Disruption of an N-acetyltransferase gene in the silkworm reveals a novel role in pigmentation

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SUMMARY
The pigmentation of insects has served as an excellent model for the study of morphological trait evolution and developmental biology. The melanism (mln) mutant of the silkworm Bombyx mori is notable for its strong black coloration, and is characterized by areas that are colorless in wild-type larvae (Fig. 1B). This striking phenomenon is of considerable interest in the investigation of the mechanism of fine-scale regulation of pigment metabolism and morphological divergence.

INTRODUCTION
Insects exhibit the richest diversity of all animals, and the rapid evolution of their phenotypic patterns suggests strong selective forces that promote and maintain variation. As a prototype of visible variation in insects, the melanism phenomenon is one of the most commonly cited examples of natural selection in evolutionary biology (Koch et al., 2000) relating to industrial pollution or mimicry. Melanins are generally characterized as the result of ectopic melanin deposition. In most invertebrates, melanin synthesis is achieved by the phenoloxidase (PO)-activating system and is crucial in many biological processes, including innate immunity, sclerotization and pigmentation pathways (Ashida and Brey, 1995; Soderhall and Cerenius, 1998; Andersen, 2005). As an initial step in this biochemical cascade, toxic quinone intermediates and α-quinones are produced, which can damage the hosts (Andersen, 2005; Kan et al., 2008). PO-induced melanin synthesis must therefore be tightly controlled to avoid deleterious pleiotropic effects on normal development and coloration.

The adult melanism (mln) body of the silkworm Bombyx mori shows strong overall pigmentation, whereas the wild type does not (Fig. 1A). However, black coloration appears only on the head, forelegs and tail spot of larval mln mutants, areas that are colorless in wild-type larvae (Fig. 1B). This striking phenomenon is of
MATERIALS AND METHODS
Silkworm strains
As mln is a recessive single locus trait, we designed crosses based on the single-pair backcross model (Miao et al., 2005). The mln strain (B1 was crossed with the wild-type strain Dazao. The resulting female F1 and male F1 were backcrossed with B1 to generate BC1F and BC1M populations, including 22 BC1F individuals (12 were wild type, ten were black) and 171 BC1M individuals (92 were wild type, 79 were black). Because the silkworm lacks crossing over in females (Maeda, 1939), the BC1F population was used to determine the linkage group and to screen for polymorphisms.

Additional wild-type (C108, Jingsong, Lan10, Nistari, Hua8, D1.31, D24.98 and Hainan) and mln (D104, Tchi, Huayuan, 1AHe03, 1AHDI.31 and 203) strains were used to detect and confirm the genotypes. All strains are preserved in the Zhenjiang Silicultural Research Institute of the Chinese Academy of Agricultural Sciences.

Sample preparation
Silkworms were fed with fresh mulberry and reared at 25°C. Whole bodies of individuals were homogenized in liquid nitrogen or with stainless steel drills, then genomic DNA was extracted as described (Zhan et al., 2009) and total RNA was isolated using TRIZol (Invitrogen) according to the manufacturer’s protocol. ReverTra Ace-α (TOYOBO) was used for cDNA reverse transcription.

Linkage mapping and candidate gene screening
Our previously developed simple sequence repeat (SSR) markers (Miao et al., 2005; Zhan et al., 2009) were screened in BC1F, and the co-segregated markers were then genotyped in the BC1M population as described (Miao et al., 2005). Linkage maps were generated by MAPMAKER/EXP 3.0b (Lander et al., 1987) using the Kosambi function as described (Zhan et al., 2009). SilkDB (Du et al., 2010) was used to localize the markers according to the flanking sequences, and subsequently used to predict the candidate genes based on their GLEAN model (Elsik et al., 2007; International Silkworm Genome Consortium, 2008). Open reading frames (ORFs) of the genes were cloned into pMD18-T vectors (TAKARA) and sequenced using a 3730 DNA analyzer (Applied Biosystems) with three independent replicates. Spatial expression profiles of candidate genes were obtained from the BmMDB database (Xia et al., 2007) and temporal profiles (available from GEO with accession GSE24611) were determined by the Agilent 8X15K gene expression platform. Insects were sampled daily in the larval and pupal stages. Total RNA of each sample was extracted separately and used for RNA amplification and labeling. A total of 14,623 silkworm genes for designing probes were derived from the GLEAN gene model (International Silkworm Genome Consortium, 2008). Oligonucleotide probe design, microarray construction, RNA labeling and hybridization, microarray imaging and data processing were performed by Shanghai Biochip (China).

Analysis of sequences and expression profiles
Quantitative real-time PCR was performed with SYBR Premix Ex Taq (TAKARA) on a Rotor-Gene 3000 real-time thermal cycler (Corbett Research) as described (Huang et al., 2007). BmG3PDH was amplified as a control to standardize the amount of cDNA. Primers are listed in Table S1 in the supplementary material. Annotation of candidate genes was carried out via the NCBI BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the InterProScan (http://www.ebi.ac.uk/Tools/InterProScan) online platforms. Annotation of candidate genes was carried out using a 3730 DNA analyzer (Applied Biosystems) with three independent replicates. Spatial expression profiles of candidate genes were obtained from the BmMDB database (Xia et al., 2007) and temporal profiles (available from GEO with accession GSE24611) were determined by the Agilent 8X15K gene expression platform. Insects were sampled daily in the larval and pupal stages. Total RNA of each sample was extracted separately and used for RNA amplification and labeling. A total of 14,623 silkworm genes for designing probes were derived from the GLEAN gene model (International Silkworm Genome Consortium, 2008). Oligonucleotide probe design, microarray construction, RNA labeling and hybridization, microarray imaging and data processing were performed by Shanghai Biochip (China).

Protein expression, purification and detection
Prokaryotic expression of recombinant protein was performed as follows. The intact ORFs (predicted for lm and sm) were cloned into pET-22b (Novagen) fused with a C-terminal 6-His tag and amplified in E. coli (DE3). The recombinant vectors were confirmed and transformed into E. coli DE3 and fusion proteins were expressed after IPTG induction at 37°C. An additional approach for expression of lm included another donor vector, pGEX-4t-3 (GE Healthcare), fused with glutathione S-transferase (GST). The recombinant protein was also expressed in Sf9 insect cells using a Bac-to-Bac baculovirus expression system (Invitrogen). pFastBac HT-A constructs containing intact corresponding ORFs and 6×HIS were generated and transformed into DH10Bac E. coli for transposition into the bacmids. Cellfectin (Invitrogen) was used for transfection of Sf9 cells with the confirmed recombinant bacmid. The third generation of the amplified recombinant baculovirus stock was used to infect Sf9 cells to express the recombinant protein. E. coli or cell pellets were rinsed and resuspended in 1× PBS, then homogenized by sonication in lysis buffer (pH 7.5) containing 200 mM HEPEs, 20 mM NaOH, 1 mM EDTA, 1 mM DTT, 100 μg/ml PMSF, 10% glycerol, 0.01% Nonidet P40 and Protease Inhibitor Cocktail (GE Healthcare). The samples were centrifuged at 16,000 rpm (28,672 g) for 20 minutes at 4°C, and then the recombinant proteins were purified from the supernatant using Ni-NTA Superflow cartridges (Qiagen). Proteins were eluted with Tris-HCl buffer (50 mM Tris, 100 mM NaCl, pH 7.5) for enzymatic assay.

Recombinant proteins were separated by 12% SDS-PAGE at 80 mA for 2 hours. Western blotting was performed by semi-dry transfer to Hybond-C nitrocellulose membrane (Amersham Biosciences) for 90 minutes at 0.8 mA/cm². Each membrane was then treated as follows: (1) blocked with 5% milk in TBST (2.5 mM Tris, 15 mM NaCl, 0.2 mM KCl, 0.1% Tween 20, pH 7.4) with gentle shaking for 3 hours; (2) incubated in mouse anti-HIS antibody (1:3000 dilution, Sigma) at 4°C overnight; and (3) washed three times in TBST with gentle shaking for 10 minutes; (4) incubated in TBST with a 1:1000 goat HRP-conjugated anti-mouse IgG (H+L) antibody (ProteinTech Group) at 4°C for 1 hour; and (5) washed three times in TBST with gentle shaking for 10 minutes. HRP activity was detected using a chemiluminescent substrate (Millipore), and the films and blots were imaged using a Kodak 102 medical X-ray processor.

AAANt activity assay
The assay procedure employed standard protocols (Borjigin et al., 1995; Ichihara et al., 2001) with some modification. The 25 μl reaction mixture contained 0.5 μl 50 mM trypamine or dopamine (Sigma-Aldrich), 5 μl 5 mM acetyl-CoA [10 mM H3-labeled (PerkinElmer) mixed with 5 μl unlabeled (Sigma-Aldrich) acetyl-CoA to give 1:3 hot:cold], purified protein and Tris-HCl buffer. After incubation at 37°C for the appropriate time, the reaction was stopped by adding 125 μl 5% acetic acid. Then, 600 μl of a toluene/isoamyl alcohol mixture was added (97:3 for the tryptamine substrate, 3:2 for the dopamine substrate), followed by 30 seconds of vortexing and a 5-minute centrifugation at 800 g. Then, 400 μl of the organic phase was transferred to a scintillation vial containing 1.2 ml liquid scintillation cocktail (PerkinElmer). Radioactivity was measured with a liquid scintillation counter (LS-6500, Beckman).

RNAs
For RNAi experiments, double-stranded (ds) RNAs were synthesized from PCR templates using the MEGAscript T7 Transcription Kit (Ambion) according to the manufacturer’s protocol. Primers used to generate PCR templates contained a T7 promoter at the 5’ end, and the amplified regions corresponded to CDNA of nucleotides 133-536 of C11 and 57-596 of C12. Injection of dsRNA into pupae (day 4 or 5) was performed as described.
dsRNA for enhanced green fluorescent protein (EGFP, from Clontech pEGFP-N1, nucleotides 74-619) was used as a control. Ten insects (five male and five female) were sampled as a group for analysis of gene expression at 0, 24, 48 and 72 hours after injection by real-time PCR, as described above.

RESULTS

Mapping the mln locus of Bombyx mori

We initially screened polymorphisms from 518 SSR markers within the female backcross BC1F population. The genotyping results demonstrated that the markers on chromosome 18 co-segregated with the black color trait, which was consistent with previous results (Miao et al., 2005). Eight available markers (see Table S1 in the supplementary material) on chromosome 18 were then genotyped on 171 BC1M segregants. Based on the linkage analysis (LOD=5), we localized the mutation site between S1808 and S1807 (Fig. 2A). These two closely linked markers corresponded to 8,223,821 and 8,871,281 bp, respectively, of nscaf2902 according to the SilkDB database (Duan et al., 2010) (Fig. 2B). Thus, the candidate region was narrowed to ~650 kb.

Identification of the mln candidate gene

A total of 13 predicted genes were found in the region of interest (Fig. 2C and see Table S2 in the supplementary material). Based on comparisons of coding sequences, seven candidates were excluded (Fig. 2C): C2, C12 and C13 of mln (b04) were identical to those in the wild-type strain Dazao; C3 and C6 only exhibited several silent SNPs, using Dazao as a reference; and SNPs detected in C1 and C8 appeared specific to Dazao, as mln shared the same genotype at these sites with another wild-type strain, Jingsong. We also detected an insertion in C7 of mln but not Dazao, but this mutation was found not to be mln specific after more wild-type and
mln strains were examined. In addition to the sequence variations, the expression pattern of the candidate gene should associate with substantial expression on the larval head and tail spot, and over the entire body of the adult (Fig. 1A,B). Based on available microarray data (Xia et al., 2007), both stage- and tissue-specific expression profiles suggested that candidates C4, C5, C7, C9 and C10 could be excluded because of their unrelated patterns (Fig. 2D). Sequence analysis revealed a 126 bp deletion in C11 in the mln genomes (Fig. 2C) and the level of C11 transcripts was significantly elevated in adult stages and relatively high in the head and integument (Fig. 2C). In the mutant, the deletion in exon 4 caused the frameshift mutation of C11 is responsible for the observed phenotype. Using real-time PCR, we further investigated the expression pattern of C11, including in the tail spot. As expected, C11 was strongly expressed in the head, integument and tail spot (see Fig. S1 in the supplementary material), where black coloration was evident in the larvae of mln strains.

**Sequence analysis of mln**

According to the annotation, C11 represents Bm-iAANAT, a previously cloned silkworm gene that encodes an AANAT (Tsugehara et al., 2007). Bm-iAANAT consists of five exons and spans a ~3.9 kb region on nscaf2902 (Fig. 3A). Genomic sequencing of mln revealed a 126 bp deletion of the distal part of exon 4 and the beginning of the next downstream intron, and a 30 bp tandem insertion of the following 30 bp sequence (Fig. 3B). We obtained cDNA from mln mutants and identified two co-existing transcripts in various strains and stages (Fig. 3C). The short mutant (sm) ORF (deposited in GenBank with accession GU479039) was spliced from exon 3 to exon 5 and completely lacked exon 4, whereas the long mutant (lm) ORF (deposited in GenBank with accession GU479038) contained the defective exon 4 and an additional 15 bp from the downstream intron (Fig. 3C). Based on the GT/AG rule, we believe that the lack of the original splice site and the import of GT from the following 15 bp insertion are likely to have resulted in alternative splicing, a pattern that has been observed previously in another silkworm mutant (Futahashi et al., 2008). Wild-type Bm-iAANAT encodes 261 amino acids, with a putative GCN5-related N-acetyltransferase domain (IPR000182, InterProScan) at residues 53-259 and a coenzyme A-binding pocket structure (cd04301, NCBI BLASTx) at residues 180-195 (Fig. 3C). In the mln mutant, the deletion in exon 4 caused the frameshift mutations for sm and lm from residues 132 and 179, respectively. Multiple alignments suggest that both of these mutated proteins most likely lack the function of the wild-type.

**Phylogenetic analysis of AANAT**

AANAT genes have been cloned in many organisms (Klein, 2007; Tsugehara et al., 2007). They have generally been reported as penultimate, rate-limiting enzymes in the reaction of serotonin with acetyl-CoA to form N-acetylserotonin, playing a major role in the regulation of melatonin circadian rhythm in vertebrates (Ferry et al., 2000). However, Drosophila melanogaster AANAT1 (Dat)
Fig. 3. Differences in Bm-iAANAT between the wild type and mln. (A) Boxes indicate the exons (Ex) of Bm-iAANAT, the positions of their start points on nscF2902 are shown above. The lines connecting boxes indicate introns, and the numbers represent their length. A deletion in mln was found in exon 4 and the next downstream intron, as indicated by the dotted box and shortened length of the intron. (B) An enlarged view of the deletion region. The 126 bp deletion in the mln genome is indicated by dotted lines. The positions of the start point of exon 4, the start point of the deletion, the point of the original splice junction, and the end point of the deletion are shown above. An additional 30 bp insertion into the mln genome is marked by brackets and its base sequence shown. (C) Bm-iAANAT transcripts in the wild type and mln. Two parallel transcripts in mln, termed lm and sm, are indicated by the splicing of exon boxes. The red box indicates the mln-specific insertion from the red base portion of the insertion in the genome as shown in B. The black rectangles crossing the exon boxes indicate the altered protein sequences caused by a frameshift mutation. A GCN5-related N-acetyltransferase domain (orange) and the conserved CoA-binding pocket (green) are highlighted. The PCR patterns of the transcripts in mln and wild-type are shown beneath. The primer set used for amplification is indicated by the pair of black arrowheads above the boxes. The detection panel used cDNA from fifth instar day 3 larvae as follows (from left to right): Tchi, Huayuan, z03 (male), z03 (female), 1AH203 (male), 1AH203 (female), 1AH1D31 (male), 1AH1D31 (female), I04 and I04 (adult) in the mln panel and Dazao, C108, Jingsong, Lan10, Nistari, Hainanji, Huaa, D24.98, D1.31 (male) and D1.31 (female) in the wild-type panel. M is a DNA marker, showing (top to bottom) 2000, 1000, 750 and 500 bp.

represents a novel gene family that is unrelated to known acetyltransferases, except for two weakly conserved amino acid motifs (Hintermann et al., 1996). Our phylogenetic analysis, based on amino acid sequences of AANAT, showed that the Chinese tussah moth (Antheraea pernyi) enzyme exhibits the highest degree of homology with the silkworm enzyme. Independent insect clusters were derived from Protista and were subsequently well separated from vertebrate lineages (see Fig. S2 in the supplementary material), which suggests the novel role of AANAT in insects. This evolutionary pattern is consistent with that of genes responsible for other visible silkworm traits, such as BmKYNU, a kynureninase gene that determines the red blood (rb) mutant (Meng et al., 2009a).

Enzymatic properties of Bm-iAANAT in mln

The phylogenetic analysis suggests that AANAT might have additional functional domains. To further investigate the enzymatic properties of Bm-iAANAT in mln mutants, we expressed three recombinant proteins and compared AANAT activity between normal (wt) and two mutant (lm and sm) types. Overexpression of wt and sm was detected in a bacterial expression system; however, expression of lm was not observed. Therefore, we used SF9, a cell line derived from pupal ovarian tissue of Spodoptera frugiperda, to produce this mutant protein. SDS-PAGE and immunoblot analysis demonstrated that the sizes of the purified proteins were exactly as predicted (Fig. 4A). The relative molecular mass of the bacterially expressed lm recombinant protein was slightly lower than that expressed in insect cells, indicating some post-translational modification in eukaryotic cells.

We assayed wt and mutant recombinant AANAT proteins for AANAT activity using tritium-labeled acetyl-CoA and tryptamine as substrates. Radiolabeled product formation was proportional to the incubation time and the amount of protein in the reaction mixtures containing wt recombinant protein. By contrast, the AANAT activity of sm and lm remained significantly lower, even when the amount of protein or reaction time was greatly increased (Fig. 4B and see Table S3 in the supplementary material). We also used dopamine, another monoamine, as a substrate for the enzymatic assay, and this confirmed the inactivity of AANAT in
have also been identified in the Swallowtail butterfly and Red flour phenotypic traits for upstream enzymes, such as TH and DDC, branches that promote melanin formation (Fig. 1). Several ectopic melanin formation (Fig. 1), or on yellow (Wittkopp et al., 2002a; Wittkopp et al., 2002b; Gompe et al., 2005), which is involved in branches that promote melanin formation (Fig. 1). Several ectopic phenotypic traits for upstream enzymes, such as TH and DDC, have also been identified in the Swallowtail butterfly and Red flour beetle (Koch et al., 2000; Futahashi and Fujiwara, 2005; Arakane et al., 2009). In a parallel pathway to ebony, AANAT catalyzes the conversion of dopamine to NADA, but no mutant phenotype has been observed (Brodbeck et al., 1998). AANAT was previously considered to have a major role in sclerotization (Walter et al., 1996; Andersen, 2005; Gibert et al., 2007). We provide the first phenotypic demonstration of the contribution of AANAT to insect color patterns.

Taking into account the previous lines of phenotypic evidence, the mutation of each gene in the melanin synthesis pathway appears to confer a unique or complex color pattern in insects. Many or all of the genes involved are pleiotropic, as melanism has roles in multiple parts of the body and in other physiological processes, such as innate immunity and development. Thus, the independent and stable phenotypic defects are likely to be due to functional redundancy among the gene family members in insects. Recent research has shown that the yellow-e protein determines the silkworm bts mutant (Ito et al., 2010), which exhibits a similar

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R, regional melanization; O, overall melanization; No, no ectopic phenotype.

**RNAi verification**

The entire body of mln adults is black. To verify whether a deficiency of Bm-iAANAT is responsible for the black color pattern of the integument, we performed RNAi experiments to suppress the expression of the corresponding gene in wild-type insects. After injecting dsAANAT into pupae, we observed that the cuticle of the majority of wild-type adults (90 of 96 males and 71 of 112 females; Table 1) turned partly (Fig. 5A,B) or completely (Fig. 5B-E) black. It is conceivable that the phenotype produced by RNAi-mediated repression of C11 is less severe than that caused by the mln mutation. We also noticed that RNAi of C11 was more effective with males than females. In the RNAi experiment, we injected equal amounts of dsRNA into female and male pupae, but because the male pupae are usually smaller than female pupae, the males effectively received a higher dose, causing the effect of RNAi to be more evident. Real-time PCR revealed that the level of Bm-iAANAT transcription was significantly downregulated on the third day after dsRNA injection (Fig. 5F). We also injected dsAANAT into mln mutants, but observed no change in the melanin phenotype. These findings provide additional evidence that the black coloration of mln results from the loss of its AANAT function.

**DISCUSSION**

**Dat** is reported to be involved in melanin metabolism rather than in time keeping (Wright, 1987; Wittkopp et al., 2003a; Wittkopp et al., 2003b). In this process (Fig. 1), tyrosine is hydroxylated to DOPA by tyrosine hydroxylase (TH), and DOPA can then be transformed into dopamine by decarboxylation by DOPA decarboxylase (DDC). Dopamine is a key compound for both sclerotization and melanin formation (Andersen, 2005). The black color patterns of insects are the result of ectopic melanin deposition, which reflects the differential spatial regulation of four parallel branches from the core dopamine pathway (Wright, 1987; True et al., 1999; Wittkopp et al., 2002a). The downstream products of dopamine – N-acetyl dopamine (NADA) and N-β-alanyl dopamine (NBAD) – can both serve as precursors in cuticle sclerotization (True, 2003; Wittkopp et al., 2003a), as an alternative to producing DOPA-melanin or dopamine-melanin pigments (Fig. 1). For decades, fruit fly pigmentation studies have focused on ebony (Wittkopp et al., 2002a; Kohn and Wittkopp, 2007; Wittkopp et al., 2009) and tan (True et al., 2005; Jeong et al., 2008; Wittkopp et al., 2009), which are involved in the NBAD branch that inhibits melanin formation (Fig. 1), or on yellow (Wittkopp et al., 2002a; Wittkopp et al., 2002b; Gompe et al., 2005), which is involved in branches that promote melanin formation (Fig. 1). Several ectopic phenotypic traits for upstream enzymes, such as TH and DDC, have also been identified in the Swallowtail butterfly and Red flour beetle (Koch et al., 2000; Futahashi and Fujiwara, 2005; Arakane et al., 2009). In a parallel pathway to ebony, AANAT catalyzes the conversion of dopamine to NADA, but no mutant phenotype has been observed (Brodbeck et al., 1998). AANAT was previously considered to have a major role in sclerotization (Walter et al., 1996; Andersen, 2005; Gibert et al., 2007). We provide the first phenotypic demonstration of the contribution of AANAT to insect color patterns.

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phenotype to mln larvae. However, our results show that the mln (b04) Bmyellow-e cDNA sequence is identical to that of the wild type, suggesting that they share a similar function in shaping silkworm larval pigmentation.

In the candidate region, C12, another NAT gene, is located adjacent to the candidate in mln, which raises the question of whether a defect of C12 might be responsible for the observed mln phenotype. We initially excluded C12 because of the lack of a coding mutation, but recent published work has shown evidence for regulatory mutation in the silkworm trait (Sakudoh et al., 2010). Comparative analysis indicated that the expression levels of C12 at the sites where melanism was seen (such as the larval head, the larval tail spot and the whole body of the adult) were essentially the same in the wild type and mln mutant (see Fig. S3A in the supplementary material). This would seem to preclude the possible occurrence of regulatory mutations in C12. In addition, we did not find a correlation between the expression pattern of C12 and the melanic phenotypes of mutants (see Fig. S3B,C in the supplementary material), which reinforces the notion that C12 is not related to the mln phenotype. RNAi of C12 in wild-type insects effectively suppressed C12 expression (see Fig. S4 in the supplementary material), but this did not lead to any ectopic melanin phenotype (Table S5 in the supplementary material).

mln phenotypes differ between larvae and adults within the same strains (Fig. 1A,B). A plausible explanation for this is that the adult and larval color patterns are, in many cases, regulated by different regulatory mutations. In addition to the melanic tissues, silkworm AAANATs are also highly expressed in the anterior and middle silk gland (Fig. 2D), suggesting a potential pleiotropic role. The differential regulation of Bm-iAAANAT in establishing pigment patterns in different tissues of larval and adult stages is intriguing, and the impact of mln phenotypes on the overall fitness of the mutants remains to be established.

Acknowledgements
We thank Drs Anjiang Tan, Hui Xiang, Xiaonan Yang and Yongzhen Xu for valuable discussions and Yajun Jiang for assistance with protein purification.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.053678/-/DC1

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