Modular cis-regulatory logic of yellow gene expression in silkmoth larvae

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Abstract

Colour patterns in butterflies and moths are crucial traits for adaptation. Previous investigations have highlighted genes responsible for pigmentation (e.g. yellow and ebony). However, the mechanisms by which these genes are regulated in lepidopteran insects remain poorly understood. To elucidate this, molecular studies involving dipterans have largely analysed the cis-regulatory regions of pigmentation genes and have revealed cis-regulatory modularity. Here, we used well-developed transgenic techniques in Bombyx mori and demonstrated that cis-regulatory modularity controls tissue-specific expression of the yellow gene. We first identified which body parts are regulated by the yellow gene via black pigmentation. We then isolated three discrete regulatory elements driving tissue-specific gene expression in three regions of B. mori larvae. Finally, we found that there is no apparent sequence conservation of cis-regulatory regions between B. mori and Drosophila melanogaster, and no expression driven by the regulatory regions of one species when introduced into the other species. Therefore, the trans-regulatory landscapes of the yellow gene differ significantly between the two taxa. The results of this study confirm that lepidopteran species use cis-regulatory modules to control gene expression related to pigmentation, and represent a powerful cadre of transgenic tools for studying evolutionary developmental mechanisms.

Keywords: cis-regulatory modules, yellow, Lepidoptera.

Introduction

Lepidopteran coloration and patterns play key roles in survival during both larval and adult stages (True, 2003; Wittkopp and Beldade, 2009). These include camouflage (e.g. tree-bark crypsis (Cook et al., 2012), leaf masquerade (Suzuki, 2013; Suzuki et al., 2014)) and mimicry (e.g. Batesian mimicry (Kunte et al., 2014; Nishikawa et al., 2015) and Müllerian mimicry (Benson, 1972)). Several genes encode for these adaptive colorations (e.g. yellow (Zhang et al., 2017) and tan (Futahashi et al., 2010)). As species capable of camouflage and mimicry show various colour patterns, such pigmentation genes must be expressed in several body parts. Previous studies have closely investigated the cis-regulatory regions of pigmentation genes in Diptera and have demonstrated their cis-regulatory modularity (Wittkopp, 2006; Rebeiz and Williams, 2017). In particular, the yellow gene of Drosophila and its cis-regulatory region have been used as a model system for identification, functional evolution, and connection to an evolving trans-landscape of a pleiotropic gene (Gompel et al., 2005; Jeong et al., 2006; Prud'homme et al., 2006; Werner et al., 2010; Ordway et al., 2014; Camino et al., 2015; Roeske et al., 2018). The modularity of gene regulation is thought to avoid pleiotropic constraints in evolutionary divergence (Stern, 2000; Davidson, 2001; Carroll et al., 2004; Carroll, 2005; 2008; Wittkopp, 2006; Wray, 2007; Stern and Orgazozo, 2008). Unlike the increasing understanding of regulatory mechanisms of pigmentation in Diptera, the functional validation of the cis-regulatory regions in lepidopterans is limited because of genomic positional effects and the labour-intensive and large-scale nature of constructing strains. Most studies on regulatory analyses in lepidopteran species have focused exclusively on identifying regulatory regions short distances
away from their correspondent genes (eg Sakurai et al., 2011). Consequently, no cis-regulatory regions operating remotely from their associated genes have been experimentally isolated, and thus, the modularity of cis-regulatory regions has never been tested.

Transgenic technologies can be used to test the capability of a given DNA sequence to drive the expression of a reporter gene, such as green fluorescent protein (GFP; Wimmer, 2003). To date, transgenic techniques have been developed for use in several insects [eg the fruit fly Drosophila melanogaster; the red flour beetle Tribolium castaneum (Lorenzen et al., 2003); and the mosquito Anopheles gambiae (Grossman et al., 2001)]. The silkmoth, Bombyx mori, has proven to be an excellent model insect (Goldsmith et al., 2005), which evolved from Bombyx mandarina approximately 4100 years ago (Sun et al., 2012). This species can be used in conjunction with established transgenic techniques (Tamura et al., 2000) in a variety of applications (eg Robinson et al., 2004; Sakurai et al., 2011; Daimon et al., 2015). As the cis-regulatory modularity of a gene can be tested by dissecting it into smaller templates and examining tissue-specific expressions of corresponding genes (eg Koshikawa et al., 2015), the Bombyx transgenic system exhibits immense potential for functional evaluation, and could be suitable as the first lepidopteran species to be used in such an analysis.

Thus, our first objective was to focus on the cis-regulatory structure of the yellow gene in B. mori, which is associated with melanin pigmentation and is widely investigated in both lepidopteran (Futahashi et al., 2008; Zhang et al., 2017) and other insect species (Wittkopp et al., 2002; Arakane et al., 2010). The yellow gene has been previously identified in B. mori, and has been shown to be homologous to yellow from other insect species (Futahashi et al., 2008; Ferguson et al., 2011). A Δyellow mutant of B. mori (k12) was isolated (Toyama, 1909), and the mutated genomic region identified (Futahashi et al., 2008). Using this mutant, previous studies revealed that the yellow gene is associated with the hair, epidermis, trachea, spiracle, pupal colour and head capsule in fifth-instar larvae and adults (Table 1).

Furthermore, the underlying basis of the conservation of cis-regulatory mechanisms is poorly understood. Interestingly, in D. melanogaster larvae, yellow was shown to be associated with hairs (thoracic and abdominal microsetae) and mouthparts (Kornezos and Chia, 1992), which are regulated in a 3.1-kb segment of the 5′ genomic region of the gene (Martin et al., 1989). These reports beg the question as to whether the DNA sequence of the cis-regulatory region and the trans-regulatory landscape are conserved between B. mori and D. melanogaster. Furthermore, more interesting is the question of whether or not the functional encodings are conserved in the face of DNA sequence evolution. In the present study, the trans-regulatory landscape is defined as an upstream molecular mechanism activating cis-regulatory regions sufficiently to drive gene expression. Although B. mori and D. melanogaster are distantly diverged, elucidating the degree of conservation of these regulatory processes would provide valuable insight into the evolution of the regulatory mechanisms governing the yellow gene.

Here we used a transgenic reporter assay to show cis-regulatory modularity of the yellow gene in larvae of the silkmoth, B. mori. To achieve this, we first used a Δyellow mutant of B. mori and determined which body parts are pigmented black by the yellow gene. We then examined the modular structure of the cis-regulatory region by observing the tissue-specific expression of the yellow gene using a reporter assay. Lastly, we performed reciprocal tests on the regulatory genomic regions between B. mori and D. melanogaster and demonstrated that they could not function in other species, suggesting that the trans-regulatory landscapes of the yellow gene differ significantly between the two taxa.

**Results**

The yellow gene is associated with black pigmentation in several body parts

Previous studies focused mainly on pigmentation in fifth-instar larvae and adults (Table 1). It was, therefore, unknown whether these observations were applicable to first-instar (embryonic) larvae. To confirm the previous reports, we used a Δyellow mutant of B. mori (k12), which exhibits a splicing deficiency in the yellow gene (Futahashi et al., 2008). We compared wild-type and Δyellow silkmoth larvae at three embryonic stages to determine which pigmented body parts require the activity of the yellow gene (Fig. 1). In the earliest stage, there were no differences between wild-type and Δyellow silkmoth larvae (Fig. 1A, D). We then observed that as the embryonic stages progressed, not only did the differences between them increase, but black pigmentation was absent in the trachea in Δyellow larvae (Fig. 1B, B′, E, E′). In the next embryonic stage, we found that black pigmentation was absent in the head capsule (Fig. 1C, F),...
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the hair and the thoracic legs (Fig. 1C, C’, F, F’) in Δyellow larvae. In summary, we found that several body parts of B. mori are pigmented through the effects of the yellow gene.

Three yellow cis-regulatory elements (CREs) control tissue-specific gene expression

To identify the number and location of the CREs that control melanin pigmentation in embryonic larvae, we isolated 22 kb of B. mori DNA, which included a major part of the yellow locus. This sequence spanned the 5’ end of the yellow translation initiation site through much of the large second intron. We then subdivided the noncoding sequences in this region into six DNA fragments (Fig. 2A) and determined whether they drove reporter gene expression in transgenic B. mori. To search for yellow CREs active at larval stages, we used a transgenic reporter gene assay. To explore the distant noncoding regions, we used the native yellow gene promoter (Supporting Information Fig. S1), and found that, without additional genomic sequences, this 1.2-kb region did not confer reporter expression (Fig. S2), thereby highlighting its suitability for use as a basal promoter.

As a result of the transgenic reporter gene assay, we observed that three of the six constructs drove reporter expression (Fig. 2B–D), whereas the remaining three did not (Fig. S3). These three tissues were the thoracic leg (Fig. 2B), the larval hair (Fig. 2C) and the trachea (Fig. 2D). As this study employed a piggyBac transposon system, the DNA fragments required for the reporter assay were inserted into arbitrary genomic regions. In order to rule out the possibility that the genomic positions in which the DNA fragments were inserted accounted for the reporter assay results, it was necessary to check whether GFP reporter expression could be reproduced in other transgenic lines. We investigated several lines for each

Figure 1. Melanin pigmentation in Bombyx mori embryonic larvae. (A–C) B. mori wild-type (WT) yellow (p50 strain). (D–F) B. mori mutant yellow (k12 strain). Each species is presented in three developmental stages [stage 1 (A, D); stage 2 (B, B’, E, E’); stage 3 (C, C’, F, F’)]. B’ and E’ show the trachea magnified from B and E. C’ and F’ show the hair (left) and thoracic legs (right) magnified from C and F. Development proceeds in order from stage 1 to 2 to 3. Dotted red lines indicate the position slightly below the tracheas (B’, E’; note the tracheas in B and E are not illustrated by colours). Arrowheads indicate one of the hairs (white arrowheads in C and F; black arrowheads in C’ and F’). Arrows indicate the thoracic legs (white arrows in C and F; black arrows in C’ and F’). Asterisks indicate the head capsules (C, F). (A–F) Scale bars = 500 µm. (B’, C’, E’, F’) Scale bars = 125 µm. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 2. Three yellow cis-regulatory elements (CREs) encode tissue-specific expression in Bombyx mori. (A) Genome structure around the yellow gene. For CRE analyses, the 22-kb yellow locus was dissected into six fragments (I–VI). Top bars indicate the six DNA fragments screened for using reporter assays in embryonic larvae. The blue bar represents the thoracic leg CRE (fragment III), the turquoise bar marks the location of the hair CRE (fragment V) and the red bar exhibits the trachea CRE (fragment VI). At the bottom, the black bar represents the basal promoter for the reporter assay and the triangle represents the translation start site, ATG. In transgenic B. mori embryonic larvae, green fluorescent protein reporter protein expression was driven by the thoracic leg CRE (B), the hair CRE (C) and the trachea CRE (D). Scale bars = 500 µm.
cis-regulatory region and confirmed that all three CREs exhibited mutually consistent tissue-specific expression as follows: all two of two observed lines showed specific expression of the thoracic leg (Fig. S4), all six of six observed lines showed specific expression of the larval hair (Fig. S5), nine of 10 observed lines showed specific expression of the thoracic leg (Fig. S6). These results indicate that the regulatory regions of the yellow gene are modularized, and each module induces tissue-specific expression (Fig. 2).

CREs require long genomic regions
To elucidate the organization of the three CREs and localize their functional sequences, we further dissected these elements into smaller fragments. We made a series of shorter constructs for four subregions (1154, 2634, 3961 or 2227 bp) of the hair-specific CRE (5493 bp; Fig. S7A) to determine which subregion was required. We found that no constructs drove reporter expression in the developing embryo (Fig. S7B–E). These results suggest that the longer region is required for the expression of the yellow gene in larval hair. We also investigated which subregions (1735, 2082 or 1897 bp) of the thoracic leg-specific CRE (3787 bp) were necessary to drive gene expression and found that no constructs drove reporter expression in the developing embryo (Fig. S8). In summary, we found that a long genomic region is required to regulate yellow gene expression in B. mori.

The trans-regulatory landscape of B. mori differs significantly from that of D. melanogaster
A functional analysis of the cis-regulatory region of the yellow gene revealed that three CREs are sufficient to drive gene expression throughout embryonic larvae (Fig. 2). Interestingly, previous reports using D. melanogaster larvae revealed that 3.1 kb of the 5′ genomic region of the yellow gene – the genomic position of which is similar to that of B. mori – regulates melanin pigmentation in the hairs (Fig. 4; Martin et al., 1989). This begs the question as to whether the DNA sequence of the cis-regulatory region and the trans-regulatory landscape are conserved between B. mori and D. melanogaster. In the present study, the trans-regulatory landscape is an upstream molecular mechanism activating cis-regulatory regions sufficiently to drive gene expression.

To address this question, we first investigated the sequence similarity between the corresponding 5′ genomic regions of B. mori and D. melanogaster. No sequence conservation was detected (Fig. 3A). Nevertheless, some studies have suggested that the same genomic position exhibits similar expression patterns across species even when the sequences are not conserved (Cande et al., 2009; Kalay and Wittkopp, 2010). To rule out this possibility, we conducted reciprocal tests for the hair-specific CRE of both B. mori and D. melanogaster. The hair-specific CRE of B. mori was not active in the developing embryo of D. melanogaster (Fig. 3B, C). Similarly, the hair-specific CREs in D. melanogaster drove no reporter expression in B. mori. These results suggest that the trans-regulatory landscape of B. mori hair-specific CREs differs significantly from that of D. melanogaster.

Discussion
This study presents the first functional analysis directed at elucidating the cis-regulatory modularity of genes in Lepidoptera. Colour divergence in this insect order is thought to be the product of the regulatory evolution of genes coding for pigmentation and those controlling them (Jiggins et al., 2017). Although we have considerably increased our understanding of colour divergence, the functional evaluation of the underlying regulatory structures remains poorly understood. Here, we generated several transgenic lines and have provided functional validation of cis-regulatory modularity in the yellow gene. Colour divergence of insects is based on various pigmentation in several tissues. Pigmentation genes have been found to be used recursively for tissue-specific pigmentation, and these multiple uses place a pleiotropic constraint on protein-coding sequence evolution. Based on our results, we suggest that the cis-regulatory modularity of the pigmentation gene yellow has been a means to bypass pleiotropic constraints and facilitate gene expression and colour divergence.

The present study showed that many silkmoth larva body parts are pigmented through the expression and use of the yellow gene (Fig. 1). Nevertheless, we were not able to locate the CREs responsible for regulating the pigmentation of certain body parts (ie the head and the epidermis; Fig. 2). There are a few possible explanations for this result: (1) the CREs for these body parts are very distant from the genomic regions we investigated (22 kb in total); (2) expression of the yellow gene in these body parts requires the combinatorial regulation of several distinct genomic regions; or (3) the regulatory regions controlling yellow gene expression must be longer (eg ~100 kb) than those investigated in this study (~5 kb). Transposition efficiency has been shown to significantly decrease with an increase in the length of piggyBac (Ding et al., 2005). In any case, the complete identification of yellow CREs will thus require further investigation, and may require the construction of transgenic lines with longer genomic regions using bacteriophage phiC31 integrase (Yonemura et al., 2013). In Drosophila, phiC31-mediated transgenesis integrates DNA fragments up to 133 kb long from...
Bacterial Artificial Chromosome clones (Venken et al., 2006). Although the present study did not completely identify all possible CREs controlling yellow gene expression in silkmoth larvae, it clearly demonstrates that cis-regulatory modularity is involved in the regulation of yellow gene expression.

Interestingly, we found that a long genomic region is required to regulate yellow gene expression in B. mori, whereas CREs are usually restricted to ~500–2000 bp in other insect species (eg D. melanogaster, T. castaneum, A. gambiae; Cande et al., 2009). Furthermore, as transcription factor binding sites are several tens of base pairs long, it is reasonable to assume that CREs would be a few hundred base pairs long as they are collections of binding sites (Spitz and Furlong, 2012). One possible reason for the apparently longer genomic region required to regulate yellow gene expression in B. mori could be the difference in genome size between B. mori and D. melanogaster: the genome of B. mori (482 Mb) is three times larger than that of D. melanogaster (175 Mb) [cf. T. castaneum (166 Mb) and A. gambiae (265 Mb)]. The numerous repetitive sequences (eg transposons) in the B. mori genome account, in part, for its large size (Mita et al., 2004; Osanai-Futahashi et al., 2008), as has been observed in other lepidopterans (Papa et al., 2008). As previously suggested, numerous repetitive sequences may facilitate the extraordinary colour and pattern divergence seen in Lepidoptera.

How did the CREs of the yellow gene evolve? Previous studies have offered two contrasting hypotheses: (1) de novo evolution and (2) inheritance from a common ancestor (Rebeiz et al., 2011). Whereas the yellow gene CREs have rapid evolutionary turnovers (Kalay and Wittkopp, 2010), it has been proposed that the yellow cis-regulatory modules are conserved and inherited with some modifications (Gompel et al., 2005; Jeong et al., 2006; Prud’homme et al., 2006; Werner et al., 2010). Our experimental findings may support the de novo evolution hypothesis because no CRE sequence conservation was observed and the reciprocal tests demonstrated no reporter expression between B. mori and D. melanogaster. Furthermore, as there were no previous reports indicating yellow gene expression in the trachea of Drosophila larvae, and Drosophila larvae do not have thoracic legs, the results of our experiments did not substantiate the hypothesis that the three CREs in B. mori evolved from a common ancestor of the two species. Nevertheless, the results of this study do not completely rule out the possibility that the yellow CREs were inherited from a common ancestor and experienced substantial sequence turnovers until their sequence homology became undetectable. Because of its rapid turnover rate, yellow CREs have a limitation for tracing their origins between distantly related insects, such as B. mori and D. melanogaster. Comparison amongst B. mori and other lepidopterans may provide additional evidence for seeking the origins of B. mori yellow CREs.

The practical application of new technologies increases our understanding of the regulatory mechanisms underlying colour pattern divergence. Transgenic techniques have also been applied to construct transgenic strains in other lepidopteran species (Marcus et al., 2004; Hahghhat-Khah et al., 2015). Although previous studies have not investigated the regulatory region of pigmentation genes in lepidopteran insects, the regulatory regions of upstream regulatory genes that govern pattern formation, including transcription factors such as distal-less (Carroll et al., 1994; Brakefeld et al., 1996; Zhang and Reed, 2016), optix (Reed et al., 2011), cortex (Nadeau et al., 2016); and morphogens such as wingless (Martin and Reed, 2010; Yamaguchi et al., 2013) and wntA (Martin et al., 2012; Gallant et al., 2014; Martin and Reed, 2014) were suggested. Previous implications on such regulatory regions have only been supported by empirical evidence derived from sequence-based observational methods such as Genome-wide association study (Nadeau et al., 2016) and chromatin immunoprecipitation sequencing (Lewis et al., 2016). The integration of transgenic technologies demonstrated in the present study into...
sequence-informed approaches would provide systematic insights for understanding regulatory mechanisms of lepidopteran colour patterns. As these technologies progress further, they can be exploited in other aspects of lepidopteran evo-devo research [eg the identification of CREs that define the nymphalid ground plan (NGP) of moth and butterfly wing patterns (ngpCREs)]. We hope that the methods used in the present study can be applied to other nonmodel systems to further elucidate the regulatory mechanisms that drive evolutionary important traits.

**Experimental procedures**

**Silkworm strains**

Silkworms were fed either mulberry leaves or an artificial diet (Nihon Kosan Kogyo, Yokohama, Japan) and maintained under a 12 h light/12 h dark photoperiod at 25–27 °C as previously described (Tsubota et al., 2014). Both nondiapause (w1-pnd) and diapause (w-1) silkworm strains were used in this study. Silkworms were kept at the Transgenic Silkworm Research Unit of the Division of Biotechnology at the Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan. The ch^{112} mutant strains were provided by the Silkworm Stock Center, located at Kyushu University, and supported by the National BioResource Project.

**Cloning and transgenic strategy of B. mori**

The *B. mori* yellow locus sequence was amplified from genomic DNA via PCR using specific primers designed from available genome sequences (Table S1). PCR was performed using KOD-Plus-Neo DNA polymerase (Toyobo, Tokyo, Japan) under the following conditions: 35 cycles at 94 °C for 2 min, 98 °C for 10 s and 68 °C for 30 s/kb. PCR products were subcloned into a pTA2 vector (TArget Clone™, Toyobo, Tokyo, Japan) and sequenced with an ABI3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA). The DNA fragments of yellow used for reporter constructs were cloned into the vector pBacGFP-3xP3DsRed (Tsubota et al., 2014). For the reporter assay, the native yellow promoter, initiator, and TATA were used (Fig. S1). We confirmed that this 1.2-kb genomic region lacks GFP reporter expression (Fig. S2).

**D. melanogaster transgenic experiments**

To investigate possible reciprocal CRE exchange between *B. mori* and *D. melanogaster*, the *B. mori* hair CRE was amplified by PCR using specific primers (Table S1) from a cloned fragment in a pTA2 plasmid and ExTaq DNA polymerase (Takara, Tokyo, Japan) under the following conditions: 30 cycles at 98 °C for 10 s, 55 °C for 30 s and 72 °C for 5 min. The PCR product was subcloned into a pS3aG vector (Williams et al., 2008). These constructs were injected into *D. melanogaster* (yellow) strain embryos and introduced into the genome via the P element function of pS3aG (BestGene Inc., Chino Hills, CA, USA). The hair CRE of *D. melanogaster* (SB fragment in Martin et al., 1989) was amplified by PCR from genomic DNA of the Canton-S strain using specific primers (Table S1) and ExTaq DNA polymerase under the following conditions: 30 cycles at 98 °C for 10 s, 55 °C for 30 s and 72 °C for 3 min.

**Imaging**

For all *B. mori* reporter lines, embryonic larvae were extracted from their eggs, mounted without fixation, and immediately viewed using bright-field imaging with an Olympus SDF PLAPO 1XPF objective dry lens mounted on an Olympus SZX16 microscope equipped with an Olympus DP71 camera (Olympus Japan Co., Tokyo, Japan). For *D. melanogaster* transgenic lines, transgenic embryos were dechorionated, fixed in 4% v/v paraformaldehyde:n-heptane (1:1), and mounted on glass slides.

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Leica M205FA microscope and a DFC 700T camera (Wetzlar, Hesse, Germany) were used for imaging.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1. Genomic structure around the transcription start site of the yellow gene in Bombyx mori. The genomic region between 1 and 1156 bp was used as a basal promoter for the cis-regulatory element reporter assays.

Figure S2. The basal promoter region of the yellow gene, including the TATA box initiator and the transcription start site, induces no green fluorescent protein reporter expression. (A) Reporter assay of fragment I. (B) Reporter assay of fragment II. (C) Reporter assay of fragment IV. Scale bar = 500 μm.

Figure S3. Three fragments of the 5 genomic region of the yellow gene do not induce green fluorescent protein reporter expression. (A) Reporter assay of fragment I. (B) Reporter assay of fragment II. (C) Reporter assay of fragment IV. Scale bar = 500 μm.

Figure S4. Green fluorescent protein (GFP) reporter expression induced by the thoracic leg cis-regulatory element (CRE) of the yellow gene. Transgenic silmoth lines were generated using PiggyBac. The thoracic leg CRE-GFP reporter unit was inserted into arbitrary genomic regions. We verified reproducibility by determining whether several lines expressed identically. We constructed two lines and confirmed the result in the thoracic leg CRE illustrated here. Panel (A) is the same image as that in Fig. 2B. Scale bar = 500 μm.

Figure S5. Green fluorescent protein (GFP) reporter expression induced by the hair cis-regulatory element (CRE) of the yellow gene. Transgenic silmoth lines were generated using PiggyBac. The hair CRE-GFP reporter unit was inserted into arbitrary genomic regions. We verified reproducibility by determining whether several lines expressed identically. We constructed six lines and confirmed the result in the hair CRE illustrated here. Panel (B) is the same image as that in Fig. 2C. Scale bar = 500 μm.

Figure S6. Green fluorescent protein (GFP) reporter expression induced by the trachea cis-regulatory element (CRE) of the yellow gene. Transgenic silmoth lines were generated using PiggyBac. The trachea CRE-GFP reporter unit was inserted into arbitrary genomic regions. We verified reproducibility by determining whether several lines expressed identically. We constructed 10 lines and confirmed the result in the trachea CRE illustrated here. Panel (E) is the same image as that in Fig. 2D. Scale bar = 500 μm.

Figure S7. Green fluorescent protein reporter expression induced by four shorter fragments of the yellow gene hair cis-regulatory element. Scale bar = 500 μm.

Figure S8. Green fluorescent protein reporter expression induced by three shorter fragments of the yellow gene thoracic leg cis-regulatory element. Scale bar = 500 μm.

Table S1. List of primers used in the study.

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