Note

Natural Variation of *ebony* Gene Controlling Thoracic Pigmentation in *Drosophila melanogaster*

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ABSTRACT

We identified the causal genetic variation for the difference in the thoracic trident pigmentation intensity between two wild-derived strains of *Drosophila melanogaster*. It was found to be the difference in expression level of *ebony*, which codes for an enzyme in the melanin-synthesis pathway and has pleiotropic effects on vision and behavior.

INTRASPECIFIC variation in the pattern and intensity of pigmentation exists widely in diverse insect taxa (reviewed in TRUE 2003; GRAY and MCKINNON 2006). In *Drosophila melanogaster*, variation in thoracic trident pigmentation intensity has been long understood (MORGAN and BRIDGES 1919; PLOUGH and IVES 1934, 1935; JACOBS 1960; DAVID *et al.* 1985; MORETEAU *et al.* 1995; GIBERT *et al.* 2000, 2004; MUNJAL *et al.* 1997; WITTKOPP *et al.* 2002). Some authors predicted that *ebony* is responsible for this phenotypic variation, mainly from the observation of mutants showing a dark trident pattern on their thorax segments (JACOBS 1960; WITTKOPP *et al.* 2002). *ebony* mutants are also known to have defects in neuronal activities at photoreceptor terminals (HOTTA and BENZER 1969; HEISENBERG 1971; BORYCZ *et al.* 2002), arrhythmic locomotor activity (NEWBY and JACKSON 1991), and unusual sexual behavior (KYRIACOU *et al.* 1978; KYRIACOU 1981). These pleiotropic effects are likely to stem from the EBONY expression in the nervous systems (HOVEMANN *et al.* 1998; RICHARDT *et al.* 2002, 2003; WAGNER *et al.* 2007); thus, variation of this gene is likely to have some impact on individual fitness under natural conditions.

Despite the widely accepted prediction, no evidence directly links the *ebony* gene to the thoracic trident pigmentation variation in natural populations. We performed a genetic mapping using two wild-derived strains to identify the causal genetic differences underlying this naturally occurring phenotypic variation.

To conduct a systematic genetic mapping, we used two representative wild-derived inbred lines, Mel6 (G59) and TW1 (G23), which originated from Benin, West Africa, and Taiwan, respectively. These lines were subjected to 59 and 23 generations of sib mating, respectively, to construct nearly isogenic lines. Figure 1, A and B, shows the thoracic pigmentation pattern in these strains. The trident pattern shaped by dark pigmentation clearly appears in TW1 but not in Mel6. We checked the phenotype at three different temperatures—18°, 20°, and 25°—since temperature is known to affect the intensity of pigmentation (DAVID *et al.* 1985; MORETEAU *et al.* 1995; GIBERT *et al.* 2000, 2004, 2007; MUNJAL *et al.* 1997). The pigmentation of the TW1 line darkens when reared in lower temperatures, but no obvious trident shape appeared in Mel6 in the temperature range we tested (data not shown). The phenotype of TW1 is completely recessive over Mel6 at least when reared at 25°.

Chromosomal substitution lines were constructed (Figure 1C) to investigate which chromosomes have effects on the pigmentation pattern. Thoracic trident phenotypes of these lines indicate that only the third chromosome contributes to the phenotypic difference (Figure 1C). Next, we constructed 73 third chromosome recombinant lines in the Mel6 homozygous background. These lines were made homozygous using balancer chromosomes, and they were genotyped for 17 molecular markers. On the basis of the comparison of
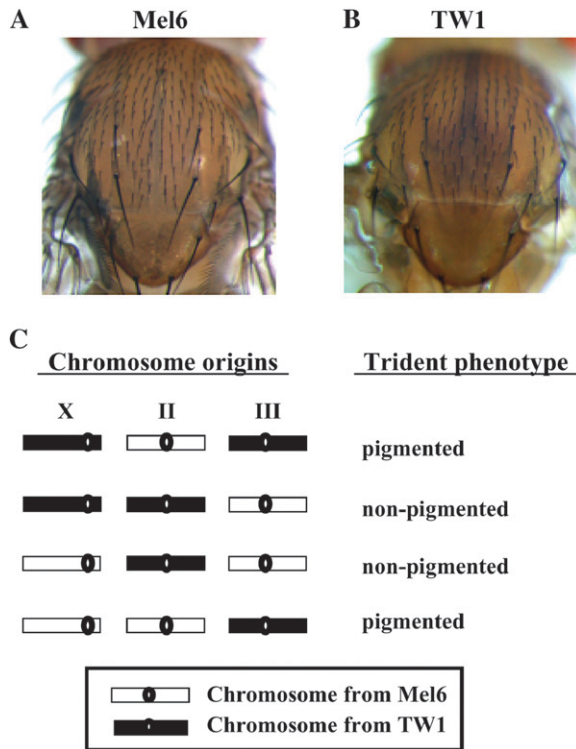
FIGURE 1.—Thoracic trident pigmentation pattern and its association with the third chromosome. (A) Thorax segment of Mel6 (G59) originated from Benin, West Africa. (B) Thorax segment of TW1 (G23) originated from Taiwan. Mel6 (G59) and TW1 (G23) were subjected to 23 and 59 generations of sib mating, respectively, to construct nearly isogenic lines. (C) Trident phenotypes of the whole-chromosome substitution lines between Mel6 and TW1. Chromosome origins and the trident phenotypes of the lines are indicated in the left and right columns, respectively. These lines were generated using balancer chromosomes by a similar scheme used in HOLLOCHER *et al.* (1997). Pigmentation intensity was examined under the microscope.

genotypes and phenotypes of these recombinants, there is likely to be only one major segregating factor between *Rh2* and *Gr93f* for the trident pigmentation variation (Figure 2A).

Finer breakpoint investigation on six recombinant lines that had their breakpoints between markers *Rh2* and *Gr93f* showed that the critical region lies between markers *r-l* and *tin* (Figure 2B). Note that one line in Figure 2A that showed nonpigmented phenotype but had TW1 genotype at *Gr93f* is represented as MT10-64 in Figure 2B. This line actually had Mel6 genotype at most of the marker positions between *Rh2* and *Gr93f*. To further map the gene, we used a laboratory population that was made from a cross between Mel6 and TW1 and experienced over 20 generations of free recombination. We chose 116 individuals showing the trident phenotype and screened for those that had recombination breakpoints between *r-l* and *tin*. Ten individuals met these criteria. Figure 3 shows the recombination breakpoints of the three most informative recombinants. From these results, we narrowed down the critical region for the responsible gene to ∼75 kb between *CG7000* and *CG5892*.

The ∼75-kb region included seven annotated genes including *ebony* (*CG3331*), a strong candidate gene. We sequenced the coding regions of *ebony* gene from TW1 (AB301907) and Mel6 (AB301908). There were two amino acid differences between those sequences. Although we cannot completely exclude the possibility, it is less likely that these differences cause some effect on the phenotype, since those changes were not consistent with the phenotype in other inbred lines collected in Iriomote, Japan, or in other worldwide areas (data not shown).

Immunocytochemical and reporter gene assays have shown that EBONY is expressed in multiple tissues including the epidermal cells of thorax segment (HOVEMANN *et al.* 1998; RICHARDT *et al.* 2002, 2003; WITTKOPP *et al.* 2002; GIBERT *et al.* 2007; WAGNER *et al.* 2007), in which the highest level of the EBONY expression is found in cells that produce the trident pigmentation in *ebony* mutants (WITTKOPP *et al.* 2002). Also, *e-lacZ* transformants showed high expression of the *ebony* transcripts at the trident producing cells (GIBERT *et al.* 2007). To see if the expression level of *ebony* is lower in the thorax of TW1 than that of Mel6, we performed quantitative reverse transcriptase PCR (qRT–PCR) as follows. Adult flies within 1 hr of eclosion were dissected under the microscope; this is when the pigmentation of the adult cuticle initiates. For each of Mel6 and TW1 strain, three replicate RNA were extracted from thoraces of 12 individuals using the QIAGEN RNeasy kit (QIAGEN, Valencia, CA) with subsequent DNase I digestion procedure. The first stranded cDNA was synthesized by using 2 μg of total RNA with SYBR ExScript RT–PCR kit (Perfect Real Time) from Takara-bio (Tokyo) in a 40-μl reaction volume. qRT–PCR was performed with SYBR ExScript RT–PCR kit (Perfect Real Time, Takara-bio) on a Thermal Cycler Dice TP800 from Takara-bio. For each cDNA, two qRT–PCR reactions were conducted.

The expression levels of *ebony* were measured three times with RNA samples extracted on different days. The expression level standardized by *Actin57B* (*CG10067*) was 1.69 ± 0.13 (SD) fold higher in the thorax of Mel6 when compared to that of TW1. This difference was statistically significant (t in pairwise Student's t-test = 16.16, d.f. = 2, $P < 0.005$). The same conclusion was obtained by using *rp49* (*CG7939*) as the internal control (ratio of expression levels, Mel6/TW1 = 1.33 ± 0.06 (SD); $t = 5.08$, d.f. = 2, $P < 0.05$). This finding is consistent with the hypothesis that the thoracic trident pigmentation variation is caused by the difference in *ebony* expression level: low expression in the pigmented strain and high expression in the nonpigmented strain.

We also performed a complementation test using *ebony* mutants. TW1 showed pigmented phenotype under the heterozygous condition with the e^1 mutant (Figure 4A), whereas Mel6 showed nonpigmented phenotype under
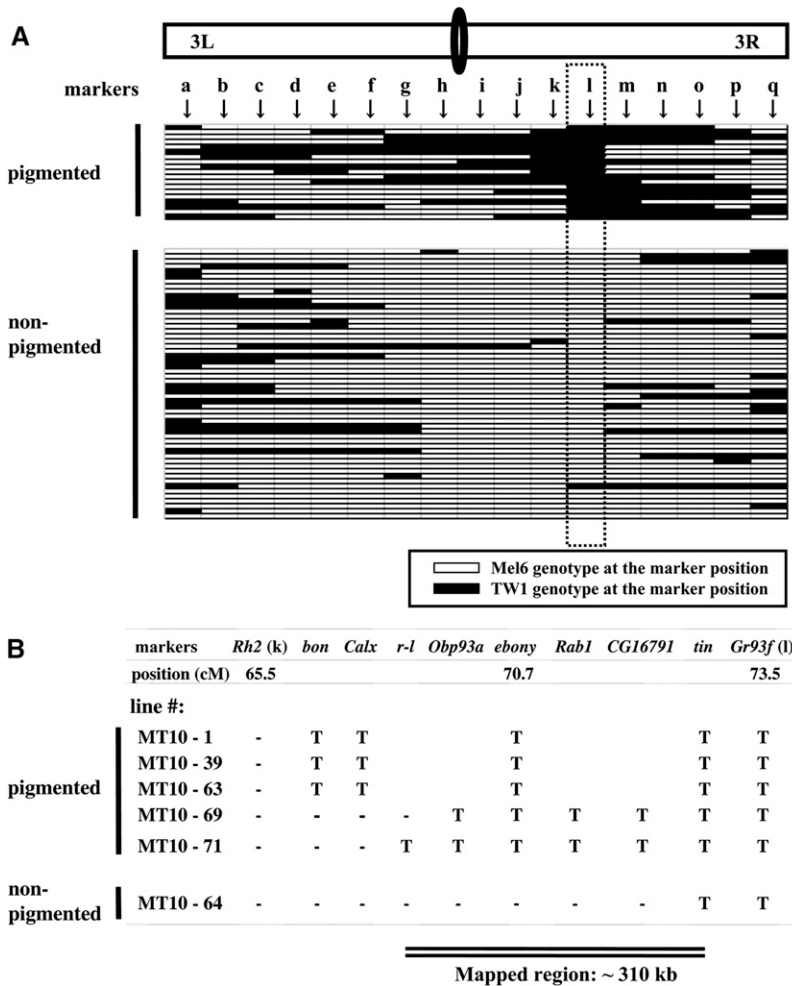
FIGURE 2.—Genetic mapping of the thoracic trident pigmentation phenotype using 73 homozygous recombinant lines constructed between TW1 and Mel6. (A) Genotypes of the pigmented ($n = 19$) and nonpigmented ($n = 54$) phenotype lines at 17 restriction fragment length polymorphism (RFLP) markers (a–q) on the third chromosome: a, *Gr61d*; b, *gry*; c, *Gr64A*; d, *lama*; e, *Gr65c*; f, *Dhpr*; g, *Gr68d*; h, *Or74a*; i, *desat2*; j, *pnr*; k, *Rh2*; l, *Gr93f*; m, *RpS3*; n, *ssh*; o, *Pr*; p, *Gr98a*; q, *Ptx*. Horizontal bars indicate genotypes of the recombinant lines. The dotted square indicates the genotypes at *Gr93f* marker that had almost complete association with the phenotype ($r^2 = 0.93$). Note that one exceptional line that showed nonpigmented phenotype but had TW1 genotype at *Gr93f* is represented as MT10-64 in B. RFLP markers were developed by direct sequencing PCR fragments of ∼600–900 bp from Mel6 and TW1. Further information on RFLP markers can be provided upon request. Primers were designed by Primer3 software (ROZEN and SKALETSKY 2000) from the publicly available genome sequence (ADAMS *et al.* 2000). DNA was extracted from one male each from Mel6 and TW1 using the GeneElute Mammalian Genomic DNA kit (Sigma-Aldrich, St. Louis). PCR products were obtained from these genomic samples by the AmpliTaq Gold PCR kit (Applied Biosystems, Foster City, CA) and were purified using MultiScreen-FB filter plates (Millipore, Billerica, MA). These purified products were directly sequenced on both strands using the BigDye terminator cycle sequencing kit version 3 (Applied Biosystems). The third chromosome recombinant lines were constructed from F_2 backcrossed individuals in the Mel6 homozygous background. The line was backcrossed twice (including the F_2 backcross) to Mel6 and had two generations of recombination opportunity. The lines were made homozygous using balancers in the last step. Note that these third chromosome recombinants possess the X and the second chromosomes from Mel6. (B) Genotypes at eight additional RFLP markers in six recombinant lines from A that had their breakpoints between markers *Rh2* (k) and *Gr93f* (l). T indicates TW1 genotype and "-" indicates Mel6 genotype. Two critical lines, MT10-69 and MT10-64, indicate that the ∼310-kb region between markers *r-l* and *tin* is responsible for the phenotype.

markers	Rh2 (k)	bon	Calx	r-l	Obp93a	ebony	Rab1	CG16791	tin	Gr93f (l)
position (cM)	65.5					70.7				73.5
line #:										
MT10 - 1	-	T	T			T			T	T
MT10 - 39	-	T	T			T			T	T
MT10 - 63	-	T	T			T			T	T
MT10 - 69	-	-	-	-	T	T	T	T	T	T
MT10 - 71	-	-	-	T	T	T	T	T	T	T
MT10 - 64	-	-	-	-	-	-	-	-	T	T

Mapped region: ∼ 310 kb

the same condition (Figure 4B). The results were almost identical when we used e^{AFA}, *Df(3R)e-H4*, and *Df(3R)e-R1* as *ebony* mutants (data not shown). These results indicated that *ebony* is allelic to the locus we mapped.

Next, we designed another experiment to show that the *ebony* transcript is necessary to give rise to the nonpigmented-thorax phenotype. It is already known that loss-of-function mutations of *ebony* show pigmented phenotype. However, those mutant strains have various genetic backgrounds. To cleanly examine the effect of this single gene, we conducted an inducible RNAi experiment using GAL4-UAS system, which allowed us to knockdown this specific gene in a controlled genetic background (w^{1118}). Figure 4, C and D, shows that the trident pigmentation appears when RNAi is induced by a ubiquitously expressed *Actin5C* GAL4 driver. This result showed that the reduced expression level of this single gene can produce the pigmented-trident phenotype in a controlled genetic background.

We have shown that the difference in expression level of *ebony* is responsible for the variation in thoracic trident pigmentation between the two representative lines. On the other hand, there are reports of mutations in other loci, which give rise to this trident-pigmented phenotype. In the early years of Drosophila genetics, MORGAN and BRIDGES (1919) reported *with* (*with trident*) on 2-55 and Bridges discovered *ptg* (*pentagon*) around the same time (LINDSLEY and GRELL 1968). PLOUGH and IVES (1934, 1935) reported another locus, *tri* (*trident*). Nevertheless, none of these have been molecularly identified. These reports and phenotypes of other body color mutants such as *black* (LINDSLEY and ZIMM 1992) suggest that *ebony* may not be the only segregating locus for the trident pigmentation variation in natural populations. Curiously, among the worldwide populations, latitudinal cline of the pigmentation intensity has been reported (DAVID *et al.* 1985; MUNJAL *et al.* 1997). It is intriguing to investigate how much of
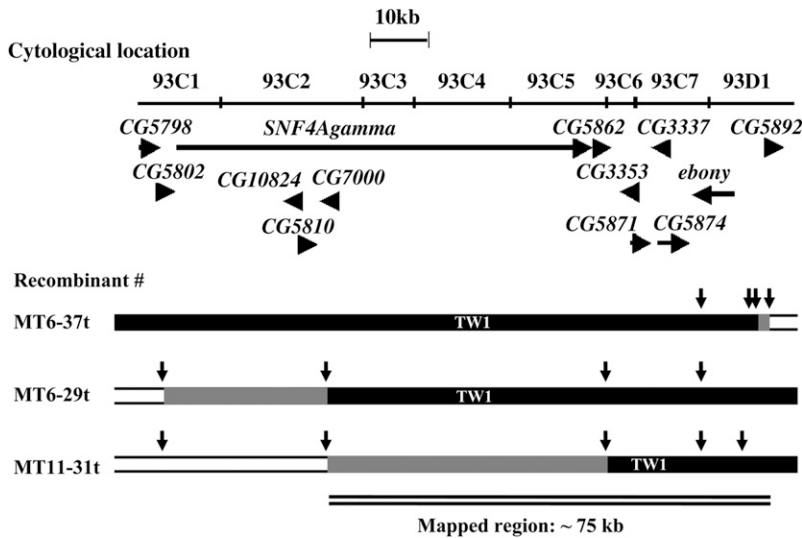
FIGURE 3.—Recombination breakpoints of the three most informative recombinants between Mel6 and TW1. The recombinants were screened from a laboratory population that experienced over 20 generations of recombination. These third chromosome recombinants had the X and the second chromosomes from Mel6. Horizontal arrows indicate annotated ORFs with their transcriptional directions. Vertical arrowheads indicate positions of the markers genotyped. Open and solid bars represent chromosome segments from Mel6 and TW1, respectively. Shaded bars indicate regions where recombinant breakpoints reside. The markers were genotyped by directly sequencing PCR fragments of ∼600–900 bp. The thoracic tridents of the three recombinants were all pigmented. Note that the critical interval of ∼75kb between *CG7000* and *CG5892* includes the candidate gene *ebony*.

this clinal variation can be explained by the expression level variation of the *ebony* gene.

Recently, POOL and AQUADRO (2007) investigated female abdominal pigmentation in the lines from Africa. They compared the DNA sequences of the *ebony* gene region and found that darkest lines sequenced had nearly identical haplotypes spanning 14.5 kb upstream of the protein-coding exons of *ebony*. Neither of the two lines that we used in our study had dramatically dark abdominal pigmentation as seen in the darkest scored individuals photographed in their study. However, it is certainly of our interest to examine *ebony*'s contributions to both abdominal and thoracic pigmentation and also whether the haplotype block found in the darkest African lines is present in other worldwide samples with various intensities of abdominal and thoracic pigmentation. The next step would be to investigate DNA sequences from worldwide strains and population samples to identify the specific site(s), region(s), or haplotype(s) responsible for the pigmentation variations in this species.

Many of the insect pigmentation patterns are controlled by the melanin-synthesis pathway, in which *ebony* has a catalytic role. This pathway involves dopamine, which is also utilized as a neurotransmitter. In Drosophila, the genes controlling this dopamine pathway, such
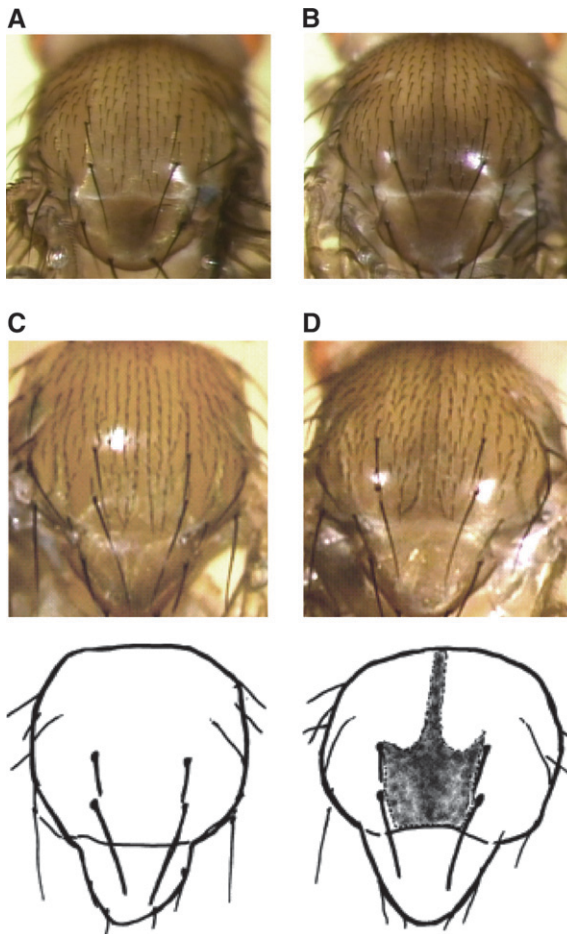

FIGURE 4.—Thoracic trident pigmentation phenotype obtained from complementation test with *ebony* and *ebony* RNAi knockdown experiment. (A) Dorsal view of the thorax segment of the Mel6/e^1 heterozygote. (B) Dorsal view of the thorax segment of the TW1/e^1 heterozygote. The flies in A and B were reared at 25°. The results were almost identical when e^{AFA}, *Df(3R)e-H4*, and *Df(3R)e-R1* were used as *ebony* mutants (data not shown). (C) Dorsal view of the thorax segment of the wild-type control, w^{1118}; *P{w^{+mc} = Actin5C-GAL4}/+*. (D) Dorsal view of the thorax segment of the RNAi knockdown individual, w^{1118}; *P{UAS-ebony-IR}/+*; *P{w^{+mc} = Actin5C-GAL4}/+*. The insert, *P{UAS-ebony-IR}* contains two fragments of 500 bp *ebony* cDNA as an inverted repeat (IR). These lines were crossed to w^{1118}; *P{w^{+mc} = Act5C–GAL4}17bFO1/+* strain to induce IR RNA in the whole body. w^{1118}; *P{w^{+mc} = Act5C–GAL4}17bFO1/+* was obtained by backcrossing *y¹ w*; P{w^{+mc} = Act5C–GAL4}17bFO1/TM6B, Tb¹* to w^{1118} strain for 10 generations. Adult flies carrying one copy of the *UAS-IR* construct and one copy of the GAL4 driver were investigated for the thorax pigmentation. The flies in C and D were reared at 20°.

as *ebony* and *tan*, are known to catalyze reactions of histamine at the photoreceptor terminals (RICHARDT *et al.* 2002, 2003; BORYCZ *et al.* 2002; TRUE *et al.* 2005). Taking these factors into account, the natural variation that we identified in the expression level of *ebony* provides an excellent model system for studying the association between color morphology and visually affected behavior in insects.

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