

piggyBac-based insertional mutagenesis in *Tribolium castaneum* using donor/helper hybrids

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Abstract

We describe an efficient method for generating new *piggyBac* insertions in the germline of F₁ hybrid *Tribolium castaneum* derived from crosses between transgenic helper and donor strains. Helper strains carried single *Minos* elements encoding *piggyBac* transposase. The donor strain carried a single *piggyBac* element inserted into an actin gene, expanding the eye-specific, 3xP3-EGFP (enhanced green fluorescent protein) reporter expression domain to include muscle. Remobilization of the donor element is accompanied by loss of muscle fluorescence but retention of eye fluorescence. In a pilot screen, the *piggyBac* donor was remobilized in 84% of the hybrid crosses, generating hundreds of new lethal, enhancer-trap, semisterile and other insertions. The jumpstarter system described herein makes genome-wide, saturation insertional mutagenesis a realistic goal in this coleopteran species.

Keywords: *piggyBac*, *Minos*, germline transformation, EGFP, enhancer trap.

Introduction

The genome of the red flour beetle, *Tribolium castaneum*, has been sequenced, and a working assembly (Tcas_2.0) has been released by the Human Genome Sequencing Center, Baylor College of Medicine, USA (<http://www.hgsc.bcm.tmc.edu/projects/tribolium/>). The addition of this insect to the 'genome club' is of particular interest in view of its status as a significant agronomic pest, a generalist feeder, a representative of the largest animal order (the Coleoptera) and an important model species for research in evolutionary and developmental biology, genetics and pest science. Inter-

preting this wealth of genomic data will require sophisticated genetic dissection to facilitate studies of gene function.

Various applications of transposon-mediated germline transformation have provided a versatile toolkit for functional analysis of the genome of *Drosophila melanogaster* (Cooley *et al.*, 1988a; Robertson *et al.*, 1988; Wilson *et al.*, 1989; Brand & Perrimon, 1993). We and our collaborators have reported previously on the design and use of transposon-based transformation systems in *T. castaneum* based on embryo injection of transformation constructs (Berghammer *et al.*, 1999; Lorenzen *et al.*, 2002a,b, 2003; Pavlopoulos *et al.*, 2004), but injection-based transformation is impractical for large-scale projects such as genome-wide insertional mutagenesis. We therefore have developed an *in vivo* system for germline transformation, i.e. one based on genetic hybridization.

A report by Horn *et al.* (2003) presents a useful design for transposon-based technologies for nondrosophilid insects, namely an efficient, broad-spectrum system for insertional mutagenesis. This design, tested and validated in *D. melanogaster*, is patterned after the hybrid dysgenesis-inspired, P-element-based jumpstarter system (Kidwell, 1979; Rubin & Spradling, 1982; Cooley *et al.*, 1988b). The system of Horn *et al.* utilizes two different transposons (various combinations of *piggyBac*, *Hermes*, *Minos* or *Mos1*) to generate the donor and helper strains. The donor provides the non-autonomous element to be remobilized, while the helper supplies a stable source of the required transposase. Remobilization of the donor element is activated by simple genetic hybridization of donor and helper strains. Each new transgenic line is stable after segregation of the helper element, providing for easy strain maintenance.

To adapt this approach for insertional mutagenesis in *T. castaneum* we chose *piggyBac* (Cary *et al.*, 1989; Fraser, 2000), a member of the TTAA family of elements, as donor, and *Minos* (Franz & Savakis, 1991), a member of the Tc1/mariner family, to establish helper lines. The choice of *piggyBac* as donor was based both on its high level of activity in *T. castaneum* (Berghammer *et al.*, 1999) and on the availability of a *piggyBac* insertion (Fig-19) that had already proven useful as a donor in injection-based remobilization experiments (Lorenzen *et al.*, 2003). *Minos* has also been shown to be functional in *T. castaneum* (Pavlopoulos *et al.*, 2004) and was therefore chosen as the

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framework for building helper constructs. Analysis of the *T. castaneum* genome sequence (BLASTN for termini and TBLASTN for transposase) indicates that this beetle lacks closely related *Minos* and *piggyBac* elements. Here we describe: (i) the establishment of *T. castaneum* helper lines using a DsRed-marked *Minos* element that provides a stable source of *piggyBac* transposase; (ii) the production of donor/helper hybrids carrying both the *Minos*-flanked helper and the Pig-19 donor; and (iii) the use of such hybrids to effect the postintegration remobilization of the non-autonomous Pig-19 donor insertion at very high rates. Finally, we report the results of a pilot screen to determine the feasibility of genome-wide insertional mutagenesis in this beetle.

Results

Production of helper lines

To establish *Minos*-based helper lines, germline transformation was performed in a white-eyed *T. castaneum* strain (*pearl*) by coinjection of a *Minos* helper plasmid (Klinakis *et al.*, 2000) and a DsRed-marked *Minos* element that provides a source of *piggyBac* transposase (Horn *et al.*, 2003). Of 65 injected G_0 embryos surviving to the adult stage, eight produced transformed progeny. Seven of the eight resulting helper lines were homozygous viable, the M59 line being the only exception. One line showed strong position-effect variegation of eye fluorescence and was terminated.

Effect of helpers on donor element remobilization

In order to identify the most useful strains for further development, the seven remaining helper lines were tested for activity in helper/donor hybrids. All seven helpers were highly active, generating new germline insertions at a rate of 97% (63 out of 65 hybrids tested from all seven lines) (Table 1). Based on remobilization efficiencies (percentages of hybrids giving rise to at least one new transformant), all helpers catalysed remobilization equally well. This measure of efficiency was relevant because we wanted to limit our recovery of new transformants to a single F_2 individual per F_1 hybrid. We thereby avoided potential clusters of identical insertions derived from premeiotic remobilization events, ensuring the independent derivation of each insertion line. We further compared the transformation frequencies (percentages of F_2 progeny bearing new insertions) associated with each helper. The transposition frequencies ranged from 0 to 23.6% among the 63 hybrid beetles that produced such offspring, with the most active helpers (M25 and M26) giving five-fold to six-fold higher average transposition frequencies than the least active helpers (M50 and M59). In contrast, there were no remobilization events in any of 3527 F_2 progeny tested from 20 control crosses lacking the helper, demonstrating the stability of

Table 1. Activity of *Minos*-based helper lines*

Helper line	Efficiency (No. active/total)†	Total N‡	Transformation frequency§ (range)	Standard error
M22	8/9	1015	7.3% (0.0–16.3)	1.8
M25	10/10	1220	11.6% (6.5–23.6)	1.8
M26¶	9/9	816	13.8% (9.4–18.9)	1.1
M27	10/10	1429	8.5% (4.0–13.8)	1.0
M40	8/8	1389	6.9% (1.8–17.3)	1.5
M50	10/10	1785	2.5% (0.8–5.6)	0.6
M59**	8/9	1295	2.3% (0.0–6.5)	0.7
Total	63/65 = 97%			
none	0/20	3527	0	0

*For each helper line five hybrid female and five hybrid male beetles, each carrying one helper and one donor chromosome, were outcrossed to *pearl* in single pairs.

†Total number of crosses producing at least one new insertion + total number of fertile crosses.

‡Total number of F_2 progeny screened for new insertions from all crosses within each line.

§Percent of total N carrying a new insertion.

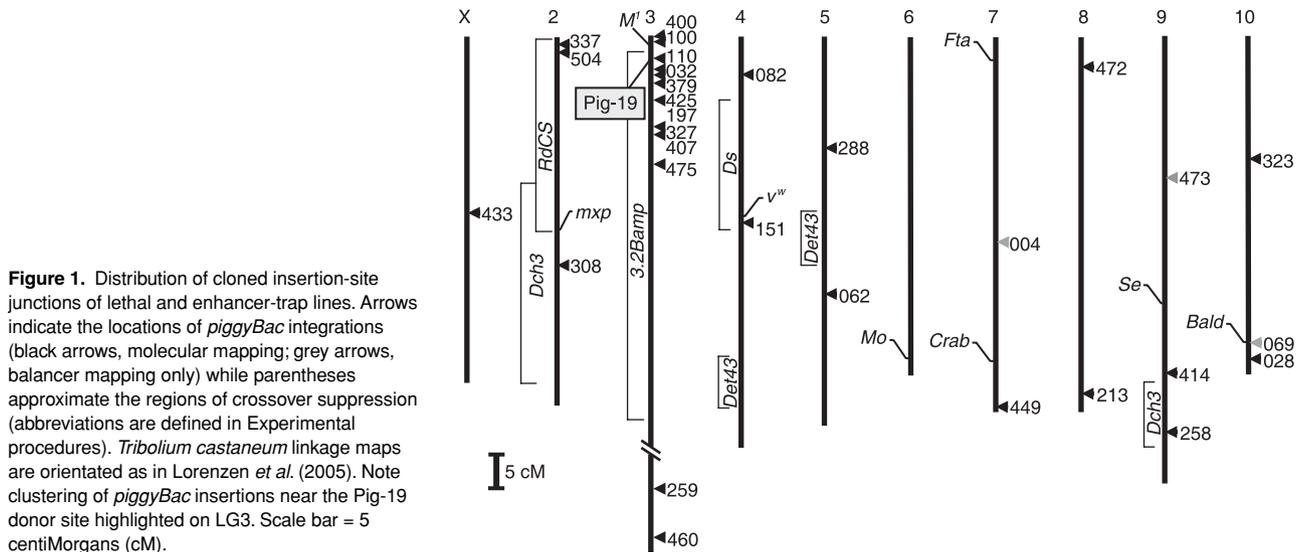
¶X-linked.

**Homozygous lethal.

the donor element in the absence of the helper chromosome. Hybridization-based remobilization was far superior to injection-based remobilization, even for hybrids carrying the least active helper. Among 131 donor beetles injected with helper plasmid as embryos for excision footprint analysis, only 37 (28%) generated new germline insertions. This contrasts with the transformation efficiency of 97% measured in hybrids, as described above.

Insertional mutagenesis in *T. castaneum*

Having confirmed that *piggyBac* transposes at very high rates in helper/donor hybrids, we then set up a pilot experiment to determine the feasibility of genome-wide insertional mutagenesis in *T. castaneum*, using one of the newly generated helper lines. For this pilot test we selected the least active helper (M59), in the hope of minimizing the percentage of new transformants that might carry more than one new insertion. A mass mating was made between M59 male beetles and donor (Pig-19) female beetles. Hybrid progeny carrying both helper and donor elements were selected, and 510 of these were individually outcrossed to white-eyed mates. Of the 490 fertile crosses, 411 produced at least one F_2 individual that showed loss of muscle expression with retention of eye-specific expression of the EGFP reporter (remobilization efficiency = 84%). This pattern of reporter expression indicates that the donor element had been mobilized from its original insertion site and reinserted elsewhere in the genome. In the absence of the helper, eye- and muscle-specific EGFP expression always cosegregated in outcrosses of Pig-19 heterozygotes (data not shown), confirming the results from the helper comparison test described above (Table 1, last row). A single line was established from each hybrid that generated a new



transformant, except in cases where two insertions could be differentiated by an enhancer-trap phenotype. A large majority of the new insertions occurred singly in the F_2 genomes, as evidenced by 1 : 1 segregation ratios in the subsequent outcross based on EGFP eye fluorescence. For F_2 female beetles, even those outcrosses yielding an excess of EGFP-expressing progeny proved to be single insertions in most cases. In these situations, the apparent distortion of the expected 1 : 1 ratio was caused not by multiple insertions, but by the presence of the maternally acting selfish gene, *Medea*, in the F_2 female beetle (see Discussion).

Lethal and sterile insertions

In order to identify recessive lethals and reveal recessive phenotypes we attempted to homozygote each of the 411 *piggyBac* insertions (see Experimental procedures). Twenty of the 411 (5%) proved to be recessive lethal and one semisterile. Integration sites were determined by amplifying, cloning and sequencing insertion-site junctions. Approximately 85% of the genome sequence assembly has been integrated into the recombination maps (unpublished). This has enabled us to determine linkage positions for most of the 21 lethal/sterile insertions (Fig. 1). The AM473 junction sequence matched two sequence scaffolds equally well, and thus could not be positioned with confidence. Of 20 insertions for which sequence scaffolds could be unambiguously identified, 17 were mapped (see Fig. 1 and Table 2). The three remaining insertions (AM004, AM069 and AM351) could not be mapped with precision, because their corresponding scaffolds are not yet associated with linkage groups.

To determine which gene (or genes) was affected in each of the lethal or sterile lines, candidate genes were identified

in a 10–100 kb region of the genome (centring on the insertion site) using BLASTX (Altschul *et al.*, 1997) and GENSCAN (Burge & Karlin, 1998). Fourteen of the 21 insertions were found to be within the transcription units of known or predicted genes. For example, the insertion site in AM323 is in the 5'UTR of a gene encoding the beta subunit of pyruvate dehydrogenase (*Drosophila* orthologue = *CG11876*) and the insertion site in the AM288 line is in an intron of the orthologue of the *Drosophila lethal(2)k0743* gene (aka *CG33130*). The association among the remaining seven insertions and their candidate genes was less clear. Because most genes in this second category have only short stretches of high similarity to candidate homologues, the complete gene structures could not be determined. In these cases it could not be determined with certainty whether the insertions interrupted transcription units.

Several of the recessive-lethal lines produced progeny with interesting developmental defects. The AM028 insertion interrupts an intron of *Tc Kruppel*, a gap gene that Cerny *et al.* (2005) characterized via both RNA interference (RNAi) and molecular dissection of a null mutation. Inspection of presumably homozygous AM028 embryos (Fig. 2B) revealed a phenotype typical of that observed in embryos mildly affected by *Tc Kruppel* RNAi. The AM100 line, which carries an insertion in an exon of *Tc Cox11*, produces a class of terminal embryos in which the head appendages are abnormally orientated toward the posterior (Fig. 2D). In contrast, AM472 homozygotes survive until adult eclosion, but die soon after as the nascent wings and elytra fail to differentiate and expand to full size and proportions (Fig. 2E). The recovery of these phenotypes in a relatively small pilot screen bodes well for future *piggyBac*-based mutagenesis in *Tribolium*.

Table 2. Map positions and candidate gene knockouts of lethal and sterile *piggyBac* insertions

Insertion	Map position*	Coordinates of insertion target†	Genetic distance from balancer‡	Nearest gene§
AM004	AAJJ01001704	13588	LG7 (13 cM from Crab, 20 cM from Fta)	<i>CG12713</i> (exon)
AM028	CM000285 (LG10; 47.1)	8392816	LG10 (4 cM from Bald)	<i>Kruppel</i> (intron)
AM062	CM000280 (LG5; 34.7)	11740678	LG5 (3 cM from Det43)	<i>CG4103</i> (exon)
AM069	AAJJ01000253	48160	LG10 (0 cM from Bald)	<i>Notch</i> or <i>CG14127</i>
AM100	CM000278 (LG3; 0.0)	1008309	LG3 (0 cM from 3.2Bamp)	<i>Cox11</i> (exon)
AM110¶	CM000278 (LG3; 1.7)	1719986	LG3 (0 cM from 3.2Bamp)	<i>MED23</i>
AM151	CM000279 (LG4; 27.4)	5041851	LG4 (0 cM from Ds)	<i>CG14535</i> (intron)
AM197	AAJJ01000897 (LG3; 7.2)	10591	LG3 (0 cM from 3.2Bamp)	<i>CG10955</i> (intron)
AM258	CM000284 (LG9; 52.8)	11611077	LG9 (0 cM from Dch3, 14 cM from Se)	<i>Goosecoid</i> (intron)
AM259	CM000278 (LG3; 82.1)	29175624	LG3 (37 cM from 3.2Bamp)	<i>MESK2</i>
AM288	CM000280 (LG5; 14.1)	3657017	LG5 (10 cM from Det43)	<i>CG33130</i> (intron)
AM308	CM000277 (LG2; 26.4)	3715731	LG2 (1 cM from RdCS)	<i>Pi3K59F</i> (intron)
AM323	CM000285 (LG10; 17.1)	4499762	LG10 (28.3 cM from Bald)	<i>Pyruvate dehydrogenase</i> (5'UTR)
AM337	CM000277 (LG2; 1.6)	3964776	LG2 (1 cM from RdCS)	<i>Eip74EF</i> (intron)
AM351	AAJJ01002477	6781	not linked	<i>CG10882</i>
AM414	CM000284 (LG9, 52.8)	11008132	LG9 (2 cM from Dch3, 13 cM from Se)	<i>Spat</i> or <i>sog</i>
AM449	CM000282 (LG7, 52.8)	16555336	not linked	<i>shavenoid</i> (intron)
AM460	CM000278 (LG3; 100.5)	31571876	not linked	<i>CG33275</i> or <i>CG33277</i>
AM472	CM000283 (LG8; 0.0)	184640	not linked	<i>CG7565</i> or <i>SnoN</i>
AM473	not determined	not determined	LG9 (18 cM from Se)	<i>Transposase</i> (exon)
AM475	CM000278 (LG3, 24.3)	8886174	LG3 (0 cM from 3.2Bamp)	<i>Nup358</i> (intron)

*GENBANK accession no. of chromosomal locus or contig determined by BLASTN of insertion junction against genome assembly, followed by position on molecular recombination map in parentheses (Lorenzen *et al.*, 2005).

†Position of insertion point on chromosomal locus or contig (GENBANK numbering system).

‡Determined by balancer linkage tests.

§Gene names refer to *Drosophila melanogaster* orthologues.

¶The AM110 insertion appears to be associated with a 150 kb deletion resulting from recombination between two nearby *piggyBac* insertions.

Balancer-assisted mapping and confirmation of homozygous lethality

To assess the distribution of *piggyBac* insertions in the genome, we tested a subset of the insertion lines for linkage to balancer chromosomes. One hundred of the 411 insertions were tested for linkage to one or more balancers in a set of five. Forty-one of these mapped to within 10 recombination units of one or another of these balancers. Twenty-three insertions that proved to be tightly linked to a balancer were subsequently tested for homozygous lethality (true-breeding of balancer/insertion *trans*-heterozygotes). All 23 were found to be viable (data not shown), confirming independent analysis carried out by progeny testing, as described in the Experimental procedures (Determination of homozygous lethality). Two balancers (*RdCS* and *Dch3*) overlap on linkage group (LG) 2, and as expected, all six insertions that showed linkage to *RdCS*, were also linked to *Dch3*. *Dch3* is a (2; 9) translocation (Beeman *et al.*, 1996), and thus balances a portion of LG9 as well as LG2. Three out of eight *Dch3*-linked insertions tested showed no linkage to *RdCS*, and these presumably represent insertions on to LG9.

Twenty lines that had previously been identified as homozygous lethal, as well as one semisterile line, were also tested for linkage to a set of balancers and other dominant markers. Twelve (57%) of these were closely

linked to at least one of these markers, and all but one of the 12 were confirmed to be homozygous lethal, while the twelfth (AM414) was confirmed to be homozygous semisterile. Map assignments based on linkage to balancer chromosomes were in agreement with mapping based on insertion-site sequences (Table 2). In most cases the insertion sites were within or near the transcription units of orthologues of vital genes, further supporting the inference of lethality.

Footprint analysis

Perfect excision is the rule for the *piggyBac* transposon in a number of eukaryotic phyla, including several insect orders in the phylum Arthropoda (Thibault *et al.*, 1999, 2004; Grossman *et al.*, 2000; Handler, 2002; Ding *et al.*, 2005). In order to determine whether this is also true in the order Coleoptera, we used the dominantly marked LG3 balancer *3.2Bamp* to isolate insertion junctions in *T. castaneum*. As the Pig-19 insertion site lies within the region of crossover suppression associated with this balancer, we were able to track postexcision chromosomes for subsequent molecular analysis. The crosses we established permitted simultaneous assay of two postexcision sites from each line. Direct sequencing of amplification products for two different G₀ lines (four postexcision chromosomes) showed that in each case the single, preinsertion TTAA site was restored after excision. Thus *piggyBac* appears to excise perfectly in this

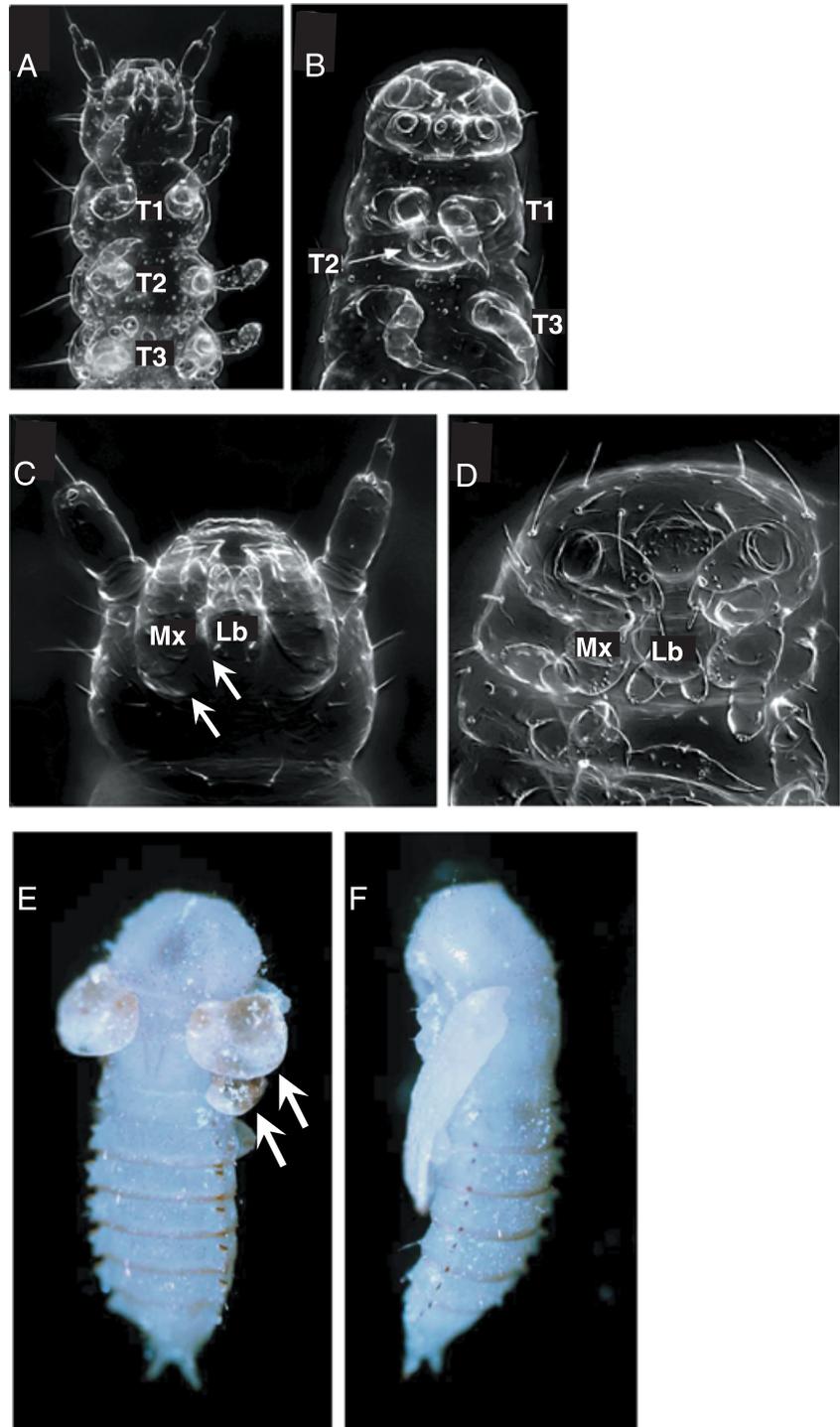


Figure 2. Mutant phenotypes of putative homozygotes from three lethal insertion lines. (A, C) Wild-type, first-instar larvae. (B) Putative AM028 homozygote. Note the extreme reduction of the second thoracic segment (T2) and the transformation of the T2 legs to labial appendages. (D) Putative AM100 homozygote. Note that the head appendages are abnormally orientated, with their distal tips pointing posteriorly instead of anteriorly. This is particularly noticeable for the maxillary (Mx) and labial (Lb) appendages. (E) Putative AM472 homozygote (pupa), shown in dorsolateral view. (F) Corresponding wild-type pupa. Arrows indicate the nascent, non-expanded elytra and wing in AM472 compared with the fully extended elytra of the wild-type. The wing is not visible in the wild-type because it is completely covered by the elytra. Wing and elytra are slightly false-coloured to enhance visualization.

coleopteran species as it does in other eukaryotes, leaving no footprint.

Enhancer-trap lines and a hotspot for piggyBac integration

Expression of the 3xP3-EGFP marker construct typically occurs in the eyes and brain in *T. castaneum* (Lorenzen *et al.*, 2003). Eighteen per cent (74/411) of the lines pro-

duced in our pilot screen also showed EGFP expression in at least one additional domain in either embryos or pupae. These additional EGFP expression domains often included the peripheral nervous system, distal limbs or appendage joints. It is possible that certain frequently observed expression patterns depend on the combined action of enhancer-like sequences adjacent to the insertion sites and on

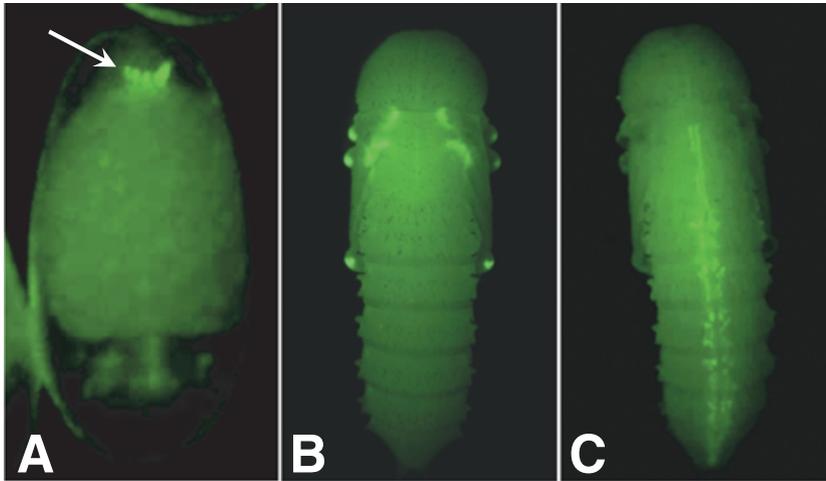


Figure 3. Enhancer-trap phenotypes of AM327 embryo and AM166 and AM032 pupae. (A) AM327-associated fluorescence is confined to the six Malpighian tubule tip cells in the developing embryo (arrows). In *Drosophila*, the Malpighian tubule tip cells play an important role in tubule elongation, signalling nearby cells to divide. (B) AM166-associated fluorescence is seen in the wing and elytral hinges and the femoral-tibial joints. (C) AM032-associated fluorescence is confined to the alary muscles and pericardial epithelium along the entire length of the dorsal vessel.

Table 3. Map positions and candidate genes for enhancer-trap insertions

Insertion	Chromosome*	Insertion point†	Linkage group	Enhancer domain	Nearest gene
AM213	CM000283	14744643	LG8 (51.4)	pericardium	<i>matrix metalloprotease 2</i>
AM032	CM000278	3154312	LG3 (4.2)	pericardium	<i>sticks-and-stones</i>
AM379	CM000278	3170044	LG3 (4.2)	pericardium	<i>sticks-and-stones</i>
AM425	CM000278	3140364‡	LG3 (4.2)	pericardium	<i>sticks-and-stones</i>
AM082	CM000279	1577563	LG4 (6.8)	embryonic pericardium	<i>tenascin accessory</i>
AM327	CM000278	6004447	LG3 (14.8)	Malpighian tubule tip cells	<i>Keren/Spitz</i> (EGF ligand)
AM400	CM000278	30786	LG3 (0.0)	distal wing	<i>Tim8</i>

*GENBANK accession no. of chromosomal locus.

†Position of insertion site on chromosomal locus (GENBANK numbering system).

‡Insertion sequence extends into NNN region of CM000278.

the internal 3xP3 sequence itself. Therefore, caution is required before ascribing such patterns entirely to host sequences flanking the insertions. A majority of the 74 enhancer-trap-like lines produced in our pilot screen appeared to fall into this category. However, several insertion lines did display EGFP expression patterns that appeared to be unrelated to 3xP3, suggesting that the EGFP marker was influenced by bona fide chromosomal enhancer sequences near the sites of *piggyBac* integration. EGFP expression in the AM327 line occurred in the Malpighian tubule tip cells of developing embryos (Fig. 3A). This insertion site was cloned and sequenced, and mapped near a gene encoding an epidermal growth factor (EGF) ligand related to *D. melanogaster* Spitz and Keren. EGFP expression in the AM166 line is seen in the wing and elytral hinges and the femoral-tibial joints (Fig. 3B). The insertion junction in this strain has not yet been identified. Five other insertions, all independently derived, were apparently influenced by pericardial enhancer(s) near the insertion sites. EGFP reporter expression in these lines was always present in the alary muscles surrounding the dorsal vessel, and along the entire length of the pericardial epithelium

(Fig. 3C). All five pericardial enhancer insertion junctions are unique, three of these being located within a 30 kb segment on LG3, near a gene with similarity to the *D. melanogaster* *sticks-and-stones* (*sns*) gene. In *Drosophila*, *sns* is required for myoblast differentiation and visceral muscle development (Galletta *et al.*, 2004). Positions of insertion sites for all sequenced enhancer-trap junctions are indicated in Fig. 1 and Table 3.

The insertion hotspot near the putative pericardial enhancer on LG3 is located only 2.5 centiMorgans (cM) away from the *piggyBac* donor site, and may be symptomatic of a more general preference of *piggyBac* for local transpositions. In addition to the pericardial enhancers, evidence based on *Medea* linkage suggests that at least 22 out of 184 new insertions examined (12%) also occurred within 12 cM of the original donor site, which is located 2 cM from the *Medea*¹ locus near the tip of chromosome 3 (see Discussion). This is approximately five-fold more frequent than would be expected from a completely random pattern of insertion, given the total amount of genetic recombination in *T. castaneum* (~570 cM; Lorenzen *et al.*, 2005).

Discussion

The jumpstarter strategy is feasible in T. castaneum

Previously we reported that the Pig-19 insertion could be remobilized via injection of helper plasmid into precellular Pig-19 embryos (Lorenzen *et al.*, 2003) with an efficiency of ~28%. We have reproduced these results using the same helper plasmid, but a larger number of Pig-19 embryos, and once again found that the remobilization efficiency is approximately 28% (37/131). In the present work we show that hybridization-based donor remobilization is not only far more efficient than that based on injection, but is also far less technically challenging because it circumvents the tedious process of generating, handling and injecting preblastoderm embryos. In the initial test using all seven helper lines, an overall efficiency of 97% (new insertions from 63 out of 65 hybrids) was achieved. In the subsequent pilot test of the M59 helper, we observed an efficiency of 84% (411 out of 490 hybrids). With our recent switch to the more active M25 or M26 helpers, we now routinely achieve a transformation efficiency of 100%, while maintaining a high frequency of single-insertion events per line. The results of this study clearly show the feasibility of hybridization-based transformation for genome-wide insertional mutagenesis in *Tribolium*.

Local versus global hops

During the analysis of data for this report, it became apparent that the maternal-effect lethal gene *Medea*¹ (Beeman *et al.*, 1992) had influenced the results. The presence of a maternally derived *Medea* allele can cause an apparent distortion of segregation ratios because of genotype-dependent selective mortality of embryos. Twenty-two out of 184 insertions detected in F₂ female beetles during the remobilization pilot test gave apparent segregation ratios > 6 : 1 of EGFP-positive to EGFP-negative progeny. In contrast, none of the 233 male beetles had a segregation ratio > 6 : 1. After completion of the experiment, we discovered that the M¹ *Medea* allele was segregating in the donor stock, and that many of the donor beetles carried the M¹ allele in *cis* with the Pig-19 donor element on LG3. In view of the close genetic linkage of *Medea*¹ and Pig-19, this arrangement would have produced a severe apparent segregation distortion (excess) of the donor chromosome in the progeny of F₂ female beetles. In the absence of a remobilization, or in the event of a short-distance hop to a closely adjacent site in *cis* with the donor site, the observed ratio of EGFP-expressing beetles to non-EGFP would be approximately 50 : 1, given that the recombination rate between *Medea*¹ and the Pig-19 insert is ~2%. As multiple hops were rare, the 22 new insertions with severely distorted segregation ratios probably reflect local transpositions within the donor chromosome. The likelihood that any new random reinsertion would occur within a 12-map-unit

segment that includes the donor site is $12/570 = 2.1\%$ because there are ~570 map units in the genome. Thus we would expect about four of the 184 reinsertions to occur within this region. If we assume that all 22 new insertions with severely distorted segregation ratios (> 6 : 1) are local hops within 12 cM of the donor insert, this means that $22/184 = 12\%$ occurred within this region, or more than five-fold higher than predicted. The inference that the distorted segregation ratios reflected local transposition was confirmed for eight of the 22 insertions by genetic mapping (the others were not tested). This actually represents an underestimate of the true frequency of local transposition, as not all F₁ female beetles carried the *Medea*¹ allele (data not shown).

Analysis of linkage of reinsertions to the LG3 balancer *3.2Bamp* also speaks to this issue. This balancer covers approximately 9% of the genome (50 cM in a 570 cM genome) and spans the Pig-19 donor site. Of 100 insertions tested, 29 (29%) were tightly linked to *3.2Bamp*, a value more than three-fold higher than that expected from a completely random pattern. Three additional insertions were linked to the balancer, but showed some recombination (10–20%).

A similar bias for local transposition was found among the lethal insertions. Four of the 20 lethal insertions (20%) mapped to the *3.2Bamp* arm of chromosome 3. This could be construed as yet more evidence for a significant preference for short-distance hops. Alternatively, it is possible that there is simply a bias for *piggyBac* insertion on LG3. Insertion-site preferences and non-preferences have been noted for the P transposable element in *Drosophila* (Simmons & Lim, 1980). Metaxakis *et al.* (2005) reported that *Minos* insertions were also non-uniformly distributed in the *D. melanogaster* genome. To distinguish amongst these possibilities, it will be necessary to remobilize a *piggyBac* insertion from another chromosome and determine the genomic distribution of the new insertion sites. In spite of this apparent preference for local transposition, the majority of insertions were not linked to the *3.2Bamp* balancer, and appeared to be widely scattered over the genome.

Lethal insertions

All 20 lethal insertions were located in or near the transcription units of known or predicted genes. Fourteen of these were clearly within transcription units, while the remaining six were associated with genes with incompletely annotated transcription units. Table 2 indicates the best candidate gene(s) for each insertion. It is possible that more than one gene could be affected by a single insertion, or that we misidentified the affected gene. Resolution of these possibilities must await future functional genomic analysis.

We were unable to confirm that all strains used in this work were completely lethal-free. However, analysis of the lethal lines suggests that background mutations are not a major issue. One of the insertions (AM028) is in an intron

of a previously characterized gene, *Tc Kruppel* (Cerny *et al.*, 2005), and the phenotype of presumed AM028 homozygotes is consistent with partial loss of function of this gene. This supports the idea that lethality is a result of insertion-site location rather than a tightly linked background mutation, at least in this strain. Furthermore, all well-characterized *T. castaneum* genes disrupted by lethal insertions are orthologues of essential genes in *D. melanogaster*, further supporting the idea that background lethals are not a major issue. Reversion of lethality via remobilization of the *piggyBac* element would reveal whether the insertion is the direct cause of lethality. However, remobilizations of non-enhancer-trap insertions will require the construction of additional, appropriately marked strains.

Future prospects for hybridization-based transformation in T. castaneum

In view of the efficiency of hybridization-based transformation in *T. castaneum*, we and our collaborators have initiated a large-scale, genome-wide insertional-mutagenesis screen, with the goal of generating 15 000 independent *piggyBac* insertions. Approximately 7000 lines have been generated already, and are currently being analysed for enhancer-trap effects, homozygous lethality/sterility, and genomic distribution. At the same time, we are developing and testing a *Minos*-based transformation system as a supplement or alternative to the *piggyBac*-based one described here. Because it has a different insertion target from *piggyBac* (TA rather than TTAA), *Minos* might, in part, complement the insertion-site preferences of *piggyBac*. Transgenic *Minos* enhancer-trap lines of *T. castaneum* are available (Pavlopoulos *et al.*, 2004), and we have already shown that the *Minos* donor element can be remobilized from one such enhancer-trap line by injection of a *Minos* helper plasmid followed by screening for loss of enhancer phenotype (unpublished observations). Future refinements of hybridization-based transformation in *T. castaneum* will involve the use of donor constructs that incorporate an improved enhancer-trap module, a gene-trap module with splice acceptor for truncation of transcripts and recombination sites for directed integration and for efficient recovery of deletions and inversions.

Experimental procedures

Donor line

The Pig-19 donor strain (Lorenzen *et al.*, 2003) is homozygous for an insertion of the pBac{3xP3-EGFPaf} cassette described by Horn & Wimmer (2000). The insertion point in this strain is in the 3' UTR of an *actin* gene at position 1.7 on LG3 (= nucleotide 1569190 on NCBI chromosome CM000278), resulting in expansion of the EGFP expression domain to include muscle tissue throughout the body, in addition to the eye-specific expression conferred by the 3xP3 promoter. EGFP expression in muscle provides an additional,

easily scored phenotype that is insertion-site-dependent. As the eye expression is insertion-site-independent, remobilization events are easily detected by loss of muscle fluorescence with retention of eye fluorescence. This strain, as well as all white-eyed strains used in this work, is homozygous for the recessive, eye-colour mutation *pearl* (Park, 1937), which prevents deposition of eye pigments, thereby improving the detectability of eye-specific fluorescence. All beetles were reared in yeast-fortified wheat flour under standard conditions (Beeman & Stuart, 1990).

Helper lines

The helper lines were established by coinjection of the *Minos*-based plasmid pMi{3xP3-DsRed, hsp70-piggyBac} (Horn *et al.*, 2003) which supplies a source of *piggyBac* transposase, and the *Minos* helper plasmid pHSS6hslLMi20 (Klinakis *et al.*, 2000) into preblastoderm *pearl* embryos at a final concentration of 440 ng/μl and 330 ng/μl, respectively. Injection procedures are described in Lorenzen *et al.* (2003). Individual helper lines were established after segregation of single, independent insertions from each G₀ line.

Minos helper tests

Helper lines were rendered homozygous when possible, and tested for their ability to catalyse *piggyBac* transposition in donor/helper hybrids *in vivo*. Single-pair crosses were made between the Pig-19 donor strain and each of the seven helper lines or a non-transgenic white-eyed control strain. For each helper line, 10 F₁ hybrid progeny carrying one copy each of the helper and donor chromosomes (verified by red and green fluorescence selection) were outcrossed in single pairs (five of each reciprocal type) to *pearl* mates. Although the *Drosophila hsp70* promoter was employed to drive *piggyBac* transposase expression in this helper, high helper activity was achieved without the need for heat shock at standard rearing temperatures used for *Tribolium*. Approximately 100–200 progeny (mean = 131) from each cross were examined for remobilization of the Pig-19 donor element, as evidenced by the retention of eye-specific EGFP expression with concomitant loss of muscle expression. Twenty corresponding, single-pair control outcrosses involving Pig-19 heterozygotes lacking the helper element were made in order to confirm the stability of the Pig-19 insertion in the absence of the *piggyBac* transposase transgene. Approximately 150–200 progeny (mean = 176) from each control cross were similarly examined for evidence of Pig-19 remobilization.

Hybridization-based remobilization of Pig-19 donor with M59 helper

Mass-crosses were made between Pig-19 homozygotes and M59 heterozygotes because the M59 insertion proved to be homozygous lethal. Single F₁ progeny inheriting both the helper and donor chromosomes were outcrossed to *pearl* mates and progeny carrying remobilized donor elements were identified as described above. A single, new insertion line was established from each F₁ hybrid after one additional outcross to *pearl*.

Determination of homozygous lethality

All 411 insertion lines were screened for homozygous lethality. After outcrossing each new F₂ transformant to *pearl*, small mass-selfcrosses (five male and eight female beetles) were made among F₃ insertion heterozygotes. One-third of the EGFP-expressing

F₄ progeny were expected to be homozygous for the insertion. For each insertion line, up to 40 insertion-bearing F₄ beetles were tested for homozygosity as follows: an insertion was regarded as potentially lethal if each of the first eight single-pair F₄ selfcrosses produced one or more non-EGFP offspring, as would be expected if the parents were both heterozygous. In this case, 12 additional confirmatory selfcrosses were made, and if none of the 40 F₄ beetles proved to be homozygous based on progeny phenotypes, the insertion was considered to be lethal. Where possible the lethals were subjected to confirmatory tests using balancer chromosomes (see below).

Balancer-assisted mapping and confirmation of homozygous lethality

The balancer chromosomes and other dominantly marked chromosomes used in this study are listed below (also see Fig. 1): *Reindeer Crossover Suppressor (RdCS)*, marked by the dominant *Reindeer* mutation (Dawson, 1984), balances a ~40 cM region of LG2 and overlaps *Dachsund-3 (Dch3 = mxp^{Dch3})* in Beeman *et al.*, 1996). *Dch3* is a (2; 9) translocation, and as such, balances portions of two different linkage groups (LG2 and LG9). *RdCS* overlaps the region balanced by *Dch3* on LG2, and is used to differentiate between LG2- and LG9-linked insertions. The dominant mutation *Blunt abdominal and metathoracic projections* (Beeman, 1986) marks *3.2Bamp*, which balances a 50 cM region (one arm) of LG3. *Displaced sternellum (Ds)*; Mocelin & Stuart, 1996) marks one of the LG4 balancers, while the dominant marker *Divergent elytral tips (Det43)* marks another. *Det43* is a (4; 5) translocation and therefore can be used to identify LG4- and LG5-linked insertions. *RdCS* and *3.2Bamp* were discovered in screens for crossover suppressors on dominantly marked chromosomes. All other balancers were discovered as dominant-visible mutations, and were subsequently shown to be homozygous lethal. All balancers were radiation-induced, using a Co⁶⁰ source. The location and approximate range of each balancer are given in Fig. 1.

Other dominant-visible, recessive-lethal markers used in these tests were: *Crab* (LG7), an EMS-induced mutation associated with shortened, thickened and bowed tibiae, conferring a crab-like appearance on affected beetles; *Fused tarsi and antennae (Fta)*; LG7; Sokoloff & St. Hilaire 1962); *Microphthalmic (Mo)*; LG6; Sokoloff, 1960); *Short elytra (Se)*; LG9); and *Bald* (LG10), an EMS-induced mutation that results in a reduced density of sensory bristles and their associated pits on the dorsal pronotum and abdominal sternites.

One hundred of the 411 new insertion lines were randomly selected and linkage crosses were initiated using the five balancer chromosomes *RdCS*, *Dch3*, *3.2Bamp*, *Ds* and *Det43*. Twenty-one additional lines already determined to be homozygous lethal (or in one case, semisterile) based on progeny phenotypes (see above) were similarly tested against the full panel of 10 balancers and dominant-visible markers for independent confirmation of lethality or semisterility, as well as mapping and balancing. Linkage to each balancer was tested in F₁ male beetles derived from crosses between white-eyed balancer and insertion lines. Such male beetles, heterozygous for balancer and insertion, were outcrossed to one to two white-eyed female beetles, and the progeny (50–100 per outcross) scored for linkage.

Screening for enhancer traps

To detect embryonic and early larval enhancer-trap phenotypes, eggs and larvae were collected from adults reared on triple-sifted

flour (50-mesh sieve) for five days. After collection, eggs and first-instar larvae were treated with 50% Clorox® bleach (2.6% sodium hypochlorite, The Clorox Company, Oakland, CA) to dechlorinate eggs, and then rinsed with water into nine-well Pyrex™ spot plates (Corning Incorporated, Corning, NY) for viewing. Pupae were examined for enhancer-trap phenotypes while scoring for eye-specific EGFP expression.

EGFP and DsRed analysis

Expression of EGFP and DsRed was observed using either a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems Inc., Wetzlar, Germany) or an Olympus SZX12 fluorescence stereomicroscope (Olympus Corporation, Tokyo, Japan). The Leica microscope was equipped with a GFP Plus filter set (excitation filter: 480/40 nm, barrier filter: 510 nm) and a TXR TEXAS RED filter set (excitation filter: 560/40 nm, barrier filter: 610 nm) which was used to detect DsRed expression. For the Olympus microscope, EGFP was detected with a 480/40 nm excitation filter and a 525/50 nm emission filter. DsRed was detected with one of two filter sets: a 545–580 nm barrier filter and a 610 nm emission filter, or a 545/30 nm excitation filter and a 620/60 nm emission filter. Photography was performed with a DXM1200F digital camera (Nikon Inc., Melville, NY).

Remobilization via microinjection, and footprint analysis

Preblastoderm embryos (G₀) from a homozygous Pig-19 stock were injected with 350 ng/μl of the *piggyBac* helper plasmid phspBac (Handler & Harrell, 1999). After adult development, 131 single-pair crosses were made between the helper-injected, Pig-19 G₀ male and *3.2Bamp* female beetles. Phenotypically *Bamp* G₁ progeny were screened for EGFP expression, and a male and female beetle, each with loss of muscle-specific expression but retention of eye-specific EGFP expression, were selected and mated *inter se*. Non-*Bamp* G₂ progeny (*trans*-heterozygous for two empty Pig-19 donor sites) were subjected to DNA sequence analysis. Excision sites were PCR-amplified from postexcision chromosomes from two independently derived lines and directly sequenced.

DNA isolation and PCR

Single-beetle DNA isolations were performed using the Wizard Genomic DNA Isolation Kit (Promega, Madison, WI, USA) using 60 μl of nuclei lysis solution and 20 μl of protein precipitation solution. For some transgenic lines, DNA was isolated from groups of five pupae or adults using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions for DNA isolation from insects (using the option for homogenization in Phosphate buffered Saline (PBS)). *piggyBac* insertion junctions were amplified using inverse PCR (Ochman *et al.*, 1988), universal PCR (Lorenzen *et al.*, 2003) or vectorette PCR (Arnold & Hodgson, 1991).

Sequence analysis

DNA templates were sequenced using an ABI 373 A DNA sequencer (College of Veterinary Medicine, Kansas State University, USA), an Applied Biosystems 3730 DNA Analyser (DNA Sequencing Facility, Department of Plant Pathology, Kansas State University) or an Applied Biosystems 3730xl DNA Analyser (Elim Biopharmaceuticals Inc., Hayward, CA). Data analysis was performed using

the Vector NTI® sequence analysis program (Invitrogen, Carlsbad, CA, USA). Insertion-site sequences (GENBANK accession numbers EF075984–EF076012) were compared to the sequenced *T. castaneum* genome (BLASTN) and the corresponding regions evaluated for known and predicted genes via BLASTX (Altschul et al., 1997) and GENSCAN (Burge & Karlin, 1998).

Acknowledgements

We thank Jonna Voorhees, Sue Haas, Canda Harvey, Terri O'Leary, Kelli Goodrich and Craig Ackerman for technical assistance, and Ernst Wimmer and Martin Klingler for providing plasmids. We also thank Yoonseong Park for sharing the primers and protocol for vectorette PCR. This work was supported by grants from the National Science Foundation (IBN031882), the National Institutes of Health (R01-HD29594) and CSREES-NRI (#2004-3560414250). This article is contribution no. 06-309-J from the Kansas Agricultural Experiment Station. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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