Developmental Biology 394 (2014) 357–366

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Evolution of Developmental Control Mechanisms

Over-expression of *Ultrabithorax* alters embryonic body plan and wing patterns in the butterfly *Bicyclus anynana*

Xiaoling Tong [a,b,*], Steven Hrycaj [c], Ondrej Podlaha [a], Aleksandar Popadic [c], Antónia Monteiro [a,d,e,**]

[a] Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511, USA
[b] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, China
[c] Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA
[d] Department of Biological Sciences, National University of Singapore, Singapore 117543
[e] Yale-NUS College, Singapore 138614

ARTICLE INFO

Article history:
Received 19 June 2014
Accepted 19 August 2014
Available online 26 August 2014

Keywords:
Ultrabithorax
Over-expression
Homeotic transformation
Wing
Eyespot
Bicyclus anynana

ABSTRACT

In insects, forewings and hindwings usually have different shapes, sizes, and color patterns. A variety of RNAi experiments across insect species have shown that the hox gene *Ultrabithorax* (*Ubx*) is necessary to promote hindwing identity. However, it remains unclear whether *Ubx* is sufficient to confer hindwing fate to forewings across insects. Here, we address this question by over-expressing *Ubx* in the butterfly *Bicyclus anynana* using a heat-shock promoter. *Ubx* whole-body over-expression during embryonic and larvae development led to body plan changes in larvae but to mere quantitative changes to adult morphology, respectively. Embryonic heat-shocks led to fused segments, loss of thoracic and abdominal limbs, and transformation of head limbs to larger appendages. Larval heat-shocks led to reduced eyespot size in the expected homeotic direction, but neither additional eyespots nor wing shape changes were observed in forewings as expected of a homeotic transformation. Interestingly, *Ubx* was found to be expressed in a novel, non-characteristic domain – in the hindwing eyespot centers. Furthermore, ectopic expression of *Ubx* on the pupal wing activated the eyespot-associated genes *spalt* and *Distal-less*, known to be directly repressed by *Ubx* in the fly's haltere and leg primordia, respectively, and led to the differentiation of black wing scales. These results suggest that *Ubx* has been co-opted into a novel eyespot gene regulatory network, and that it is capable of activating black pigmentation in butterflies.

Introduction

Insects typically have two pairs of wings that differ in size, shape, or coloration, and these differences are usually attributed to the hox protein Ultrabithorax (Ubx). Ubx is normally expressed in posterior wings during the larval stage (Akam and Martinez-Arias, 1985; Warren et al., 1994), whereas the anterior pair of wings usually does not express any homeotic gene during its development (Carroll et al., 1995). Differences in the morphology of anterior and posterior wings are usually attributed to the way Ubx interacts with multiple wing regulatory network genes to alter network output in the hindwing (Pavlopoulos and Akam, 2011; Tomoyasu et al., 2005; Weatherbee et al., 1999).

Ubx function has been explored to different degrees in a variety of insect species. Removing Ubx function in insect hindwings usually converts the identity of these appendages into that of the forewing (Lewis, 1978; Tomoyasu et al., 2005; Weatherbee et al., 1999) suggesting that Ubx function is necessary to promote hindwing identity. Fewer experiments, however, have tested whether Ubx is sufficient to alter the "hox-free" forewing into hindwing identity. Here the evidence is confined to experiments in *Drosophila* (Castelli-Gair et al., 1990; Pavlopoulos and Akam, 2011), and the butterfly *Junonia coenia* (Lewis et al., 1999). Ectopic expression of Ubx in *Drosophila* forewings led to complete wing-to-haltere transformations (Castelli-Gair et al., 1990; Pavlopoulos and Akam, 2011), suggesting that Ubx expression is sufficient to confer hindwing identity to forewings in flies. In *Junonia*, however, the experiments were less conclusive. Strong ectopic mosaic Ubx expression on the forewing imaginal disc, using a sindbis viral promotor, transformed certain forewing traits, such as scale

* Corresponding author at: State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, China.
** Corresponding author at: Department of Biological Sciences, National University of Singapore, Singapore 117543.
E-mail addresses: xiaoling.tonger@gmail.com (X. Tong), antonia.monteiro@nus.edu.sg (A. Monteiro).

http://dx.doi.org/10.1016/j.ydbio.2014.08.020

morphology and pigmentation, to hindwing identity in the adult (Lewis et al., 1999), but wing shape was unaltered. However, because only mosaic expression was achieved at ectopic levels that were significantly higher relative to endogenous hindwing Ubx levels, it remains unclear whether uniform Ubx forewing expression, at normal endogenous hindwing levels, is sufficient to produce a complete homeotic transformation of forewing to hindwing identity in *Junonia* or in other butterflies.

Here we test Ubx's sufficiency in transforming a forewing into a hindwing in the butterfly *Bicyclus anynana* using novel transgenic tools. This butterfly, unlike *J. coenia*, has fewer eyespots on the forewing (two) relative to the hindwing (seven), and eyespot number can be used as an extra marker for detecting a homeotic transformation. Unlike *J. coenia*, however, *B. anynana* expresses the hox protein Antennapedia (Antp) in the centers of each eyespot on both forewings and hindwings (Saenko et al., 2011). The forewing is, thus, not strictly speaking a hox-free tissue in this species, and these experiments also allow us to investigate how the two hox genes interact on the wing.

We generated transgenic lines of *B. anynana* carrying the complete coding sequence of *Ubx* from *J. coenia* under the control of a heat-shock promoter (Chen et al., 2011) and over-expressed *Ubx* throughout embryonic and larval development using multiple heat-shocks. We also ectopically expressed *Ubx* on the early pupal wing by means of an infra-red laser beam (to provide a spatially restricted heat-shock) and investigated the response of candidate direct target genes, *Distal-less* and *spalt*. Finally, we cloned and described additional Ubx isoforms that are present in *B. anynana*, but absent in *J. coenia*. These experiments suggest that while the *Ubx* sequence tested is not sufficient to confer hindwing identity to forewings in *B. anynana*, it can activate a gene regulatory network involved in black pigmentation.

Materials and methods

Making the Ubx over-expression transgenic line

A fragment of 762 bp of the *J. coenia Ubx* cDNA (AY074760.1) (a gift from Sean Carroll) containing the entire open reading frame was cloned into the *Pogostick* plasmid (Chen et al., 2011), a *piggyBac* based vector that drives transgene expression via a heat-shock. A mixture of the recombinant plasmid (*Pogostick-JcUbx-up1*) carrying the desired insert and a helper plasmid (pHsp82PBac) carrying a *piggyBac* transposase sequence (Horn et al., 2002) was injected into *B. anynana* embryos within 2 h after egg-laying. Positive offspring were identified by the presence of EGFP fluorescence in their eyes. Integration of *Pogostick-JcUbx-up1* into the genome of *B. anynana* was confirmed by sequencing the genomic flanking regions to the piggyBac insertion using Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995). More details are in S1 Materials and Methods.

Whole-body heat-shocks and laser ectopic heat-shocks

Ubx transgenics and wild-type butterflies were raised in a climate room at 27 °C with a 12:12 h light:dark cycle and 80% relative humidity. In a previous limited characterization of the Ubx transgenic line we discovered that Ubx mRNA levels were significantly reduced 5 h after the end of a single heat-shock (Chen et al., 2011), so multiple heat-shocks were used to assure a high level of ectopic Ubx expression in the following experiments.

Embryonic heat-shocks

Two hours after egg laying (AEL) embryos of the Ubx over-expression line and Wt were collected and given four heat-shock pulses, each consisting of a 1.5 h heat shock at 39 °C, followed by a 6.5 h period at 27 °C. The embryos either completed embryogenesis at 27 °C or were used for immunostainings.

Larval heat-shocks

Forewing and hindwing eyespot-specific gene expression patterns appear during the middle (stage 2.5) of the fifth larval instar (Oliver et al., 2012), so we monitored 4th instar larvae (Ubx and Wt) daily until they molted to the final instar. These larvae were then given four heat-shocks per day at 39 °C, each 1.5 h in duration, separated by 4.5 h intervals at 27 °C, until the pre-pupal stage. Pre-pupae were transferred to 27 °C and reared until adult emergence. Ubx and Wt larvae were treated in parallel throughout these experiments: they were set up in approximate equal numbers in the same heat shock incubator, at the same time. Controls, i.e., non-heat-shocked individuals from the same generation from each line, were reared at 27 °C throughout. Adults were sacrificed by freezing upon emergence.

Laser heat-shocks

We used an infra-red laser heat shock system, similar to the green laser system described in (Ramos et al., 2006), to ectopically express Ubx in a small cluster of cells on the dorsal surface of the pupal forewing. Pupation time was scored by time-lapse photography using a Kodak DC290 digital camera. 14–21 h old pupae were treated with the laser. We used infrared heat pulses of 25 ms, separated by 400 ms intervals, during 20 min. After heat-shock, pupae were placed inside a small cup at room temperature until adult emergence, or until dissected for immunostainings.

Real-time PCR and immunostainings

Transcript levels of ectopic *JcUbx* and endogenous *B. anynana Ubx* (*BaUbx*) before and after heat-shock were quantified by real-time PCR. After 5 days of multiple heat-shocks, the forewing and hindwing discs of 5th instar larvae (Ubx and Wt), as well as non-heat shocked controls, were dissected for RNA isolation 1–2 h after the final heat-shock. Total RNA was extracted using an RNeasy Micro kit (Qiagen) and reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied biosystems). Real-time q-PCR was performed with TaqMan Universal PCR Master Mix using the Applied Biosystems 7500 Fast Real-Time PCR System. Eukaryotic 18S rRNA was used as the endogenous control. Relative quantification of *Ubx* transcripts was obtained using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *Ubx* levels were quantified using a *Ubx* (*Ubx*_F: GCG GAG GAG ACG TAT AGA AAT GG, *Ubx*_R: GGC GGT TTT GGA ACC ATA TTT TGA T, *Ubx*_Probe: CAC GCG CTC TGT CTC A) probe, which can amplify both *BaUbx* and *JcUbx* transcripts.

Expression of *Ubx*, *Antp*, *Dll* and *spalt* was assessed using immunostainings in embryos and wings from heat-shocked as well as control animals following the protocol of Brunetti et al. (2001). We used a rabbit anti-*J. coenia* Ubx antibody (at 1:500; a gift from L. Shashidhara), a mouse monoclonal anti-Antp 4C3 (at 1:200; Developmental Studies Hybridoma Bank), a rabbit polyclonal anti-Dll (at 1:200, a gift from Grace Boekhoff-Falk) and Guinea pig polyclonal anti-Spalt (at 1:20,000) antibody (Stoehr et al., 2013). The images were captured on a Nikon 90i microscope with NIS-Elements software (Nikon Instruments, Melville, NY, USA). More details are in S1 Materials and Methods.

Morphological analysis

First instar larvae that survived the heat-shock treatment (and some that were manually “hatched”) were photographed under a stereo microscope (Nikon SMZ1500), and multiple Z-sections were