, full-length cDNA for *Bm-Ubx* was cloned by 5′ and 3′ RACE and consisted of 1,458 bp (Fig. S1; Electronic Supplementary Material). The ORF for *Bm-Ubx* encoded 254 amino acid residues (Fig. S1; Electronic Supplementary Material). As shown in Fig. 1, the entire Ubx sequences of *B. mori* showed the highest in identity to the butterfly *J. coenia* Ubx (98%) when compared with those of *J. coenia*, the red flour beetle *T. castaneum*, and *D. melanogaster*. Bm-Ubx shares 61% identity with *T. castaneum* Ubx and 53% identity with *D. melanogaster* Ubx. Conservation was highest in homeodomain and the Ubd-A peptide, in which all of the amino acid residues are identical among Ubx sequences of these insects. A transcriptional repression domain in the C-terminal region is also highly conserved (Galant and Carroll 2002; Ronshaugen et al. 2002). Thus, sequence comparison demonstrates that the cloned *B. mori* cDNA is a homolog of the known *Ubx*.

Expression of *B. mori Ubx* during embryogenesis

We examined the expression of *Bm-Ubx* transcript during embryogenesis. For embryos in stage 16 (60 h after

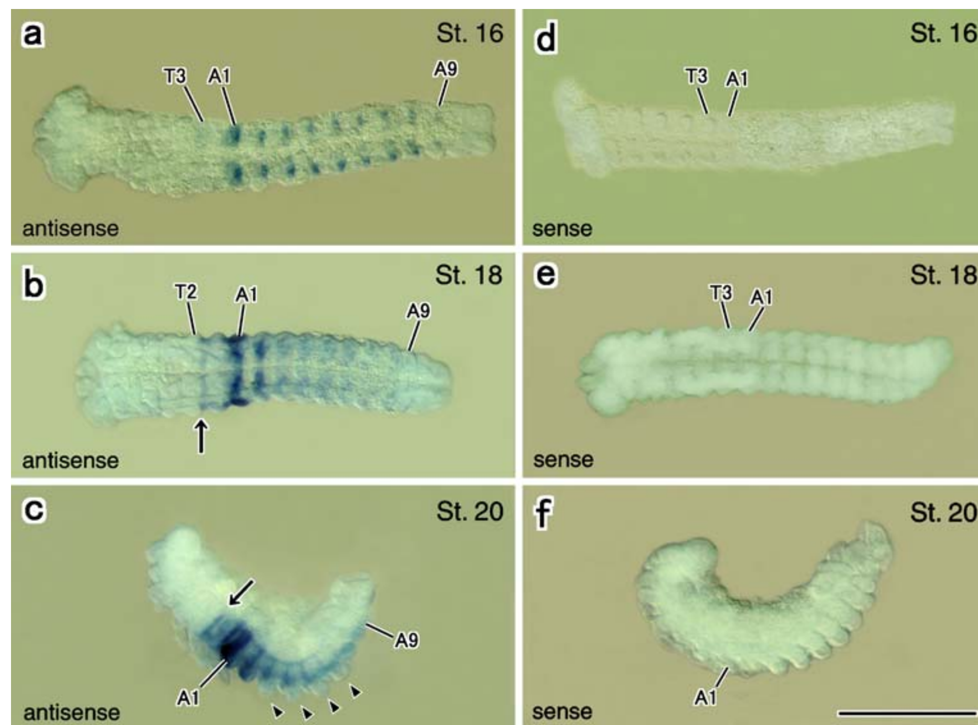

Fig. 2 Expression pattern of *Bm-Ubx* transcripts in the *B. mori* embryo. Embryos were stained with a riboprobe (*blue*). Anterior is *left*. **a** Embryo in stage 16 (60 h after oviposition). Ventral view. *Bm-Ubx* expression is detected in T3–A9. The highest levels of staining are detected in A1. The expression in T3 and A9 is weak. No expression of *Bm-Ubx* is detected in the lateral regions of T3–A9. **b** Embryo in stage 18 (80 h after oviposition). Ventral view. *Bm-Ubx* expression is detected from the posterior half in T2–A9. The highest levels of staining are detected in A1. *Arrow* indicates the anterior limit of *Bm-Ubx* expression. **c** Embryo in stage 20 (96 h after oviposition). Lateral view. The expression pattern is similar to that in stage 18. *Arrowheads* indicate proleg primordia where the signal was undetectable. **d–f** Embryos with the sense strand probe in the same stage of **a–c**, respectively. No signal is detected. *A1–9* abdominal segments, *T1–3* thoracic segment. Bar=500 μm

oviposition), *Bm-Ubx* transcripts were detected in T3–A9 (Fig. 2a). The highest levels of staining were detected in A1. The expression in T3 and A9 were weak. No expression of *Bm-Ubx* was detected in the lateral regions of T3−A9. For embryos in stage 18 (80 h after oviposition), *Bm-Ubx* expression was detected in from the posterior half of T2 to A9 (Fig. 2b). The signal in anterior A1 was the highest level with diminishing levels in the more posterior segments. Signals in A3−A9 were lower than that in stage 16. Unlike embryos in stage 16, the signals were detected in the lateral region. For embryos in stage 20 (96 h after oviposition), the expression pattern of *Bm-Ubx* was the same as in stage 18 (Fig. 2c). What has to be noticed is that the signal was undetectable in the proleg primordia of A3−A6 (Fig. 2c). For embryos in stage 22 (120 h after oviposition), *Bm-Ubx* expression could not be detected due to nonspecific background that was probably due to cuticle deposition. No signal was observed with sense strand probes in stages 16−20 (Fig. 2d–f). Therefore, the abovementioned signals obtained by an antisense probe were specific for *Bm-Ubx*.

The expression of *Bm-Ubx* in the embryonic stages was almost the same as that in the moth *M. sexta* (Zheng et al. 1999) and the butterfly *J. coenia* (Warren et al. 1994). It indicates that *Ubx* expression pattern has been highly conserved in lepidopteran insects.

Phenotypes of *Bm-Ubx* RNAi

The *B. mori* larva has three pairs of thoracic legs from the first to the third thoracic segments (T1–T3), four pairs of abdominal legs in the A3–A6 segments (prolegs), and a pair of caudal legs in A10. There are no leg-like structures in A1 and A2 (Fig. 3a, b).

We injected *Bm-Ubx* dsRNA into eggs within 5 h of oviposition. We dissected embryos in the last developmental stage (just before hatching), because many embryos could not hatch. In *Bm-Ubx* RNAi embryos, the additional pair of leg-like protuberances in A1 was observed (Fig. 3c, d). These protuberances had setae and shallow grooves, suggesting segments, and corresponded in position to the thoracic leg (Fig. 3g). Furthermore, this protuberance had a claw-like structure on the tip so that it was more similar to a thoracic-type leg than an abdominal-type leg (proleg; Fig. 3e−g). This result is the same as in *O. fasciafus* (Mahfooz et al. 2007) and *T. castaneum* (Lewis et al. 2000). In *Ubx* RNAi *O. fascifus* nymphs, however, A1 bears

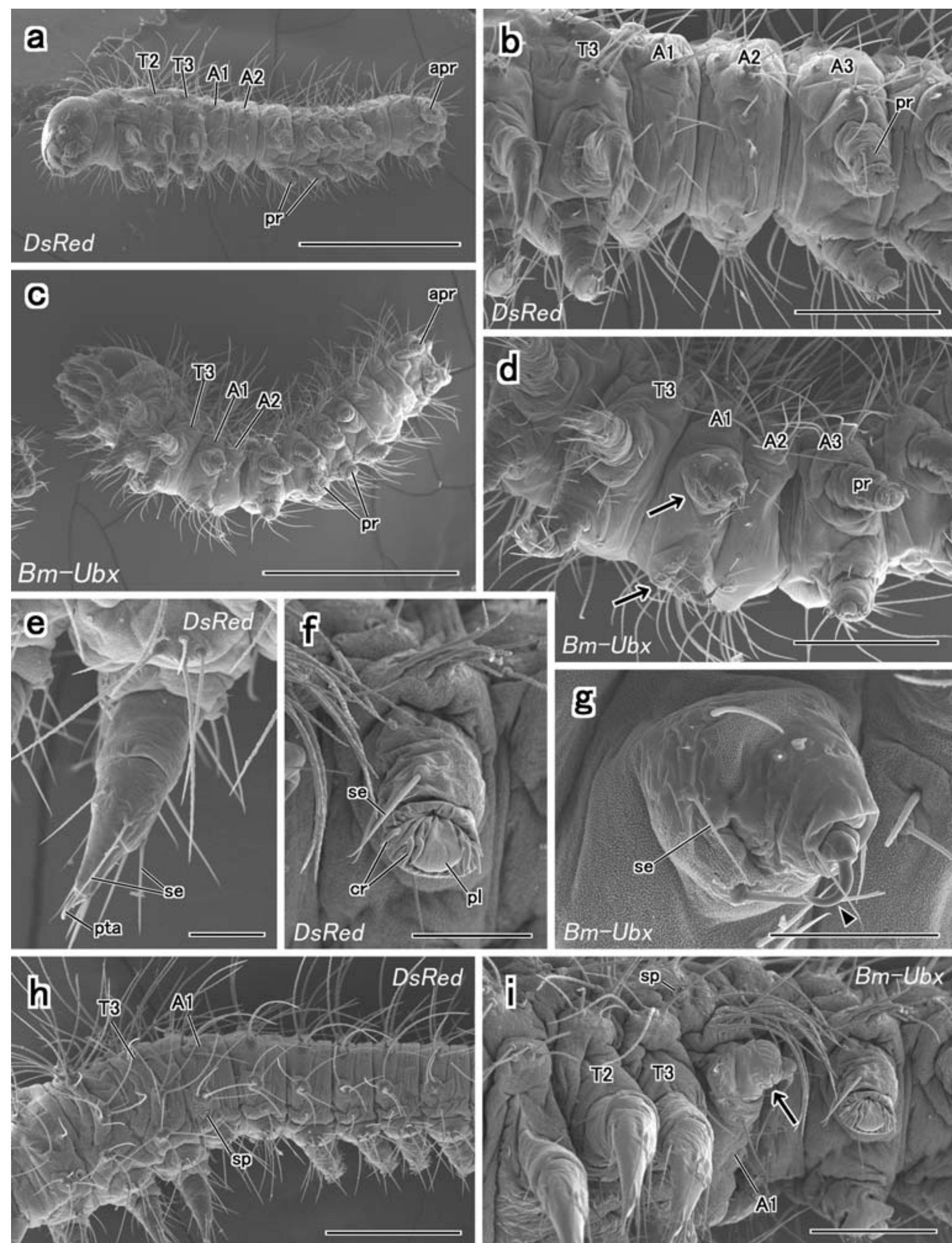

Fig. 3 RNAi of *Bm-Ubx*. **a** A lateroventral view of a *DsRed* dsRNA injected embryo. Bar= 1 mm. **b** Enlargement of the same embryo in Fig. A. Bar=300 µm. **c** A lateroventral view of a *Bm-Ubx* RNAi embryo. Bar=1 mm. **d** Enlargement of the same embryo in **c**. Bar=300 µm. **e** Thoracic leg of *DsRed* dsRNA injected embryo. Bar=100 µm. **f** Abdominal leg (proleg) of *DsRed* dsRNA injected embryo. Bar=100 µm. **g** Enlargement of the same embryo in **d**. Additional pair of legs in A1. Bar=100 µm. **h** Lateral view of a *DsRed* dsRNA injected embryo. Spiracles in T1 and A1–A8 (A7 and A8 not shown). Bar= 500 µm. **i** Lateroventral view of a *Bm-Ubx* RNAi embryo. Spiracle remains in A1. Bar=300 µm. *A1–9* abdominal segments, *apr* anal proleg, *cr* crochets, *pl* planta, *se* setae, *pr* proleg, *pta* pretarsus (claws), *sp* spiracle, *T1–3* thoracic segment, *arrows* additional pair of leg, *arrowhead* claw-like structure

ectopic T3-like dorsal pigmentation (Angelini et al. 2005; Mahfooz et al. 2007). In *T. castaneum*, *Utx* (*Ubx* ortholog in *T. castaneum*) mutant larvae lack the A1 spiracle (as wild type *T. castaneum* larvae have spiracles in T2 and all abdominal segments, but lack them in T1 and T3). It was concluded that these larvae had transformations of the A1 segment toward the T3 segment (Angelini et al. 2005; Lewis 2000). In *Bm-Ubx* RNAi embryos, however, spiracles in A1 remained (Fig. 3i), as wild-type larvae have spiracles in T1 and in A1–A8, but are lacking in T2 and T3

Table 1 Effect of *Bm-Ubx* RNAi on *B. mori* embryos

Injection	Experiment	Number of injected embryos	Number of developed embryos	
			Normal	*Ubx* phenotype
Bm-Ubx dsRNA	#1[a]	227	15	3 (16.7%)
	#2[b]	225	23	12 (34.3%)
DsRed dsRNA		152	25	0

[a] *Bm-Ubx*-ORF (the 765-bp PCR product) was injected

[b] *Bm-Ubx*-HDless (the 445-bp PCR product) was injected

(Fig. 3h). In E^{Cw} homozygous larvae, ectopic A2-like dorsal pigmentation and a degenerated spiracle formed in the A1 segment (Hirokawa 1998). Therefore, in *Bombyx*, the *Bm-Ubx* RNAi phenotype might not simply be interpreted as "entire transformations of A1 segment toward thoracic identity" as in *T. castaneum* and *O. fasciafus*. On the contrary, the regulation mechanism of the A1 identity by *Bm-Ubx* may differ between the dorsal and ventral parts.

There was no presentation of a protuberance in A2 of *Ubx* RNAi embryos (Fig. 3d). This phenotype indicates that *Dll* in A2 is not repressed by *Ubx* singularly in *B. mori*. It might be that *Ubx* and *abd-A* act redundantly to inhibit appendage development in A2 in *Bombyx* such as in *Drosophila* (Castelli-Gair and Akam 1995; Vachon et al. 1992). Recently, however, it has been reported that *abd-A* RNAi does not cause extra appendage formation in A2 in *Bombyx* (Pan et al. 2009; Tomita and Kikuchi 2009). So it is highly possible that neither *Ubx* nor *abd-A* is important for appendage suppression in A2. Also in A3–A9, where *Bm-Ubx* expressed, there was no notable alteration in morphology, and the prolegs in A3–A6 remained the same as in the wild type. These morphologies are probably regulated by *abd-A*, which is essential for proleg development (Pan et al. 2009; Tomita and Kikuchi 2009). In addition, the phenotype observed in our study closely resembled E^{Cw} homozygous larvae. Thus, E^{Cw} may be associated with the loss-of-function of *Bm-Ubx*.

In our present work, it was found that *Bm-Ubx* suppresses leg development in A1. In two other lepidopterans, *M. sexta* and *J. coenia* (Zheng et al. 1999; Warren et al. 1994), *Ubx* expression pattern is almost the same as in *B. mori* (in particular, expression was highest in A1), and so these results indicate that *Ubx* in lepidopterans functions as a suppressor of leg development in A1. We support the idea that the strong repressive interaction between *Ubx* and *Dll* evolved in the dipteran/lepidopteran lineage (Palopoli and Patel 1998), since *Ubx* acts as a modifier rather than a suppressor of the abdominal appendage in *T. castaneum* (Lewis et al. 2000). Because *Ubx* and *abd-A*, and *Ubx* and *Abd-B*, overlap in their expression (Tomita and Kikuchi 2009), simultaneous RNAi analysis is necessary to elucidate the exact regulation of abdominal appendages.

Embryonic RNAi efficiency in *B. mori*

In this study, two dsRNA constructs were used for *Bm-Ubx*: a 765-bp fragment including the homeobox (*Bm-Ubx*-ORF) and a 445-bp fragment excluding the homeobox (*Bm-Ubx*-HDless; Fig. S1; Electronic Supplementary Material). It is possible that *Bm-Ubx*-ORF dsRNA could cross-react with *Bm-abd-A* transcripts because the amino acid sequence in homeodomain of *Ubx* and *abd-A* are resembled in many insects (the homology of them was 89% in *B. mori*). Thus, in order to elucidate the specific function for *Bm-Ubx*, we used the two constructs, mentioned above, for RNAi. The phenotype observed in these studies was indistinguishable. Additionally, no detectable effect was observed in *DsRed* RNAi larvae as the control (Fig. 3a, b). Therefore, phenotypes induced by *Bm-Ubx* RNAi must be specific for *Bm-Ubx*. This specific phenotype was ectopic A1 appendages, which were observed in 16.7% and 34.3% of developed embryos that were injected with two different constructs (Fig. 3c, d, g, Table 1). This result indicates that *Bm-Ubx* is a suppressor of leg development in A1. In this experiment, it is likely that *B. mori* embryos died during early embryogenesis due to physical damage from injection of dsRNA. Although improvement with respect to survival rate is required, we conclude that the embryonic RNAi method is effective in *B. mori*.

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