ABSTRACT  Transposable elements are widely used as vectors for integrating transgenes into the genome of insects. However, the random nature of transposon vector integrations often results in mutations and makes transgene expression subject to variable genomic position effects. This makes reliable quantitative comparisons of different transgenes difficult and development of highly fit transgenic strains laborious. Tools for site-specific transgene targeting are essential for functional genomic comparisons and to develop the most advanced transgenic insect strains for applied use. Here we describe a recombinase-mediated cassette exchange gene targeting system based on Cre/loxP that is highly efficient in Drosophila, and for the first time in a non-drosophilid, the tephritid fly, Anastrepha suspensa. This system allowed a comparison of the Drosophila constitutive polyubiquitin promoter and the artificial 3xP3 tissue-specific promoter in the same genomic context within each species, showing that the widely used 3xP3 promoter is apparently nonfunctional in the tephritid fly.

Transposable elements have proven to be highly efficient vectors for the germline transformation of a variety of insect species. This began initially with use of the P element for fundamental studies in Drosophila melanogaster and more than a decade later, for both basic and applied studies in economically and medically important species using the Hermes, Minos, mariner, and piggyBac elements (Handler 2002). Despite the routine use of these vector systems in five orders of insects, there are significant drawbacks to the use of transposon-based vectors due to the random nature of their genomic integration (Schetelig et al. 2009b; Trauner et al. 2009). Insertional mutations within coding and regulatory regions can disrupt vital gene functions that are highly useful for genomic functional analysis but can be detrimental to fitness and viability in transgenic strains created for the control of pest populations or to beneficial insects used as predators or bioreactors.

KEYWORDS
site-specific recombination
genomic targeting
Anastrepha suspensa
insect pest management
3xP3 promoter

Most integrations are also subject to site-specific genomic position effects that can diminish or alter transgene expression, making functional comparisons unreliable, and the creation of strains having optimal transgene expression for applied use unnecessarily difficult (Horn et al. 2000; Schetelig et al. 2009a).

Strategies to address these issues have been developed for Drosophila using the site-specific recombination systems phiC31/attP, Cre/loxP, and FLP/FRT for genomic targeting (Groth et al. 2004; Horn and Handler 2005; Oberstein et al. 2005; Venken et al. 2006; Gao et al. 2008). For all systems, target-acceptor recombination sites were first integrated genomically using a transposon vector and were then targeted for recombination with reciprocal recombination site(s) within donor plasmids coinjected with a recombinase or integrase helper. Of these, only the integrase phiC31 has been used to insert attB-containing plasmids into attP landing sites in non-drosophilid genomes, including two Aedes species (Nimmo et al. 2006; Labbé et al. 2010) and the Mediterranean fruit fly, Ceratitis capitata (Schetelig et al. 2009b). Although the phiC31 system provided the first transgenic system for non-drosophilids, it is limited by its unidirectional integration of the entire donor plasmid, typically including bacterial sequences as well as antibiotic resistance genes, and the potential for insertions into pseudo-attP sites within the genome (Groth et al. 2004). The FLP/FRT and Cre/loxP
An inbred wild-type (WT) colony of *D. melanogaster*, which is not restricted by these limitations (Horn and Handler 2005; Oberstein et al. 2005; Wimmer 2005). RMCE is based upon double recombination events, mediated by a recombinase, between two small heterospecific recombination sites within a genomic target site and a plasmid donor sequence. Depending on the number and position of independent recombination sites, RMCE allows for multiple insertion/deletion events of specific sequences at a single locus. In this fashion, transgene cassettes can be compared functionally in the same genomic context, and repetitively modified by the sequential deletion and addition of sequences.

The Flp/FRT recombinase system from the two-micron plasmid of yeast (Andrews et al. 1986) and the Cre/loxP system from bacteriophage (Siegal and Hartl 1996) both have 34-bp recombination sites consisting of two 13-bp inverted flanking repeats, separated by an 8-bp core, that specifically recombine with one another in the presence of Flp or Cre recombinase, respectively. Mutations in the 8-bp core create heterospecific sites, which are incompatible because only identical sites can recombine. Both Flp and loxP systems exhibited RMCE at relatively high frequencies in *Drosophila*, observed by the exchange of markers, although unexpectedly, single insertional recombination was also observed (Horn and Handler 2005; Oberstein et al. 2005). A combination of these two systems for RMCE has also been successfully tested in cell culture that expands the possibilities for modification of any single locus (Anderson et al. 2012). RMCE in non-drosophilids would be an important technique, especially to generate transgenic strains for insect population control, using target sites known to be nonsusceptible to mutational and position effects. The ability to improve such strains by gene replacement and addition would be a highly efficient alternative to creating completely new strains by transposon-mediated transformations that would require extensive evaluation for strain fitness and transgene expression for risk assessment evaluation. Importantly, transposon vectors used to create optimal target sites could be efficiently stabilized by a postintegration immobilization process providing enhanced environmental safety for transgenic strains created for field release applications (Handler et al. 2004; Schetelig et al. 2009b).

In support of this goal, we describe an RMCE system for a nondrosophilid species, the Caribbean fruit fly, *Anastrepha suspensa*, using the heterospecific loxN and lox2272 recombination sites (Araki et al. 2002; Livet et al. 2007) with a *D. melanogaster* hsp70-regulated Cre-recombinase. The efficacy of this system was tested in a comparative functional analysis of the 3xP3 artificial promoter, proving that this widely used promoter derived from the highly conserved *Pax-6/eyeless* system is, thus far, uniquely non-functional in a tephrilitid species.

**MATERIALS AND METHODS**

**Insect rearing**

An inbred wild-type (WT) colony of *A. suspensa* (Homestead, FL) and the *Drosophila melanogaster white* (w) (w1118) mutant strain were maintained at 25°C and reared under standard laboratory conditions (Saul 1982; Roberts 1986). All embryonic, larval, and pupal stages of *A. suspensa* were reared at 27°C and 60% humidity on a 12-12 hr light:dark cycle.

**Cloning**

The vector pBXLIJ_PUB6EGFP_TREhs343-Cctra-LAlhidAc2_loxN-3xP3-FRT-AmCyF-lox2272_loxP_attP235 (TRE-Cctra-LAlhidAc2; #443) was described previously (Schetelig and Handler 2012b). The Cre-helper plasmid phsp70-Cre (#445) was generated by recombining three fragments using the GeneArt Seamless Cloning Kit (Invitrogen) as follows: (1) a 3.7-kb EcoRV-digested fragment of *phsp-pBac* (Handler and Harrell 1999), containing the hsp70 promoter; (2) a 0.8-kb polymerase chain reaction (PCR) fragment of Cre, isolated with primer pair P815-P816 on a Cre-containing plasmid generously provided by Dr. J. Livet (Inserm); and (3) a 0.6-kb PCR fragment of the piggyBac 3′-UTR, isolated using primer pair P817-P818 on *phsp-pBac*. The Platinum Taq Polymerase was used for PCRs with the following conditions: 1 min at 95°C; 30 cycles of 15 sec at 94°C, 30 sec at 55°C, 1 min at 72°C; and 2 min at 72°C.

The construct pSl_1oxN-PUBDsRed-lox2272 was generated by ligating the 3.7-kb pSl_1oxN-lox2272 *Hpnl-Smal* digested fragment from M879 and the 2.9-kb PUBDsRed.T3 *Hpnl-Smal*-digested fragment from #1425 (Schetelig and Handler 2012a). M879 was created by ligating the *Xhol*-digested fragment loxN-3xP3FRTAmCyF-lox2272 from M746 (Schetelig and Handler 2012b) into the *Xhol-Sall* cut pSLfa1180fa (Horn and Wimmer 2000).

**Germline transformation**

Germline transformation experiments were performed by microinjection of the piggyBac target site construct #443 (500 ng/µL) with the *phsp-pBac* transposase helper plasmid (200 ng/µL) into WT *Drosophila* embryos as described previously (Handler and Harrell 1999; Handler 2000). G1 offspring were selected by enhanced green fluorescent protein (EGFP) epifluorescence using a Leica MZ FLIII microscope and a YFP filter set (ex: 500/20; em: 535/30). Independent homozygous strains were established by single pair inbreeding for successive generations with testing by segregation analysis of transplants outcrossed to WT flies. Transgenic *A. suspensa* lines carrying the #443 piggyBac cassette were generated and described earlier as a lethal effector construct (Schetelig and Handler 2012b).

**Recombinase-mediated cassette exchange**

Cre-RMCE was achieved by transformation, in which the RMCE donor plasmid pSL_1oxN-PUBDsRed-lox2272 (250 ng/µL) and the helper plasmid *phsp70-Cre* (150 ng/µL) were co-injected into RMCE target line embryos, without subsequent heat shock. Male or female 2-day-old adults of *D. melanogaster* or *A. suspensa* were mated individually to three virgin w females or males (D. melanogaster) and WT virgin females or males (*A. suspensa*), respectively. Their progeny were screened for the presence of eye and body markers by epifluorescence microscopy. Three subsequent backcrosses of transgenic males or females to WT females or males were first performed to verify a transgenic/WT progeny ratio of 1:1, and the same fluorescent marker tissue specificity consistent with single vector integrations. Independent homozygous strains were then established by single pair inbreeding for successive generations with testing by segregation analysis of transplants outcrossed to WT flies. Three filter sets (Leica) were used for fluorescent marker detection: TTxRed for DsRed (ex: 560/40; em: 610 LP), CFP for AmCyF (ex: 436/20; em: 480/40), and YFP for EGFP (ex: 500/20; em: 535/30).

**Verification of RMCE and expression analysis**

RMCE was verified by isolating the complete *loxN-Marker-lox2272* cassette from genomic DNA by PCR using primer pair P898/P899 (P898: ACGGGAAGTAGCGCCTCAAGCGACGACCCGTACGACGACTTGTACAGCCATGG; P899: GAGCGCGCACAACGCGACGCCGTACGACGACTTGTACAGCCATGG). Fragments were subcloned into the pCR4 vector (Life Technologies) and sequenced (Macrogen). All primers were designed using Geneious 5.6 software (Biomatters). Total RNA was isolated from adult heads using TRReagent (Molecular Research Center) with 1 µg of total RNA used for cDNA synthesis with the iScriptTM cDNA synthesis kit (BioRad). For both genomic DNA
and adult head cDNA, PCRs were performed targeting the AmCyan (primer pair P915: TCCACACCTCCTACAAGACCAAG / P916: GGTCAAGCTGACGCTGTGTGC) and EGFP (primer pair P913: CAGAACACCCCCATCGCGAGGC / P914: TACTTGTACAGTCGTCATCG) sequences.

Real-time quantitative PCR (qPCR) was performed on ~100 ng of cDNA, quantified on a NanoDrop 2000 (Thermo Scientific), using the iQ SYBR Green Supermix in a Chromo4 real-time PCR detector (BioRad). PCR cycling conditions were: 95° for 5 min; 45 cycles of 95° for 15 sec, 60° for 10 sec, and 72° for 10 sec with a plate read at the end of each cycle. All reactions were performed on three biological replicates. Gene specific primers for AmCyan (P915/P916) and EGFP (P913/P914) were used.

Amplified products from randomly selected samples were analyzed on a 2% agarose gel, subsequently cloned into pCR4-TOPO vector, and sequenced to confirm specificity of the AmCyan and EGFP amplifications. For absolute quantification, the AmCyan transcript levels were normalized against EGFP and compared with a standard curve against the AmCyan-carrying #443 plasmid. Calculations were performed using the Opticon Monitor 3 software (BioRad) and the standard curve was prepared on the 12,576-bp long plasmid #443 using five dilutions (300,000; 30,000; 3000; 300; 30 copies) according to Applied Biosystems (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042486.pdf).

RESULTS

Validation of markers and Cre/lox vector and helper plasmids in D. melanogaster

Before Cre-mediated cassette exchange in a non-drosophilid insect was tested, all components were functionally verified in D. melanogaster, for which lox-site RMCE was previously demonstrated, but with the use of a P vector and markers specific to that species (Oberstein et al. 2005). A piggyBac target-site transformation vector, pBXLII_PUbEGFP_TREhs43-Cctra-AlhidAla2_loxN-3xP3-FRT-AmCyan_lox2272_loxP_attP235 (#443), carrying the 3xP3-AmCyan eye marker flanked by the heterospecific lox sites loxN and lox2272 (Livet et al. 2007), together with the fluorescent marker PuB-nls-EGFP (Handler and Harrell 1999), was used for germline transformation of D. melanogaster w− flies (Figure 1A). Of 220 eggs injected, 18 adults survived that were backcrossed to w− males or virgin females. Sixteen crosses were fertile yielding two transgenic lines, Dm-F2A and Dm-M8A, that were identified by screening for both blue fluorescent eyes/ocelli (3xP3-AmCyan marker) and green fluorescent adult body tissue.

Figure 1 Recombination-mediated cassette exchange. A schematic of the piggyBac target site vector is shown on top, with images of the Drosophila (D.m.) and Anastrepha (A.s.) transformant strains below, carrying a green fluorescent body marker and an eye-specific blue fluorescent marker (A). A schematic of the target site after successful RMCE is shown, where the blue fluorescent marker is exchanged for a red body marker (B). All flies were observed under brightfield conditions (left) and epifluorescent microscopy with the filter sets YFP, CYGFP, and TxRed (right).
(PUb-nls-EGFP marker), using epifluorescence microscopy (Figure 1). The two independent lines were separately inbred to homozygosity.

To catalyze lox site recombination, the helper plasmid psph-Cre was generated having the Cre recombinase gene under DmHsp70 promoter control. The helper was coinjected with the donor vector, psL_loxN-PUbDsRed-lox2272, into homozygous Dm-F2A preblastoderm embryos. A total of 109 embryos were injected, from which 17 adults emerged that were single-pair mated to W females. Eleven matings were sterile, whereas six matings led to viable offspring. In two of these six crosses (Dm-loxM1, Dm-loxM8), successful cassette exchange was observed by loss of the blue fluorescent eye/ocelli marker expression that was replaced by whole-body expression of DsRed (Figure 1B). In addition to precise RMCE of the eye/ocelli marker expression that was replaced by whole-body expression of DsRed (Figure 1B). Therefore the integration site and composition of the construct were not responsible for the absence of the blue fluorescence in the eyes of A. suspensa.

The lines were then analyzed molecularly on two levels. First, PCR fragments of AmCyan and EGFP were isolated, subcloned, and sequenced to qualitatively reveal the absence or presence of the markers. This was performed on genomic and cDNA from the original lines, as well as lines created by RMCE (Figure 4A). Amplicons from genomic DNA confirmed that the AmCyan marker gene was present in the lines As-M3 and Dm-F2A as expected. In contrast, transcripts could not be amplified from As-M3 cDNA but were amplified from Dm-F2A. As a control, the EGFP marker was amplified from all genomic and cDNA samples of transgenic Anastrepha and Drosophila flies, whereas no amplicons were detected in WT strains tested. This finding led us to presume that expression levels of AmCyan under 3xP3 artificial promoter control were highly reduced in A. suspensa and possibly nonexistent.

Absolute real-time qPCR was then used to investigate differences in transcript copy number from the AmCyan transgene in all transgenic lines (Figure 4B). No copies were detected in the line As-M3, which is consistent with the absence of blue fluorescence in the eyes (Figure 1A). The control line, As-loxF7, in which the 3xP3-AmCyan was exchanged by RMCE for PUb-DsRed, and the Anastrepha WT strain did not express AmCyan. This clearly demonstrated that AmCyan is not expressed in A. suspensa. In the positive control Drosophila strain, Dm-F2A (visible blue eyes with epifluorescence microscopy), 1139 copies of AmCyan were detected and none in the lines Dm-loxM1, as well as the D. melanogaster WT line in which 3xP3-AmCyan does not exist.

**DISCUSSION**

Here we have shown genomic targeting of recombinant DNA to a specific locus by RMCE between heterospecific lox sites in a non-

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**Cre/lox RMCE in A. suspensa**

Five transgenic A. suspensa lines carrying the piggyBac vector, #443 were generated previously (Schetelig and Handler 2012b), and, interestingly, 3xP3-driven AmCyan could not be detected in any of the lines whereas the PUb-driven EGFP marker was clearly visible. RMCE was tested in the As-M3 line by injecting the psph-Cre helper and the pSL_loxN-PUbDsRed-lox2272 donor vector into dechorionated preblastoderm embryos. A total of 230 embryos were injected in two independent experiments (#1: 115; #2: 115), from which 12 adults (#1: 5; #2: 7) emerged that were single-pair mated to A. suspensa WT males or virgin females. Four matings (#1: 1; #2: 3) were sterile whereas eight matings led to viable offspring. In two (#1: 1; #2: 1) of the eight crosses (As-loxM1, As-loxM7) the additional expression of DsRed in whole flies indicated successful RMCE (Figure 1). The precise cassette exchange in both lines was then verified by amplifying and sequencing the complete PUb-DsRed marker cassette flanked by the heterospecific lox sites (Figure 2).

**3xP3 promoter analysis in A. suspensa**

In addition to establishing RMCE in a tephritid, the construct #443 also allowed a determination of 3xP3 promoter function in A. suspensa, in comparison with D. melanogaster. The strategy was to use the double-marked construct to compare expression of the 3xP3- and the D. melanogaster polyubiquitin (PUb)-regulated markers in the same genomic context within each species. As shown in the Drosophila and Anastrepha transgenic lines in Figure 1, the PUb-regulated markers are clearly visible in both species, whereas 3xP3-AmCyan expression is exclusively detected in ommatidia and ocelli of Drosophila, but in neither tissue in A. suspensa. One particular difference between the species is their eye pigmentation. Drosophila strains were generated from a white eye mutant strain, w1118, whereas transgenic Anastrepha lines had red pigmented WT eyes. To determine whether pigmentation may have masked detection of 3xP3-regulated marker expression in Anastrepha, Drosophila strains were backcrossed to the WT Oregon-R strain. The resulting transgenic flies having red pigmented eyes, did show weak AmCyan expression in the pseudopupil and strong blue fluorescence in the ocelli (Figure 3) consistent with earlier studies (Berghammer et al. 1999). However, after exchanging 3xP3-AmCyan for PUb-DsRed by RMCE, both species exhibited red fluorescence in the whole body (Figure 1B). Therefore the integration site and composition of the construct were not responsible for the absence of the blue fluorescence in the eyes of A. suspensa.

The lines were then analyzed molecularly on two levels. First, PCR fragments of AmCyan and EGFP were isolated, subcloned, and sequenced to qualitatively reveal the absence or presence of the markers. This was performed on genomic and cDNA from the original lines, as well as lines created by RMCE (Figure 4A). Amplicons from genomic DNA confirmed that the AmCyan marker gene was present in the lines As-M3 and Dm-F2A as expected. In contrast, transcripts could not be amplified from As-M3 cDNA but were amplified from Dm-F2A. As a control, the EGFP marker was amplified from all genomic and cDNA samples of transgenic Anastrepha and Drosophila flies, whereas no amplicons were detected in WT strains tested. This finding led us to presume that expression levels of AmCyan under 3xP3 artificial promoter control were highly reduced in A. suspensa and possibly nonexistent.

Absolute real-time qPCR was then used to investigate differences in transcript copy number from the AmCyan transgene in all transgenic lines (Figure 4B). No copies were detected in the line As-M3, which is consistent with the absence of blue fluorescence in the eyes (Figure 1A). The control line, As-loxF7, in which the 3xP3-AmCyan was exchanged by RMCE for PUb-DsRed, and the Anastrepha WT strain did not express AmCyan. This clearly demonstrated that AmCyan is not expressed in A. suspensa. In the positive control Drosophila strain, Dm-F2A (visible blue eyes with epifluorescence microscopy), 1139 copies of AmCyan were detected and none in the lines Dm-loxM1, as well as the D. melanogaster WT line in which 3xP3-AmCyan does not exist.

**DISCUSSION**

Here we have shown genomic targeting of recombinant DNA to a specific locus by RMCE between heterospecific lox sites in a non-
The pseudopupil indicate 3xP3-marker function (B). Magnified images of the ocelli are inset at the bottom right of each whole-body image.

drosophilid insect species. Compared with the phiC31/attP system, which has been established as a unidirectional targeting system in mosquitoes and a tephritid species, RMCE methodology is particularly advantageous as a means to not only target recombinant DNA constructs to a specific genomic site, but to subsequently modify the construct by sequence addition, deletion, or exchange. This significantly advances our ability to functionally characterize genes of interest and facilitates the creation and improvement of transgenic strains for applied use. A particular advantage is that cassette exchange can be performed for comparative gene expression analysis in strains for applied use. A particular advantage is that cassette exchange of a transgenic line should be strictly to select optimal transformant lines.

Differences between the system presented here and the firstlox RMCE in Drosophila (Oberstein et al. 2005), beyond use of the P element vector and markers specific to Drosophila, include the source of Cre recombinase and the successful use ofloxN in A. suspensa and D. melanogaster. Alterations between theloxN,lox2272, and the originalloxP site are two mutations in the 8-bp core at positions two and seven (Livet et al. 2007), and we found RMCE usingloxN to be strictly heterospecific in both species. The non-exchange loss of 3xP3-AmCyan in the Drosophila line Dm-loxM1b is not understood, but was presumably caused by an imprecise excision or break between thelox sites.

In the process of achieving RMCE, the use of a helper plasmid having Cre recombinase, fused to the Drosophila hsp70 promoter, proved to be more stable than capped recombinase mRNA that tended to degrade quickly and negatively affected the survival rate of injected embryos. The hsp70-Cre helper plasmid allowed for highly efficient RMCE, up to 33%, compared to 5–9% efficiency in D. melanogaster reported earlier using an hsp70/Mos1-regulated recombinase. Together, the results clearly show that DNA-based expression of Cre recombinase can be highly effective in catalyzing in-vivo RMCE usingloxN andlox2272 recombination sites in A. suspensa, that should be extended to other tephritid species, if not other insects, as well.

In the process of testing RMCE we compared the 3xP3 eyespecific promoter and the polyubiquitin (PUb) constitutive promoter in the same genomic context. The artificial 3xP3 promoter, originally tested in D. melanogaster, contains three binding sites forFbx6/eyeless homodimers upstream to a TATA box. This is an evolutionarily highly conserved system that was described as the master regulator of eye development throughout the animal kingdom (Gehring 2002). This view is consistent with the very broad function of 3xP3 (Sheng et al. 1997; Berghammer et al. 1999) as a promoter for fluorescent protein genes, which have been successfully used as an adult eye and ocelli marker for transgenesis inDrosophila, the housefly, beetles, butterflies, mosquitoes, and even flatworms (Horn et al. 2000; Hediger et al. 2001; Kokozka et al. 2001; Gonzalez-Estevez et al. 2003; Lorenzen et al. 2003; Marcus et al. 2004). It also promotes expression in the larval nervous system, which has been highly useful in identifying silkmoth transormants (Thomas et al. 2002). Nevertheless, several attempts by us and other laboratories to use 3xP3-fluorescent protein markers in the tephritidsC. capitata, A. suspensa, andAnastrepha ludens have failed to produce an identifiable transgenic phenotype (includingC. capitata white eye host strains). After exchanging the nonfunctional 3xP3-driven AmCyan cassette for a ubiquitously expressedPUb-drivenDsRed marker by RMCE, we found thatDsRed was visible by epifluorescence microscopy in the whole body ofA. suspensa. Therefore, the lack of visible fluorescence from 3xP3-AmCyan in A. suspensa was not related to a defect or genomic position effect because the same construct functioned in Drosophila, and an equally positionedPUb promoter expressed the red fluorescent protein unambiguously in both species. The lack of promoter function for 3xP3 in Anastrepha was further verified by qPCR that showed the absence ofAmCyan transcripts whereas, in contrast, a high transcript level was obtained from the same 3xP3-marker in the Drosophila lines.
The 3xP3 promoter consists of three P3 binding elements and a minimal promoter from the Drosophila hsp70 gene. Cross-species expression differences due to this minimal promoter are unlikely because it has been used successfully in A. suspensa and another tephritid, Ceratitis capitata (Schetelig et al. 2009a; Schetelig and Handler 2012a). A modification of the original 3xP3-regulated markers developed in Drosophila (Berghammer et al. 1999) is the insertion of an 84-bp linker, which includes an FRT recombination site, in between the promoter and the fluorescent protein gene. The modified 3xP3-FRT-AmCyan construct was used in the RMCE vector, and although it clearly was functional in Drosophila, it has not been tested in species known to express 3xP3-regulated markers lacking the FRT insert. Thus, the present data cannot formally exclude a negative effect of the inserted sequence on 3xP3 function in non-drosophilids.

To our knowledge this is the first report indicating that 3xP3 is nonfunctional in an insect species and possibly an insect family. Given its routine use for marker expression in lepidoptera, coleoptera, and several dipteran species, this is unexpected and not simple to explain. It certainly raises the possibility for differences in the highly conserved mechanism of eye development between tephritids and other insects, which might range from control by a distinct gene in Anastrepha, to a variation in binding sites for the P3/RSC1 elements. An in-depth analysis for this mechanism in tephritid species will be required, and RMCE methodology should play a pivotal role in the comparative studies necessary to elucidate our understanding of eyeless function.

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