<table>
<thead>
<tr>
<th>Title</th>
<th>Gain of cis-regulatory activities underlies novel domains of wingless gene expression in Drosophila.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Koshikawa, Shigeyuki; Giorgianni, Matt W; Vaccaro, Kathy; Kassner, Victoria A; Yoder, John H; Werner, Thomas; Carroll, Sean B</td>
</tr>
<tr>
<td>Citation</td>
<td>Proceedings of the National Academy of Sciences of the United States of America (2015), 112(24): 7524-7529</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2015-06-01</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/198266">http://hdl.handle.net/2433/198266</a></td>
</tr>
<tr>
<td>Rights</td>
<td>© 2015 National Academy of Sciences; This is not the published version. Please cite only the published version. この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。</td>
</tr>
<tr>
<td>Type</td>
<td>Journal Article</td>
</tr>
<tr>
<td>Textversion</td>
<td>author</td>
</tr>
</tbody>
</table>
The gain of cis-regulatory activities underlies novel domains of wingless gene expression in Drosophila.

Shigeyuki Koshikawa¹,², Matt W. Giorgianni¹, Kathy Vaccaro¹, Victoria A. Kassner¹, John H. Yoder³, Thomas Werner⁴ and Sean B. Carroll¹,*.

¹ Howard Hughes Medical Institute, Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin, USA, ² The Hakubi Center for Advanced Research and Graduate School of Science, Kyoto University, Japan, ³ Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama, USA, ⁴ Department of Biological Sciences, Michigan Technological University, Houghton, Michigan, USA

* corresponding author

Abstract

Changes in gene expression during animal development are largely responsible for the evolution of morphological diversity. However, the genetic and molecular mechanisms responsible for the origins of new gene expression domains have been difficult to elucidate. Here, we sought to identify molecular events underlying the origins of three novel features of wingless (wg) gene expression that are associated with distinct pigmentation patterns in Drosophila guttifera. We compared the activity of cis-regulatory sequences (enhancers) across the wg locus in D. guttifera and D. melanogaster and found strong functional conservation among the enhancers that control similar patterns of wg expression in larval imaginal discs that are essential for appendage development. For pupal tissues, however, we found three novel wg enhancer activities in D. guttifera associated with novel domains of wg expression, including two enhancers located surprisingly far away in an intron of the distant Wnt10 gene. Detailed analysis of one enhancer (the vein-tip enhancer) revealed that it overlapped with a region controlling wg expression in wing crossveins (crossvein enhancer) in D. guttifera and other species. Our results indicate that one novel domain of wg expression in D. guttifera wings evolved by co-opting pre-existing regulatory sequences governing gene activity in the developing wing. We suggest that the modification of existing enhancers is a common path to the evolution of new gene expression domains and enhancers.
Introduction

As animals have adapted to diverse habitats, they have evolved many new and different kinds of body parts. One of the major outstanding questions in evolutionary biology is: what kinds of mechanisms underlie the origin of morphological novelties? It is well established that the regulatory genes responsible for the formation and patterning of animal bodies and body parts, the so-called “toolkit” genes for animal development, are shared and highly conserved among most animal phyla. (1-4). The fact that very different forms are generated by similar sets of developmental genes, and a large body of empirical, comparative studies, have led to the general consensus that divergence in the expression and regulation of toolkit genes and the genes they control largely underlies morphological diversity (5-9).

Similarly, several studies have revealed that new features of regulatory gene expression are associated with the evolution of morphological novelties, such as new color pattern elements on insect wings (10-14). How new patterns of regulatory gene expression evolve, however, has been more difficult to elucidate. In principle, new patterns of gene expression may evolve through: i) changes in the deployment of upstream trans-acting regulatory factors; ii) changes in the cis-regulatory sequences of the genes themselves; or iii) a combination of these mechanisms. For example, the novel, male-specific wing spot in Drosophila biarmipes and a few close relatives evolved through a combination of changes in the spatial expression of the trans-acting Distal-less (Dll) transcription factor and the evolution of Dll and other binding sites in a cis-regulatory element of at least one pigmentation gene (15, 16). In this case, the Dll protein is said to have been co-opted in the evolution of a new morphological trait.

However, the mechanism underlying the co-option of Dll is not known in this case, nor for any other instances of the co-option of regulatory genes. It is not known, for instance, whether new features of gene expression evolve via the de novo origin of enhancers, or through the transposition or modification of existing enhancers. One distinguishing feature shared by most developmental regulatory gene loci is that, like Dll (17, 18), they often contain vast cis-regulatory regions harboring numerous independent enhancers. To complicate matters, some of these enhancers may be located far away in other genes. The diversity of enhancers belonging to individual regulatory genes is explicit evidence that gene function has expanded in the course of evolution by accumulating additional enhancers, but understanding how this occurs presents significant experimental challenges. To further our understanding of the molecular basis of gene expression novelties, it is necessary both to identify the novel enhancers in the species of interest, and to ascertain their structural and functional relationships to sequences in other species lacking
the specific domains of gene expression (19).

The Wg protein is a secreted signaling molecule that acts as a morphogen in the development of numerous structures and pattern elements in Drosophila and other animals. (20-23). Here, we have traced the molecular basis of three novel features of wingless (wg) gene expression in Drosophila guttifera that are associated with three distinct features of adult pigmentation. By searching through the wg and adjacent loci of both D. guttifera and D. melanogaster, we found three novel enhancer activities in D. guttifera. We show that one of these enhancers, the novel vein-tip enhancer in D. guttifera, is nestled within a conserved enhancer in other species. We propose that the new enhancer activity evolved through the modification of the preexisting enhancer.

Results

Novel wg expression domains in the D. guttifera pupal wing

Regulatory genes coordinate important developmental events, thus their expression patterns are constrained and usually conserved, particularly among closely related species. wg expression patterns in larval imaginal discs (wing disc, eye-antennal disc, and leg disc) of D. melanogaster and D. guttifera adhere to this generality and are essentially identical (Fig. S1). In both species, wg expression were virtually identical in the developing wing pouches and the future nota of wing discs (Figs. S1A and S1D), the anterior-ventral parts of antennae, ventral and dorsal sides of eye discs (Figs. S1B and S1E), and anterior-ventral parts of leg discs (Figs. S1C and S1F).

In contrast, in the developing pupal wings of Drosophila guttifera, wg is expressed in two domains that are not present in D. melanogaster pupal wings (14, Fig. 1). Whereas in D. melanogaster, wg is expressed in cells along the developing wing margin (henceforth "margin") and crossveins ("crossveins", Fig. 1A, arrows), in D. guttifera (Fig. 1B) wg is also expressed at the tips of longitudinal veins ("vein tip", asterisks) and in precursors of the campaniform sensilla ("campaniform sensilla", arrowheads). None of the other several species closely related to D. guttifera within the D. quinaria species group (D. deflecta, D. nigromaculata, D. palustris and D. quinaria) exhibited wg expression in the developing campaniform sensilla or vein-tips (Fig. S2). Both novel wg expression domains correlate with color pattern formation in D. guttifera (Fig. 1D), but not in D. melanogaster (Fig. 1C).

The wg enhancers active in imaginal discs are conserved between species
Our primary task was to identify the enhancers responsible for these novel features of \emph{wg} expression in \emph{D. guttifera}. Because we could not predict where novel enhancers might be located, our approach was to identify functional enhancers across the entire \emph{D. guttifera} \emph{wg} region and to compare their activity and structure with the homologous regions of the \emph{D. melanogaster} \emph{wg} region. This approach offered the added benefit of enabling a comparison of the overall organization of the cis-regulatory regions of the \emph{wg} locus of the two species. Because we could not assume that \emph{D. guttifera} enhancers would have the same activity in \emph{D. melanogaster} (the usual host for transgenic methods in \emph{Drosophila}) as in \emph{D. guttifera}, we constructed reporter genes with DNA from each species and injected them into their species of origin. The \emph{D. melanogaster} \emph{wg} locus sequence had been determined previously (20, 22, 24). From a draft assembly of the \emph{D. guttifera} genome and PCR amplification, we located the \emph{D. guttifera} \emph{wg} locus on a 64 kb long contig. We systematically fused 0.3-10kb (average 4.0kb) non-coding segments of each species’ \emph{wg} region to an EGFP/DsRed reporter gene (Fig. S3).

We first monitored the larval imaginal discs for reporter protein activity, and we were able to confirm or identify several orthologous \emph{wg} enhancers (Fig. 2) including: i) a previously reported enhancer driving wing pouch expression in the 5’ region in both species (Figs 2B and 2E; the \emph{spade^{dog}} (\emph{spdfg}) region in \emph{D. melanogaster}; 25); ii) enhancers active in the eye-antennal discs and leg discs that are located in the 3’ region of \emph{wg} gene (Figs 2C and 2F); and iii) an enhancer in the 3’ region of the \emph{Wnt6} gene (Figs. 2D and 2G). These results are consistent with a recent survey describing a large collection of imaginal disc enhancers of \emph{D. melanogaster} (Flylight; 26). Because the \emph{Wnt6} expression pattern is mostly similar to that of \emph{wg} (27), and the clustering of four Wnt genes is conserved in the \emph{Drosophila} genus, the loci have been inferred to share regulatory elements (28). We note that the overall position and order of imaginal disc enhancers is largely colinear across the 60-74 kb region in both species (Fig. 2A). This result indicates that there have not been any significant inversions or other rearrangements across the region in either lineage since the two species diverged from a common ancestor approximately 63 million years ago (the divergence between the subgenus \emph{Drosophila} and subgenus \emph{Sophophora}, 29).

\textbf{The \emph{D. guttifera} \emph{wg} locus contains a novel vein-tip enhancer}

In our search for enhancers that regulate the \emph{D. guttifera}-specific \emph{wg} expression domains in pupal wings, we identified two enhancers located 3’ of the \emph{D. guttifera} \emph{wg} gene: a crossveins enhancer (\emph{gutCV-T}) and margin enhancer (\emph{gutME}), which together account for the conserved \emph{wg}-expression domains (Figs. 3A-C; see also Fig. 1B). We also identified the
orthologous enhancers, melCV and melME, from *D. melanogaster*, which drove reporter expression in the crossveins and wing margin, respectively (Fig. 3D and 3E). Importantly, in addition to the conserved crossvein expression, gutCV-T also drove reporter expression in the developing wing tips, where the wing veins meet the margin, which is part of the novel *wg* expression pattern in *D. guttifera* (Fig. 3B).

The difference in activities between the orthologous melCV and gutCV-T enhancers of *D. melanogaster* and *D. guttifera* could be due to differences in trans-acting regulatory factors expressed in each wing, differences in cis-regulatory sequences between the enhancers, or both. To determine which might be the case, we carried out a simple cis-trans test by introducing the gutCV-T enhancer into *D. melanogaster*. The gutCV-T fragment drove reporter protein expression in both the crossveins and vein-tips in pupal *D. melanogaster* wings (Fig. 3F). This result indicates that the trans-acting factors necessary for the vein-tip expression pattern are present in both species. Thus, the differences in activities between the gutCV-T and melCV enhancers must reside in their cis-regulatory sequences.

We also isolated and tested the orthologous cis-regulatory region from *D. deflecta*, which is one of the most closely related species to *D. guttifera* but does not have *wg* expression in the vein tips (Fig. S2C). This *D. deflecta* crossveins enhancer (defCV) drove reporter protein expression in the wing crossveins in *D. guttifera*, but showed no activity in the vein tips (Fig. 3G). This result indicates that the vein-tip enhancer activity is unique to *D. guttifera*, and that the novel feature of *wg* expression in *D. guttifera* wing vein tips arose through the evolution of cis-regulatory sequences in the *D. guttifera* lineage, after it split off from a common ancestor shared with *D. deflecta*.

**The *D. guttifera*-specific vein-tip enhancer is nestled within the crossvein enhancer**

The 2.4 kb gutCV-T enhancer, which drove both crossvein and vein-tip expression in *D. guttifera* (Fig. 3B), shares numerous collinear, highly-conserved blocks of sequence with both the 1.8 kb melCV fragment from the *D. melanogaster* locus and the 1.7 kb defCV fragment from the *D. deflecta* locus, which both lack vein-tip activity (Fig. 3D and 3G; Fig. 4A). We considered two possibilities to explain how the novel vein tip expression of gutCV-T may have evolved within the domain of the crossvein enhancer: i) a distinct enhancer element, able to independently drive expression in the vein tips, inserted into the *D. guttifera* *wg* locus (by chance next to another pupal wing enhancer); or ii) a novel activity arose within the crossvein enhancer that utilized and is dependent upon pre-existing sites in the crossvein enhancer. To attempt to distinguish these
possibilities, we compared the *D. guttifera*, *D. deflecta*, and *D. melanogaster* sequences and searched for major insertions or regions unique to *D. guttifera*. Indeed, we found that the *D. guttifera* fragment is over 900 bp longer than the orthologous *D. melanogaster* sequence and 600 bp longer than the orthologous *D. deflecta* sequence (Fig. 4A). This size difference is largely due to a region in the less conserved 5’ end of the gutCV-T enhancer. This additional sequence did not show any similarity to known transposable elements (when tested by blastn against the NCBI nucleotide collection). To test whether this region might contain a distinct enhancer, we divided gutCV-T into two fragments; the insert-containing 5’ 1653bp (gutCVT5) and the 3’, highly-conserved 756bp fragment (gutCVT-core) (Fig. 4A). While the gutCVT5 fragment showed no activity, the gutCVT-core fragment drove expression in both the crossoveins and vein tips (Figs. 4B and 4C). These results reveal that the novel activity in the gutCVT enhancer arose within the 3’ 756bp region.

To examine how this region may have acquired its unique vein tip activity, we compared it in detail with the orthologous *D. deflecta* sequence that lacks vein tip activity. The *D. guttifera* CVT-core region is 83% similar to the orthologous *D. deflecta* region, with many large blocks of identical sequence and just a few small (<10bp) insertions or deletions (Fig. S4). This pattern of sequence homology indicates that the novel domain of *wg* expression in the vein tips of *D. guttifera* is likely due to a small number of nucleotide changes and/or small indels nestled within the well-conserved crossvein enhancer.

**The campaniform sensillum and thoracic stripe enhancers are in the distant Wnt10 region**

During our initial search for *wg* enhancer activities in *D. guttifera*, we were puzzled by our inability to find an enhancer for the novel patterns of *wg* expression in the developing wing campaniform sensilla, which contributes several spots to the overall polka-dotted wing pattern (14). Therefore, we expanded our search into adjacent *Wnt* loci just in case they might contain enhancers that regulate *wg* transcription. Using seven additional scaffolds, we extended the region analyzed to include a 174 kb region containing the *Wnt4*, *Wnt6*, and *Wnt10* genes. We were surprised to find two more distinct enhancer activities in the *Wnt10* region, more than 69kb away from the *wg* transcription start site and separated from it by the *Wnt6* locus (Fig. 5A; Fig. S3; see also Fig. 2). One 5kb fragment within the second intron of the *Wnt10* gene (gutCS; Fig. 5A) drove reporter expression in the campaniform sensilla and along the anterior margin of the pupal wing (Fig. 5B, arrowheads). Because *wg* is the only gene in this *Wnt* cluster that is expressed in campaniform sensilla (Fig. S5), we conclude that this enhancer controls *wg*
expression.

A second, partially overlapping 4.3 kb fragment (gutTS, Fig. 5A) drove reporter expression in a series of thoracic stripes (Fig. 5C) that correspond well with the adult thoracic striped pigmentation pattern (Fig. 5D). We were not able to confirm by in situ hybridization that this reflects a native wg expression domain because gene probes did not yield reliable signals in pupal thoracic body wall tissues. However, we performed RT-PCR on thoracic body wall total RNA to ascertain which Wnt genes were active in this tissue. Only wg showed strong expression while the other, adjacent Wnt genes (Wnt4, Wnt6, and Wnt10) exhibited weak or no expression (Fig. S6). These results, and the strong correlation with thoracic pigmentation, indicate that wg is expressed in the thorax and regulated by the gutTS enhancer.

**Cis-regulatory sequence evolution is partly responsible for novel distant enhancer activities**

We next sought to identify the relative contribution of cis-acting and trans-acting regulatory factors in the evolution of the *D. guttifera* gutCS and gutTS enhancer activities. We conducted reciprocal tests of the *D. guttifera* and homologous *D. melanogaster* sequence in the other species’ genetic background. Contrary to the gutCV-T enhancer, the *D. guttifera* CS enhancer was not active in *D. melanogaster* wings, indicating a role for trans-acting factors in enhancer activity in *D. guttifera* (Fig. S7C). In addition, the homologous *D. melanogaster* fragment (45.7% similarity) was not active in either *D. guttifera* or *D. melanogaster*, indicating an additional contribution of cis-regulatory changes in the gutCS enhancer (Fig. S7A and S7B). Taken together, these results indicate that both cis-regulatory and trans-regulatory changes were responsible for the evolution of the novel wg expression domain in campaniform sensilla.

We performed a similar set of reciprocal experiments with the gutTS enhancer and homologous *D. melanogaster* sequence (Fig. S8). The homologous fragment from *D. melanogaster* (46.3% similarity) was inactive in both *D. melanogaster* (Fig. S8A) and *D. guttifera* (Fig. S8B), whereas the *D. guttifera* TS enhancer was weakly active in stripes in the *D. melanogaster* thorax (compare Fig. S8C and Fig. S8D). These results indicate that cis-regulatory changes are largely responsible for the novel activity of the gutTS enhancer and that some, but perhaps not all, of the trans-acting factors involved in regulating the enhancer are deployed in *D. melanogaster*.

**Discussion**

A large body of comparative studies has shown that changes in the spatiotemporal
expression of toolkit genes and their target genes they regulate correlate with the evolution of morphological traits. In a considerable number of instances, these spatiotemporal changes in gene expression have been demonstrated to involve the modification of enhancers (6, 7, 30-36). However, there are relatively few cases in which the origins of new enhancers have been elucidated, and none involving regulatory genes themselves.

Here, we have shown that three novel domains of wg expression in D. guttifera are governed by three novel enhancers, respectively (Fig. 6). We found that the evolution of wg cis-regulatory sequences within the D. guttifera lineage played a role in the gain of each enhancer activity, and that the evolution of trans-acting regulatory factors was also necessary for the activity of two elements (gutCS and gutTS). Detailed analysis of the D. guttifera vein-tip enhancer revealed that it evolved within another conserved enhancer, while two other enhancers (the campaniform sensilla and thoracic stripe enhancers) arose within in an intron of the distant Wnt10 locus. These results bear on our understanding of the mechanisms underlying the evolution of new enhancers and domains of gene expression.

The origin of the vein-tip enhancer via co-option of an existing enhancer

The D. guttifera vein-tip enhancer activity was localized within a 756 bp DNA segment that was also active in the developing pupal crossveins. This DNA segment is orthologous to segments of DNA in D. melanogaster and D. deflecta that were only active in the crossveins. The segments are all collinear, and contain numerous blocks of identical sequence, which suggests that the vein tip enhancer activity evolved within the pre-existing crossvein enhancer. This inference is further supported by the observation that we were unable to separate the two enhancer activities by subdivision of the 756bp fragment.

One explanation for the presence of two inseparable activities in this one fragment is that they share functional sites – i.e., binding sites for common transcription factors. Because both activities appear in the pupal wing, it is likely that they utilize common tissue-specific (wing) and temporal (pupal) inputs. The evolution of a new activity in the vein tips could have arisen through the addition of DNA-binding sites for TFs that were already present active in cells at vein tips. In this scenario, the novel enhancer activity would have resulted from the evolutionary co-option of an existing enhancer.

There is precedent for multifunctional enhancers and for this mechanism of co-option. For example, one enhancer of the D. melanogaster even-skipped gene governs two domains of gene expression that are controlled by shared inputs (37). In addition, Rebeiz et al. (19)
demonstrated that a novel optic lobe enhancer of the *Drosophila santomea Neprilysin-1* gene arose via co-option of an existing enhancer. Moreover, it was shown that co-option had occurred in just a few mutational steps. The co-option of existing elements is an attractive explanation for the evolution of novel enhancers because it requires a relatively short mutational path.

**The evolution of distant cis-regulatory elements**

One surprising property of enhancers is their ability to control gene transcription at promoters located at considerable linear distances away in the genome (38-40). For example, the enhancer that drives *Sonic hedgehog* (*Shh*) expression in the developing amniote limb bud is located in the intron of another gene approximately 1 megabase (Mb) from the *Shh* locus (41, 42). A growing body of evidence indicates that long segments of DNA are looped out in accommodating long-range enhancer-promoter interactions (43, 44). The ability of enhancers to act over such long ranges suggests that new enhancers could evolve at considerable distances from the promoters that they regulate.

Here, we identified two enhancers in an intron of the *D. guttifera Wnt10* gene that control transcription of the *wg* gene from a distance of ~70 kb, and separated by the *Wnt6* locus. Our data suggests that the gutTS enhancer preferentially regulates *wg* transcription and not *Wnt10* or *Wnt6* transcription, although we cannot offer any explanation at present for this preference. The origins of the gutCS and gutTS enhancers are not as clear as the vein tip enhancer. We did not detect any pupal enhancer activity in the orthologous DNA segments of *D. melanogaster*, so we do not have any evidence of enhancer co-option. Nor did we find any obvious insertions in these DNA segments such as a transposon. Nevertheless, the discovery of these novel, distant elements reflects the functional flexibility of cis-regulatory elements and their contribution to the evolution of gene regulation and morphological diversity.

**Materials and Methods**

**Fly strains and genomic DNA**

*Drosophila melanogaster* Canton-S (wild-type) was used for genomic DNA preparation and expression analysis of *Wnt* genes. We obtained *D. guttifera* (stock no.15130-1971.10), *D. deflecta* (15130-2018.00), *D. quinaria* (15130-2011.00), and *D. palustris* (15130-2001.00) from the Drosophila Species Stock Center at University of California, San Diego, and *D. nigromaculata* (strain no. E-14201) from EHIME-Fly, Ehime University, Japan.
Genomic DNA was extracted and purified using a squish method (45) and Genomic tip-20/G columns (Qiagen, Hilden, Germany).

**In situ hybridization**

Species specific, partial sequences of Wnt genes (*Wnt4*, *wg*, *Wnt6* and *Wnt10*) were amplified by PCR from genomic DNA and cloned into the *pGEM-TEasy* vector (Promega, Madison, WI). PCR products re-amplified from the plasmid clones were *in vitro* transcribed to produce DIG-RNA probes (35). Imaginal discs of late 3rd instar larvae and wings of P6 stage pupae (46) were subjected to *in situ* hybridization as described previously (14, 47). Specimens were mounted and imaged under a stereomicroscope SZX-16 (Olympus, Tokyo).

**Genomic sequence of the Wnt locus**

The genome sequence reads of *D. guttifera* were obtained with a Genome Analyzer IIx (Illumina, San Diego, CA), and assembled with CLC workbench (CLC Bio, Aarhus, Denmark). The *Wnt* locus of *D. guttifera* was reconstructed with seven genomic scaffolds and genomic PCR products (Accession no. KP966547, Fig. S3). For the comparison of sequences from multiple species, we used GenePalette software (48). All primers are listed in Table S1.

**EGFP/DsRed reporter assay for enhancer activity using transgenic Drosophila**

For the site-specific integration of transgenes into *D. melanogaster*, the plasmid vector S3aG (36), fly strains VK00006 (cytogenetic location 19E7)(49) and *ZH-attP-51D* (cytogenetic location 51D)(50) were used. *D. guttifera* transgenics were made according to the previously described method (14), using the cloning shuttle vector pSLfa1180fa harboring *DsRed2* or *DsRed.T4*, the *piggyBac* transposon vector *pBac(3xP3-EGFPafm)(51, 52)* and the *piggyBac* helper plasmid *phspBac* (53). Fluorescent reporter expression was observed under a stereomicroscope SZX-16 and a confocal laser-scanning microscope FV1000 (Olympus, Tokyo).

**Acknowledgements**

We thank Jane Selegue and Steve Paddock for their technical help, Henry Chung, Héloïse D. Dufour, Cédric Finet, Noah Dowell, David W. Loehlin, Thomas M. Williams, Troy Shirangi and Mark Rebeiz for fruitful discussions and comments, and Kiyokazu Agata and Naoyuki Fuse for sharing experimental facilities.
Author contributions

The authors have made the following declarations about their contributions:
Conceived and designed the experiments: SK MWG TW SBC. Performed the experiments: SK MWG KV VAK JHY TW. Analyzed the data: SK MWG VAK SBC. Contributed reagents/materials/analysis tools: SK MWG KV VAK JHY TW SBC. Contributed to the writing of the manuscript: SK MWG TW SBC.

Funding

This work was supported by a JSPS Postdoctoral Fellowship for Research Abroad (to SK) and the Howard Hughes Medical Institute (to SBC). The funders had no role in study design, data collection and analysis, the decision to publish, or preparation of the manuscript.

References


Figure Legends.

Figure 1. Unique wingless expression domains in Drosophila guttifera pupal wings correlate with adult pigment spots. (A) wg expression pattern in the pupal wing of D. melanogaster visualized by in situ hybridization. wg is expressed in the developing crossveins and along the wing margin. (B) wg expression pattern in the pupal wing of D. guttifera. wg is expressed in the campaniform sensilla (arrowheads), crossveins (arrows) and longitudinal vein tips (asterisks), and along the entire wing margin. (C) Adult wing of D. melanogaster. (D) Adult wing of D. guttifera.

Figure 2. Conserved wg cis-regulatory elements control similar gene expression patterns in Drosophila imaginal discs. (A) Schematic of enhancers plotted on the wg locus of D. melanogaster and D. guttifera. Solid vertical lines connected by horizontal gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. (B-D) D. melanogaster third instar imaginal discs showing reporter expression with D. melanogaster enhancer fragments (EGFP, green). (E-G) D. guttifera third instar imaginal discs showing very similar reporter expression patterns driven by orthologous D. guttifera enhancer fragments (DsRed, magenta). All discs are oriented with anterior to the left and dorsal on top. w: wing disc. ea: eye-antennal disc. l: leg disc. (Magnification: B–G, 200x.)

Figure 3. A novel vein tip enhancer activity in D. guttifera. (A) Schematic of pupal wing enhancers in D. guttifera and D. melanogaster. Black bars connected by gray lines represent sequences longer than 40bp with 100% nucleotide conservation between species. Inset: Schematic of wg expression in the pupal wing that is color-coded for the responsible enhancers. (B) D. guttifera pupal wing showing reporter expression from the gutCV-T enhancer in the crossveins and vein tips (DsRed, magenta). (C) D. guttifera pupal wing showing reporter expression from the gutME enhancer (DsRed, magenta) along the wing margin. (D) D. melanogaster pupal wing showing reporter expression from the melCV enhancer fragment (EGFP, green) in the crossvein. (E) D. melanogaster pupal wing showing reporter expression from the melME enhancer (EGFP, green) along the wing margin. (F) D. melanogaster pupal wing showing reporter expression from the gutCV-T enhancer (EGFP, green) in the crossveins and vein tips. (G) D. guttifera pupal wing showing reporter expression (DsRed, magenta) from the defCV enhancer in the crossveins (asterisks). (Magnification: B–G, 100x)
Figure 4. The *D. guttifera* vein-tip enhancer is nestled within a conserved crossvein enhancer. 

(A) Schematic comparing crossvein enhancer regions in *D. melanogaster*, *D. guttifera*, and *D. deflecta*. The gutCV-T enhancer (gray bar) aligned with the melCV enhancer (black bar, top) and the defCV enhancer (blue bar, bottom) using GenePalette (gray boxes connected with gray lines indicate sequences of 15 bp or longer with 100% conservation between species) and Vista Browser (50bp sliding window with percent sequence identity indicated, peaks with greater than 80% sequence identity are shaded in pink). Peaks show extent of sequence conservation in a sliding 50bp window. The gutCV-T enhancer was divided into two fragments, gutCVT5 (yellow bar) and gutCVT-core (green bar). (B) *D. melanogaster* pupal wing showing absence of reporter expression from gutCVT5 (EGFP, green). (C) *D. melanogaster* pupal wing showing reporter expression from the gutCVT-core fragment (EGFP, green) in the crossveins (arrows) and vein tips (asterisks). (Magnification: B and C, 100x.)

Figure 5. The distant *Wnt10* region contains two novel and distinct *wg* enhancers in *D. guttifera*. 

(A) Schematic showing the location of two enhancer fragments in the second intron of *Wnt10*. (B) *D. guttifera* pupal wing showing reporter expression driven by the gutCS enhancer (DsRed, magenta) in the campaniform sensilla (arrowheads). (C) *D. guttifera* pupal thorax showing a striped reporter expression pattern driven by the gutTS enhancer (DsRed, magenta). (D) Stripes of black pigmentation on the thorax of an adult *D. guttifera*. (Magnification: B, 80x.; C, 50x.; D, 32x.)

Figure 6. Three novel *wg* enhancers drive *D. guttifera*-specific pigmentation patterns. The genomic organization of the *D. guttifera* *Wnt* region is shown with colored shapes corresponding to enhancers from this study. The pupal expression domains of each enhancer are mapped by their respective color onto the pigmentation patterns of the adult animal.

Figure S1. *wg* expression patterns in imaginal discs are conserved between two *Drosophila* species. In situ hybridizations with third instar larval imaginal discs. (A) *D. melanogaster* wing disc. (B) *D. melanogaster* eye-antennal disc. (C) *D. melanogaster* leg disc. (D) *D. guttifera* wing disc. (E) *D. guttifera* eye-antennal disc. (F) *D. guttifera* leg disc. All discs are oriented with anterior to the left and dorsal on top.
Figure S2. wg expression in longitudinal vein tips and campaniform sensilla of the pupal wing is unique to D. guttifera. In situ hybridization for the wg gene is shown in pupal wings of various species. (A) Drosophila melanogaster. (B) D. guttifera. (C) D. deflecta. (D) D. nigromaculata. (E) D. palustris. (F) D. quinaria. D. melanogaster belongs to melanogaster species-group of the subgenus Sophophora, while the other species belong to the quinaria species group of the subgenus Drosophila. All probes are species-specific.

Figure S3. Map of the Wnt region of D. melanogaster and D. guttifera. Vertical bars connected with black lines indicate sequences of 40bp or longer with 100% sequence conservation between species. Red lines indicate conserved but inverted sequences. Numbered horizontal solid bars indicate DNA fragments tested by transgenic reporter assays. D. melanogaster fragments were tested in transgenic D. melanogaster using phiC31 integration. D guttifera fragments were tested in transgenic D. guttifera using the piggyBac transposon.

Figure S4. The novel activity of the D. guttifera gutCV-T enhancer arose within a conserved enhancer. Sequence alignment of the gutCV-T enhancer and the orthologous defCV enhancer (Serial Cloner v2.6.1, local alignment, and word size=15 were used). Mismatches are marked with a #. Red bars indicate identical sequence matches >10bp with the melCV-core enhancer from D. melanogaster. The extent of collinear sequence conservation (with no significant rearrangements) between the two species indicates that a small change or an accumulation of small changes is responsible for the new enhancer activity in the vein tips.

Figure S5. Expression patterns of four Wnt genes in pupal wings of D. guttifera visualised by in situ hybridizations. (A) Wnt4. (B) wingless. (C) Wnt6. (D) Wnt10.

Figure S6. wingless is the predominant Wnt gene expressed in the pupal thorax. Wnt genes expressed in the pupal thorax (top) and embryo (middle) were detected by RT-PCR and reaction products profiled by gel electrophoresis. Control reactions from genomic DNA are shown at the bottom.

Figure S7. Cis- and trans-regulatory changes are responsible for the novel campaniform sensillum expression of wg in D. guttifera. (A) In the D. melanogaster pupal wing, the melCS enhancer shows no restricted expression (EGFP, green). (B) In the D. guttifera pupal wing, the
melCS enhancer shows no expression (DsRed, magenta). (C) *D. melanogaster* pupal wing, the gutCS enhancer shows no restricted expression (EGFP, green). (E): *D. guttifera* pupal wing, the gutCS enhancer drives reporter expression in the campaniform sensillum (DsRed, magenta).

**Figure S8.** *Cis-* and *trans-*regulatory changes are responsible for the striped expression of *wg* in the pupal thorax of *D. guttifera*. (A) *D. melanogaster* pupal thorax, the melTS fragment shows no stripe expression (EGFP, green). (B) *D. guttifera* pupal thorax, the melTS fragment shows no stripe expression (DsRed, Magenta). (C) *D. melanogaster* pupal thorax, the guts enhancer is expressed in incomplete stripes (EGFP, green). (D) *D. guttifera* pupal thorax, the gutTS enhancer drives full stripe expression which corresponds to the adult pigmentation pattern (DsRed, magenta).
Figure 1

D. melanogaster

A

D. guttifera

B

C

D
Figure 2
Figure 3
Figure 4

A

B

C

melanogaster
gutCVT5
melanogaster
gutCVT-core
Figure 5

A

Wnt10

1kb

gutCS

gutTS

B

C

gutTS

gutCS

D
Figure. S1

A. D. melanogaster w
B. D. melanogaster ea
C. D. melanogaster l
D. D. guttifera w
E. D. guttifera ea
F. D. guttifera l
Figure S2

(A) *D. melanogaster*

(B) *D. guttifera*

(C) *D. deflecta*

(D) *D. nigromaculata*

(E) *D. palustris*

(F) *D. quinaria*
Figure. S3
Figure. S4

Similarity: 633/756 (83.73%)
Figure S6

- Pupa thorax cDNA
- Embryo cDNA
- Genomic DNA

Genes detected include Wnt4, wingless, Wnt6, and Actin5C.
Figure. S7

D. melanogaster

A

melCS

B

melCS

C

gutCS

D

gutCS

D. guttifera
Figure. S8

**D. melanogaster**

- A. Image labeled with melTS
- C. Image labeled with gutTS

**D. guttifera**

- B. Image labeled with melTS
- D. Image labeled with gutTS
Table S1. Primers used in the study.

<table>
<thead>
<tr>
<th>Primers for enhancer screening (Figure S3)</th>
<th>Restriction site</th>
<th>Fragment in</th>
<th>Cloning system</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCATGGCGCGCGCGGAAATGTGCCCCAACC CGA</td>
<td>AscI</td>
<td>1 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ATCACCTGCAGGCAAAACTGAAATAGAAAAAGTTTCAC</td>
<td>SbfI</td>
<td>1 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCATGGCGCGCCCTGGCAGACATAAAGTATTGAAATT</td>
<td>AscI</td>
<td>2 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ATCACCTGCAGGCTTTCTAGCTGAGGTCTATT</td>
<td>SbfI</td>
<td>2 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCATGGCGCGGCCGCAAAGGATGCGCTTTTTATGAGA</td>
<td>AscI</td>
<td>3 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ATCACCTGCAGGCAAAAAAGGATGCGCTTTTTATGAGA</td>
<td>SbfI</td>
<td>3 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCATGGCGCGCCCGGAAAACCCGGAAGCAAAACGT</td>
<td>AscI</td>
<td>4 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACACCTGCAGGCTGGCAGCATTTGATTTGTGTTAGA</td>
<td>SbfI</td>
<td>4 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCATGGCGCGCCGCCGAAAACCCGGAAGCAAAACGT</td>
<td>AscI</td>
<td>5 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACGCTTCGAGGGGTATTTTAAATTTTCTATTGTGGCT</td>
<td>SbfI</td>
<td>5 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ACTAGGCGCGCGCTTTTGACATTAAACCAGGTGTTAATTC</td>
<td>AscI</td>
<td>6 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACGCCTGCAGGCAAGCATCGCCATGTGGGCACCA</td>
<td>SbfI</td>
<td>6 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ACTTGGCGCGCCCTCATAAAAACGCAGCCATCAAACGACA</td>
<td>AscI</td>
<td>7 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACGCCTGCAGGGAGTCAGCTAGCTACTCCCCCAT</td>
<td>SbfI</td>
<td>7 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ACTTGGCGCGCCCATCTGAATCTAGCTAGCTACTCCCCAT</td>
<td>AscI</td>
<td>8 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACGCCTGCAGGCAAAAAACATCACGTAATTTGAGCA</td>
<td>SbfI</td>
<td>8 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>AACGCCTGCAGGCAAAACTGAAATAGAAAAAGTTTCAC</td>
<td>SbfI</td>
<td>1 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCATGGCGCGCGCGGAAATGTGCCCCAACC CGA</td>
<td>AscI</td>
<td>1 melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ATCACCTGCAGGCAAAACTGAAATAGAAAAAGTTTCAC</td>
<td>SbfI</td>
<td>1 melanogaster</td>
<td>S3aG</td>
</tr>
</tbody>
</table>
ACTAGGCGCGCCCAGCGTGGCTAATTAGCACAA AscI 9 melanogaster
melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 9 melanogaster melanogaster S3aG
ACTTGGCGCGCCCGGTAATATGACGTAATAAATTTTGAA AscI 10 melanogaster melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 10 melanogaster melanogaster S3aG
ACTTGGCGCGCCAGTTTTGGATGTTTTATCGCTTGATT AscI 11 melanogaster melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 11 melanogaster melanogaster S3aG
TCTTGGCGCGCCCTAATATGTGTAACACGTGTTAGT AscI 12 melanogaster melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 12 melanogaster melanogaster S3aG
TCTTGGCGCGCCGAATTCTCGTGAACCTCCCAGCA AscI 13 melanogaster melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 13 melanogaster melanogaster S3aG
TCATGGCGCGCCAAAACTGTGAAATGCTGTGAAAAGAACG AscI 14 melanogaster melanogaster S3aG
AACGCTTGCAAGGCAAAAAGATAAGAGCAGCGCCATA SbfI 14 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 15 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 15 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 16 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 16 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 17 melanogaster melanogaster S3aG
AAGGAAAAAAAGGCAGCGCCCCGATCTCCAATTCTCGAAT SbfI 17 melanogaster melanogaster S3aG
AACGCCTGCAGGGTAAGTTTACATTGAAATTTTCCATTAAATTG
melanogaster melanogaster S3aG SbfI 18
TCTTGGCGCGCCCTGAAAGAAAATGAGCCAGAAAC AscI 18
melanogaster melanogaster S3aG
AAGGAAAAAGGCGCAGCCCTTCATCGAGGCCT AscI 19
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGCGAGTGCCAACACCAGT SbfI 19
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGCTGGAACTGCTCGACGA AscI 20
melanogaster melanogaster S3aG
AAGGAAAAAGGGCGCGCCGGTTTATGAGATGCCCA SbfI 20
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGCGTTTCAAGTTCTC SbfI 21
melanogaster melanogaster S3aG
AAGGAAAAAGGGCGCGCCCATAAACAAAGTTAAAAT AscI 21
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGGAGCACAAAGACAGCCC SbfI 22
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGCAGTCGATCGTCCATCTC AscI 22
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGTGGGGCGTAGAAAGTAGCAC SbfI 23
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGCAGTCGATCGTCCATCTC AscI 23
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGGAGCACAAAGACAGCCC SbfI 24
melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTGGTATTGGTAGTGGAGCA AscI 24
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGGAGCACAAAGACAGCCC SbfI 25
melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGGGAGCACAAAGACAGCCC SbfI 25
melanogaster melanogaster S3aG
ATATGGCTGACGACATTGCTCCTAATCAATAAAACTAA SalI 26
melanogaster melanogaster S3aG
ACTAGGGCGCGCCCTACCCCACTCGAATAATTGCC AscI 26
melanogaster S3aG
AAGGAAAAACCTGCAGGCACGGATCGATGGTTTG SbfI 27 melanogaster melanogaster S3aG
AAGGAAAAAGGCGCGCCCATCCTTGTGGTGAGCCTG AscI 27 melanogaster S3aG
AAGGAAAAACCTGCAGGCACGGATCGATGGTTTG SbfI 28 melanogaster melanogaster S3aG
AAGGAAAAACCTGCAGGCACGGATCGATGGTTTG SbfI 29 melanogaster melanogaster S3aG
ACTAGGCCTGCAGGCAGGGATCGATGGTTTG AscI 29 melanogaster melanogaster S3aG
ACTAGGCCTGCAGGCAGGGATCGATGGTTTG SbfI 30 melanogaster melanogaster S3aG
ATCGCCCTGCAGGCACGGATCGATGGTTTG SbfI 30 melanogaster melanogaster S3aG
ATCGCCCTGCAGGCACGGATCGATGGTTTG SbfI 31 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTCACTGCTGAAATTTGCTTTG SbfI 31 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTCACTGCTGAAATTTGCTTTG SbfI 32 melanogaster melanogaster S3aG
ATCGCCCTGCAGGCACGGATCGATGGTTTG SbfI 32 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTCACTGCTGAAATTTGCTTTG SbfI 33 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTCACTGCTGAAATTTGCTTTG SbfI 33 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTTCACTGCTGAAATTTGCTTTG SbfI 34 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTGCTGAAATTTGCTTTG SbfI 34 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTGCTGAAATTTGCTTTG SbfI 35 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTGCTGAAATTTGCTTTG SbfI 35 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTGCTGAAATTTGCTTTG SbfI 35 melanogaster melanogaster S3aG
ACTTGGCGCGCCCTGCTGAAATTTGCTTTG SbfI 35 melanogaster melanogaster S3aG
AAGGAAAAAACCTGCAGGGGGAAGATCGGTGCACTC SbfI 36 melanogaster melanogaster S3aG
AAGGAAAAAGGCGCGCCGATCAGCTCCCCTGGACA AscI 36 melanogaster melanogaster S3aG
AACGCCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 37 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGGAGTAGCGTAAAAATGAAATTAAAC AscI 37 melanogaster melanogaster S3aG
AACGCCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 38 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGGAGTAGCGTAAAAATGAAATTAAAC AscI 38 melanogaster melanogaster S3aG
AACGCCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 39 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGGAGTAGCGTAAAAATGAAATTAAAC AscI 39 melanogaster melanogaster S3aG
AACGCCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 40 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGGAGTAGCGTAAAAATGAAATTAAAC AscI 40 melanogaster melanogaster S3aG
ATCACCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 41 melanogaster melanogaster S3aG
TCAAGGCGCCGCTGGAAAATAGGAATTATAGGATACAT AscI 41 melanogaster melanogaster S3aG
ATCACCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 42 melanogaster melanogaster S3aG
TCAAGGCGCCGCTGGAAAATAGGAATTATAGGATACAT AscI 42 melanogaster melanogaster S3aG
TCTTGGCCGCGCCGACTGCGATTTAAAATCAACTTAATTTCA AscI 43 melanogaster melanogaster S3aG
ATCACCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 43 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGGAGTAGCGTAAAAATGAAATTAAAC AscI 43 melanogaster melanogaster S3aG
ATCACCTGCAGGCTAGTAAATCAACTGAATCGCTCGTA SbfI 44 melanogaster melanogaster S3aG
ACTTGGCCGCGCCGCAACGAAAATGAGGTACAGTATTATTA AscI 44 melanogaster melanogaster S3aG

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Restriction Enzyme</th>
<th>Length</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>45</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAAGGAAATGAAATGAAATGAAATGTTTCACATAAAC</td>
<td>SacII</td>
<td>45</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>46</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACACCTCATCAACACTTTGTTTTATATG</td>
<td>SacII</td>
<td>46</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>47</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACACCTCATCAACACTTTGTTTTATATG</td>
<td>SacII</td>
<td>47</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>48</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACACCTCATCAACACTTTGTTTTATATG</td>
<td>SacII</td>
<td>48</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>49</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACACCTCATCAACACTTTGTTTTATATG</td>
<td>SacII</td>
<td>49</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>50</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACCTCAGCCGCTTTACGAGCTTTCGACTGACT</td>
<td>SacII</td>
<td>50</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>51</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACCTCAGCCGCTTTACGAGCTTTCGACTGACT</td>
<td>SacII</td>
<td>51</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>52</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACCTCAGCCGCTTTACGAGCTTTCGACTGACT</td>
<td>SacII</td>
<td>52</td>
<td>guttifera</td>
</tr>
<tr>
<td>AACGGGTACCTTCTCGATCAGCCGTACTAATGAT</td>
<td>KpnI</td>
<td>53</td>
<td>guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGTACCTCAGCCGCTTTACGAGCTTTCGACTGACT</td>
<td>SacII</td>
<td>53</td>
<td>guttifera</td>
</tr>
</tbody>
</table>
AACGGCTAGCTGCGATATGAAGATATTAAGACATGAAT  NheI  54  guttifera
  guttifera  pBac
TCAACCGCGGAGTTTTTACATTAAACAAAGAGATAAGAGA  SacII  54  guttifera
  guttifera  pBac
AACGGCTAGCCAGTAATATGAGCAGTAATAAAATTTGAAT  NheI  55  guttifera
  guttifera  pBac
TACACCGCGTCTAAGAGGTTCTAATTGAGCCAAT  SacII  55  guttifera
  guttifera  pBac
AACGGCTAGCAGTTTTGGATGTTTTATCGCTTGATT  NheI  56  guttifera
  guttifera  pBac
ACTACCGCGAAAGGAACCTACTGGTCAATCGCA  SacII  56  guttifera
  guttifera  pBac
AACGGGTACCACAGGTGATAATTATATAATTTGTGACGGA  KpnI  57  guttifera
  guttifera  pBac
AACGGCTAGCGATCATCTATATATATCTTTCATAATCCCA  NheI  58  guttifera
  guttifera  pBac
TCAACCGCGGCTCATGCTATATATGCGTTAC  SacII  58  guttifera
  guttifera  pBac
TCAACCGCGGGATCTATAGAATAGAGTATTTAAAATAACTTGA  SacII  59  guttifera
  guttifera  pBac
ATATCTCGAGCATCGATCGACATGACAGTCG  XhoI  59  guttifera
  guttifera  pBac
TCAACCGCGGAAGTAGCTTGGAAAAACTGGGATAAC  SacII  60  guttifera
  guttifera  pBac
AACGGCTAGCGAAAGAGAGTGAAAAAGCAGAGAGAAT  NheI  61  guttifera
  guttifera  pBac
AACGGCTAGCGAAARGTGAATCTGCACAACAACGAG  NheI  62  guttifera
  guttifera  pBac
AACGGGTACCACCCATGCCRTGGCATTTGCACTC  KpnI  62  guttifera
  guttifera  pBac
TCAACCGCGGAAACTTACAATTCAAAACTATTACTTGTTTTA  SacII  63  guttifera  guttifera  pBac
AACGGCTAGCGTAAAGAAGAAAATGAAATAGAAAGTGGA  NheI  63  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  64  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  64  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  65  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  65  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  66  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  66  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  67  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  67  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  68  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  68  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  69  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  69  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  70  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  70  guttifera  guttifera  pBac
TCAACCGCGGAAGATTATACACGTGTCTGTAAG  SacII  71  guttifera  guttifera  pBac
AACGGCTAGCGAATGCTGCTGATAATGGGA  NheI  71  guttifera  guttifera  pBac
TCAACCGCGGCTGACGTTTAGTCATAAAATATTCCA SacII 72 guttifera
AACGGCTAGCGGCGCTGATTCAACAAATAAACAAA NheI 72 guttifera
guttifera pBac
guttifera guttifera pBac
TCAACCGCGGTGTGGTAGAAAAATTTAAGTTTTCGATAAAATG SacII 73 guttifera
AACGGCTAGCAAAATGAAATTAAACGCGCGCTTTAATCA NheI 73 guttifera
guttifera pBac
guttifera pBac
TCAACCGCGGATTTATGACCCATTTAGATGTGCAGAA SacII 74 guttifera
AACGGCTAGCAAAATGAAATTAAACGCGCGCTTTAATCA NheI 74 guttifera
guttifera pBac
guttifera pBac
TCAACCGCGGTCTACAGATACATTAAATATCTCAAA SacII 75 guttifera
AACGGCTAGCTGGAATTTCCATTTACGGTACGAC NheI 75 guttifera
guttifera pBac
guttifera pBac
TCAACCGCGGGCTTTGATTAAAGCGCGCGTTTA SacII 76 guttifera
AACGGCTAGCGTTTGTAATGGGTTTTTTGGCATTGAA NheI 76 guttifera
guttifera pBac
guttifera pBac
TCAACCGCGGGAGTTCACAGTTAAAGTTCGAGC SacII 77 guttifera
ATCGGGCTAGCCAAATGAAACGGCACAGATGAAG NheI 77 guttifera
guttifera pBac
guttifera pBac
TCAACCGCGGGACCTTCAAATGACGTTTTATTTGC NheI 78 guttifera
guttifera pBac
TCAACCGCGGGACCTTCAAATGACGTTTTATTTGC NheI 78 guttifera
guttifera pBac
ATCAGGCTAGCCCTTGAAACGGCACAGATGAAG NheI 79 guttifera
guttifera pBac
TCAACCGCGGGACCTTCAAATGACGTTTTATTTGC XhoI 79 guttifera
guttifera pBac
TCAACCGCGGGAGATTATGACAACTTAATAGCTACAGA SacII 79 guttifera
guttifera pBac
guttifera guttifera pBac
TCAACCGCGGGAGATTATGACAACTTAATAGCTACAGA SacII 80 guttifera
guttifera pBac
guttifera pBac
AACGGCTAGCAAAATGAAATTAAATTTATGATAAGAATGACCTTTTAGA NheI 80 guttifera

TCAACCGCGGAAGTGCAACTAAATATGTAACTACTACAA SacII 81 guttifera
guttifera pBac
AACGGCTAGCGTGGGAGGCAGAAAGGATAAC NheI 81 guttifera
guttifera pBac
ATATCTCAGAGAAGTAGGCAGAAAGAAGAATCTCT XhoI 82 guttifera
guttifera pBac
AACGGCTAGCGAAATGGAGCAAAAGAAGATGCTT NheI 82 guttifera
guttifera pBac
TCAACCGCGGATGATTAAGCGTAATTTAATGAAGACAACA SacII 83
guttifera guttifera pBac
AACGGCTAGCCAATTAAACAATTCATTTATCTTAATTGTCTG NheI 83
guttifera guttifera pBac
TCAACCGCGGTGACTTTCCCATAAAATCAACACAATTTCATTTGATGT SacII 84
guttifera guttifera pBac
AACGGCTAGCGTTTACAAACCACAGCAGCA NheI 84 guttifera
guttifera pBac
TCAACCGCGGAGCGCAGCATAACGTCATAA SacII 85
guttifera guttifera pBac
AACGGCTAGCGATGAAAGCAGTATCTTATA SacII 86 guttifera
guttifera pBac
AACGGCTAGCTGCCTAACTGGCTTAGATAGCTAA NheI 86 guttifera
guttifera pBac
TCAACCGCGGATGATTAAGCGTAATTTAATGAAGACAACA SacII 87
guttifera guttifera pBac
AACGGCTAGCGGCATATAAATAGACAGTTTGAATTTATTA NheI 87 guttifera
guttifera pBac
AACGGCTAGGCTAATAATATGGATGAAGCAAATTTATTTCATTTA SacII 88
guttifera guttifera pBac
AACGGCTAGCGTTTACAAACCACAGCAGCA NheI 88 guttifera
guttifera pBac
AACGGCTAGCTGCCTAACTGGCTTAGATAGCTAA NheI 89 guttifera
guttifera pBac
<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
</tbody>
</table>

"Primers for cis/trans test (Figure 3, 4, 5, S7, S8)"

<table>
<thead>
<tr>
<th>Species host</th>
<th>Template species Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII gutCV-T guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI gutCV-T guttifera</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI gutCV-T guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII gutCV-T guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI gutCV-T guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII defCV deflecta</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI defCV deflecta</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI defCV deflecta</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII defCV deflecta</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI defCV deflecta</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII defCV deflecta</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI defCV deflecta</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI defCV deflecta</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII defCV deflecta</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI defCV deflecta</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGAA</td>
<td>SacII 92</td>
</tr>
<tr>
<td>AACGGCTAGCAGCACAACATTGCCACTTAATTAACA</td>
<td>NheI 92 guttifera</td>
</tr>
<tr>
<td>TCAACCGCGGGAGTGGGAGTTTTACGAATTGAATGT</td>
<td>SacII 93 guttifera</td>
</tr>
<tr>
<td>TCTTGGCGCGCCCTCGAGCAACATTGCCACT</td>
<td>AscI guttifera</td>
</tr>
<tr>
<td>TCAACCAGGGAAATATAATTGCTTCTAATATGCGAAATG</td>
<td>SacII 90 guttifera</td>
</tr>
<tr>
<td>AACGGCTAGCAGCAGCATGAAGCATAAATAAAGAAAG</td>
<td>NheI 90</td>
</tr>
<tr>
<td>AACGGGTACCCAGTGTTGCAAGGTTTTCCAC</td>
<td>KpnI 91 guttifera</td>
</tr>
</tbody>
</table>
| TCAACCAGGGAAAGCAGGAAGGTTGCAAGGATTGAGA...
guttifera  pBac  
ATATCTCGAGGACATTGCTCCTAATCAATAAAAACCTAA  XhoI  gutME  guttifera 
guttifera  pBac  
AACGCCCTGCAGGAATTAGTTTAGTTTCAATTTTTGCTTTG  SbfI  melME 
melanogaster  melanogaster  S3aG  
ACTTgccgccccGGCAGAAACGATTTGCGTGA  AscI  melME  melanogaster 
melanogaster  S3aG  
AACGCCCTGCAGAAAAAAAGTGATCGTGCTACATGTGT  SbfI  gutCVT5  
melanogaster  guttifera  S3aG  
TCTTGGCGGCAGCGCCAGCAGATCGCGAT  AscI  gutCVT5  
melanogaster  guttifera  S3aG  
AACGCCCTGCAGGAAAAAAAGTGATCGTGCTACATGTGT  SbfI  gutCVT-core  
melanogaster  guttifera  S3aG  
TCTTGGCGGCAGCGCCAGCAGATCGCGAT  AscI  gutCVT-core  
melanogaster  guttifera  S3aG  
TCAACCGGGGACATTGCTCCTCAATCAATAAAAACCTAA  SacII  gutCVT-core  
guttifera  guttifera  pBac  
AACGGCTAGCAGCAAAACATCGCCCCTCTAATAACAAC  NheI  gutCVT-core  
guttifera  guttifera  pBac  
ATCACCTGCAGGGCCCATAGTGTTGCGCTTGTGA  SbfI  melCS  
melanogaster  melanogaster  S3aG  
ACTTGGCGCCCGGCGCAACGAAATGGGTACAGTATT  AscI  melCS  
melanogaster  melanogaster  S3aG  
ATATCTCGAGGAGTTATGTTGCGCTTGTGA  XhoI  melCS  guttifera  
melanogaster  pBac  
TCAACCGGGGCAACGAAATGGGTACAGTATT  SacII  melCS  guttifera  
melanogaster  pBac  
ATCACCTGCAGGGCCCATAGTGTTGCGCTTGTGA  SbfI  gutCS  
melanogaster  guttifera  S3aG  
ACTTGGCGCCCGGCGCAACGAAATGGGTACAGTATT  AscI  gutCS  
melanogaster  guttifera  S3aG  
TCAACCGGGGCGCTAGTGTTGCGCTTGTGA  SacII  gutCS  guttifera  
guttifera  guttifera  pBac  
AACGGCTAGGCGGCTATATGACAGTATT  NheI  gutCS  
guttifera  guttifera  pBac  
ATCACCTGCAGGGGCGCTAGTGTTGCGCTTGTGA  SbfI  melTS
<table>
<thead>
<tr>
<th>Gene</th>
<th>Target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>melanogaster melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ACTTGGCGCGCCGCGGCAAATGCGATTTAATTGGCTGA</td>
<td>AscI melTS</td>
</tr>
<tr>
<td>melanogaster melanogaster</td>
<td>S3aG</td>
</tr>
<tr>
<td>ATATCTCGAGGAGTGAGGCGAGCGTATCTCTCTAAGAC</td>
<td>XhoI melTS guttifera</td>
</tr>
<tr>
<td>melanogaster</td>
<td>pBac</td>
</tr>
<tr>
<td>TCAACCCGCGGGCAACGAAATGGGGTACAGTATTATTA</td>
<td>SacII melTS guttifera</td>
</tr>
<tr>
<td>melanogaster</td>
<td>pBac</td>
</tr>
<tr>
<td>ATCACCTGCAGGAGTGAGGCGAGCGTATCTCTATA</td>
<td>SbfI gutTS</td>
</tr>
<tr>
<td>melanogaster guttifera</td>
<td>S3aG</td>
</tr>
<tr>
<td>TCAACCCGCGGAAGTGAGGCGAGCGTATCTCTATA</td>
<td>SacII gutTS guttifera</td>
</tr>
<tr>
<td>guttifera</td>
<td>pBac</td>
</tr>
<tr>
<td>AACGGCTAGCTGCCTAACTGGGCTTAGATAGCTAA</td>
<td>NheI gutTS guttifera</td>
</tr>
<tr>
<td>guttifera</td>
<td>pBac</td>
</tr>
</tbody>
</table>

Primers for in situ hybridization:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACGTCCAAGCAGGAGATGCCG</td>
<td>wg melanogaster</td>
</tr>
<tr>
<td>GGCGACGGCATGTTCGGGTG</td>
<td>wg melanogaster</td>
</tr>
<tr>
<td>CACGTTCAAGGGAGAGATCGCG</td>
<td>wg &quot;guttifera, deflecta, nigromaculata, palstris, quinaria&quot;</td>
</tr>
<tr>
<td>GGCGATGGCATATTGGGATGATG</td>
<td>wg &quot;guttifera, deflecta, nigromaculata, palstris, quinaria&quot;</td>
</tr>
<tr>
<td>CGAACCTTTATATCGGGACA</td>
<td>Wnt4 guttifera</td>
</tr>
<tr>
<td>GAGTCATGTCGCAATATTTCGGATG</td>
<td>Wnt4 guttifera</td>
</tr>
<tr>
<td>GCCATTCGCGAGTGCGAGATG</td>
<td>Wnt6 guttifera</td>
</tr>
<tr>
<td>CTAGAGGGCATGTGTTGACCTC</td>
<td>Wnt6 guttifera</td>
</tr>
<tr>
<td>GCCGCGGTCAATAACATGGGATG</td>
<td>Wnt10 guttifera</td>
</tr>
<tr>
<td>CCTGTATATCCGCTCCTAGAT</td>
<td>Wnt10 guttifera</td>
</tr>
</tbody>
</table>

Primers for RT-PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Template species</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAGCAGGCAACTGTGGTGGTCATGC</td>
<td>Wnt4 guttifera</td>
</tr>
<tr>
<td>GCCAATCCCTTTGGTACATGATTC</td>
<td>Wnt4 guttifera</td>
</tr>
<tr>
<td>GAGTGCAATGCCCAGGGCAT</td>
<td>wg guttifera</td>
</tr>
<tr>
<td>GGCTCCAGATAGCAATATCCCTT</td>
<td>wg guttifera</td>
</tr>
<tr>
<td>GCCATTCGCGAGTGCGATG</td>
<td>Wnt6 guttifera</td>
</tr>
</tbody>
</table>
AATTATGGTTCATGACTCTGCCGAG  Wnt6  guttifera
GTTATCGGGAAAGTGGTTTTGCG Wnt10  guttifera
CTTCAGCAGCTGCTGCAAAACTGT Wnt10  guttifera
ATGTGTGACGAAGAAGTGGCT  Act5C  guttifera
TAGATGGGCACAGTGGTGG  Act5C  guttifera