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piggyBac-mediated germline transformation in the beetle *Tribolium castaneum*

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Abstract

The lepidopteran transposable element *piggyBac* can mediate germline insertions in at least four insect orders. It therefore shows promise as a broad-spectrum transformation vector, but applications such as enhancer trapping and transposon-tag mutagenesis are still lacking. We created, cloned, sequenced and genetically mapped a set of *piggyBac* insertions in the red flour beetle, *Tribolium castaneum*. Transpositions were precise, and specifically targeted the canonical TTAA recognition sequence. We detected several novel reporter-expression domains, indicating that *piggyBac* could be used to identify enhancer regions. We also demonstrated that a primary insertion of a non-autonomous element can be efficiently remobilized to non-homologous chromosomes by injection of an immobile helper element into embryos harbouring the primary insertion. These developments suggest potential for more sophisticated methods of *piggyBac*-mediated genome manipulation.

Keywords: *piggyBac*, germline transformation, EGFP, enhancer trap, *Tribolium castaneum*.

Introduction

Advances in genomics and bioinformatics promise to deepen our understanding of the mechanisms of evolutionary diversification and to hasten the discovery and analysis of genes that regulate important biological phenomena. Such efforts will be aided by continued development of transposon-based systems for the experimental manipulation of target genomes. The lepidopteran transposon *piggyBac* (Cary *et al*., 1989) has shown excellent potential to be a broad-spectrum vector for germline transformation (Fraser, 2000). It has been shown to function in the insect orders lepidoptera (Fraser *et al*., 1995; Tamura *et al*., 2000), diptera (Handler *et al*., 1998), coleoptera (Berghammer *et al*., 1999) and most recently hymenoptera (Sumitani *et al*., 2003).

Beetles are the most species-diverse eukaryotic order, comprising 20–25% of all animal species and 15–20% of all eukaryotic species (Farrell, 1998), but there is a relative dearth of genetic and genomic knowledge of this important group. In particular, lack of versatile systems for genetic analysis in beetles, such as transposon-mediated mutagenesis (Cooley *et al*., 1988; Robertson *et al*., 1988), enhancer trapping (O'Kane & Gehring, 1987) and GAL4-based ectopic expression (Brand & Perrimon, 1993), limits the types of experimental approaches possible with coleopteran subjects. The red flour beetle, *Tribolium castaneum*, offers by far the most sophisticated system for genetic manipulation among all beetles (Stuart *et al*., 1993; Beeman *et al*., 1996; Beeman & Brown, 1999).

In previous reports we demonstrated *piggyBac* function in *T. castaneum* (Berghammer *et al*., 1999; Lorenzen *et al*., 2002a), developed native reporter systems (Lorenzen *et al*., 2002a,b), and described the use of enhanced green fluorescent protein (EGFP) as a universal selectable marker for transgenic insects (Berghammer *et al*., 1999). In the present work we undertook a more detailed molecular genetic characterization of *piggyBac* function in *T. castaneum* in order to pave the way towards development of additional *piggyBac*-based applications, including enhancer trapping, transposon-tag mutagenesis and promoter analysis.

Results

piggyBac-*mediated germline transformation of* T. castaneum

Germline transformation was tested in a white-eyed *T. castaneum* strain using an hsp70-driven transposase helper (Handler & Harrell, 1999) in conjunction with each of six *piggyBac* donor elements marked with 3xP3-EGFP (Horn & Wimmer, 2000). Among injected G_0 eggs surviving to the adult stage, transformation rates (percentages of fertile G_0 beetles producing at least one G_1 transformant)

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Table 1. Efficiency of *piggyBac*-mediated germline transformation in *T. castaneum*

	A	B	C	D	E
Transposon size (bp)	3 700	5 700	6 700	8 200	12 400
Vector plasmid size (bp)	7 300	9 300	10 300	11 800	16 000
Vector conc. ng/µl	500	413	535	560	500
Helper conc. ng/µl	375	388	400	465	375
Molar ratio (vector/helper)	1.12	0.70	0.80	0.63	0.51
Number of injected eggs	509	314	459	612	392
Egg hatch rate	44%	43%	49%	16%	37%
Survival to adult stage	30%	33%	36%	9%	31%
No. of fertile beetles	146*	95	152	50	55†
No. of independent lines	61*	38	36	12	12†
Transformation rate	56%*	40%	24%	24%	22%†
Average EGFP cluster size	30%*	24%	6%	6%	8%†
Smallest EGFP cluster size	2%*	1%	0.6%	0.5%	0.4%†
Largest EGFP cluster size	77%*	90%	23%	20%	21%†

Transformation data from five transformation experiments (A–E) using transposable donor elements of different size (*piggyBac* transposon size does not include flanking plasmid). Transformation rate = no. of G_0 crosses producing EGFP-expressing offspring/total no. of fertile G_0 adults. Note that the molar ratio (vector plasmid/helper plasmid) is smaller in the experiments with larger constructs, since roughly constant amounts of DNA were used. The last three rows detail the relative size of 'EGFP clusters', i.e. for each G_0 beetle giving rise to G_1 transformants, the proportion of EGFP-expressing G_1 offspring was calculated. Given are the average cluster size of EGFP-expressing animals, as well as the smallest and largest clusters observed for a given construct. Smaller constructs give rise to larger transformed clusters, probably due to multiple transposon insertions in the offspring of injected animals.

*Because G_0 adults were self-crossed in experiment A, the transformation rate was calculated as described in the experimental protocol. Despite the expected tendency of a self-cross to inflate cluster size relative to that of an outcross, cluster sizes in the A self-cross are similar to those in the B outcross.

†Calculations were done on the offspring of male G_0 beetles only.

ranged from 22 to 56% (Table 1), confirming our preliminary finding that *piggyBac* is an exceptionally efficient gene-transfer vector in *T. castaneum* (Berghammer *et al.*, 1999). Transformation frequencies were highest for smaller *piggyBac* donor elements (< 6 kb), but efficient transformation was observed even for a 12.4 kb element (Table 1, column E). The cluster size (percentages of transformed siblings within individual G_1 families) ranged from < 1–90% (Table 1). Segregation analysis of construct E transformants (12.4 kb donor element) indicated Mendelian segregation of single inserts.

To confirm *piggyBac* transposition into the beetle genome, several of the transformed lines were analysed via Southern DNA hybridization. Analysis of construct A transformants indicated 2–4 insertions per line (data not shown). However, because this set was derived from G_0 self-crosses, this represents an overestimate of the actual number of independent insertions per G_0.

Putative enhancer-trap lines

Expression of the 3xP3-EGFP marker construct is normally confined to the eyes, brain, a portion of the peripheral nervous system and anal plates in *Drosophila melanogaster* (Horn *et al.*, 2000), *Musca domestica* (Hediger *et al.*, 2001) and *Aedes aegypti* (Kokoza *et al.*, 2001). In *T. castaneum*, the most obvious expression is in the eyes and brain. However, several insertion lines displayed additional, novel EGFP expression patterns, suggesting that the EGFP marker was influenced by chromosomal enhancer sequences near the sites of *piggyBac* integration (Fig. 1). This is not surprising as previous reports have shown that the 3xP3 promoter can act as an enhancer detector (Horn *et al.*, 2000, 2003).

Three novel expression patterns were found among 45 transgenic lines systematically examined as adults and pupae. One such line (Pig-19) showed a striking muscle-specific pattern of expression in larvae, pupae and adults. This line has a single insertion, as evidenced by perfect cosegregation of the muscle- and eye-fluorescence phenotypes when heterozygotes are outcrossed (data not shown) and by Southern hybridization analysis (see below).

Remobilization of inserted element

We used the above-mentioned Pig-19 enhancer-trap line and the immobile helper, phspBac (Handler & Harrell, 1999), to determine whether an integrated *piggyBac* element could be remobilized after stable insertion into the *T. castaneum* genome. After injection of helper plasmid into eggs derived from this line, we screened offspring for loss of muscle expression with retention of eye expression. Such a pattern would be predicted for transposition to a different site in the genome concomitant with loss of the original enhancer-trap insertion.

Of 32 helper-injected Pig-19 animals that produced fluorescent-eyed progeny, nine gave rise to transformed lines that lacked muscle-specific EGFP expression. Such segregation of eye and muscle expression was never seen in outcrossed lines that had not been injected with helper. The remobilization rate therefore is 9/32 = 28%. The percentages of siblings harbouring such transpositions within individual G_1 families ranged from 1 to 21%.

Of the 32 helper-injected Pig-19 embryos mentioned above, 24 were heterozygous while eight were homozygous for the muscle-enhancer insertion. The nine remobilizations derived from heterozygotes and homozygotes with approximately equal frequency (7/24 = 29% and 2/8 = 25%, respectively). Importantly, the two remobilization events identified in the offspring of homozygous parents could not have been detected if the original insertion had been replicated during transposition. This indicates that *piggyBac* transposes via a cut-and-paste mechanism in *T. castaneum*.

To better characterize remobilization events, DNA was isolated from six of the nine remobilized lines and analysed by Southern hybridization analysis. The genomic DNA was digested with *Sal*I and hybridized to an *Nsi*I/*Hpa*I *piggyBac*-specific probe (Fig. 2). As the probe should hybridize only to the left insertion junction, the number of fragments visible on the autoradiogram is equal to the number of transposition

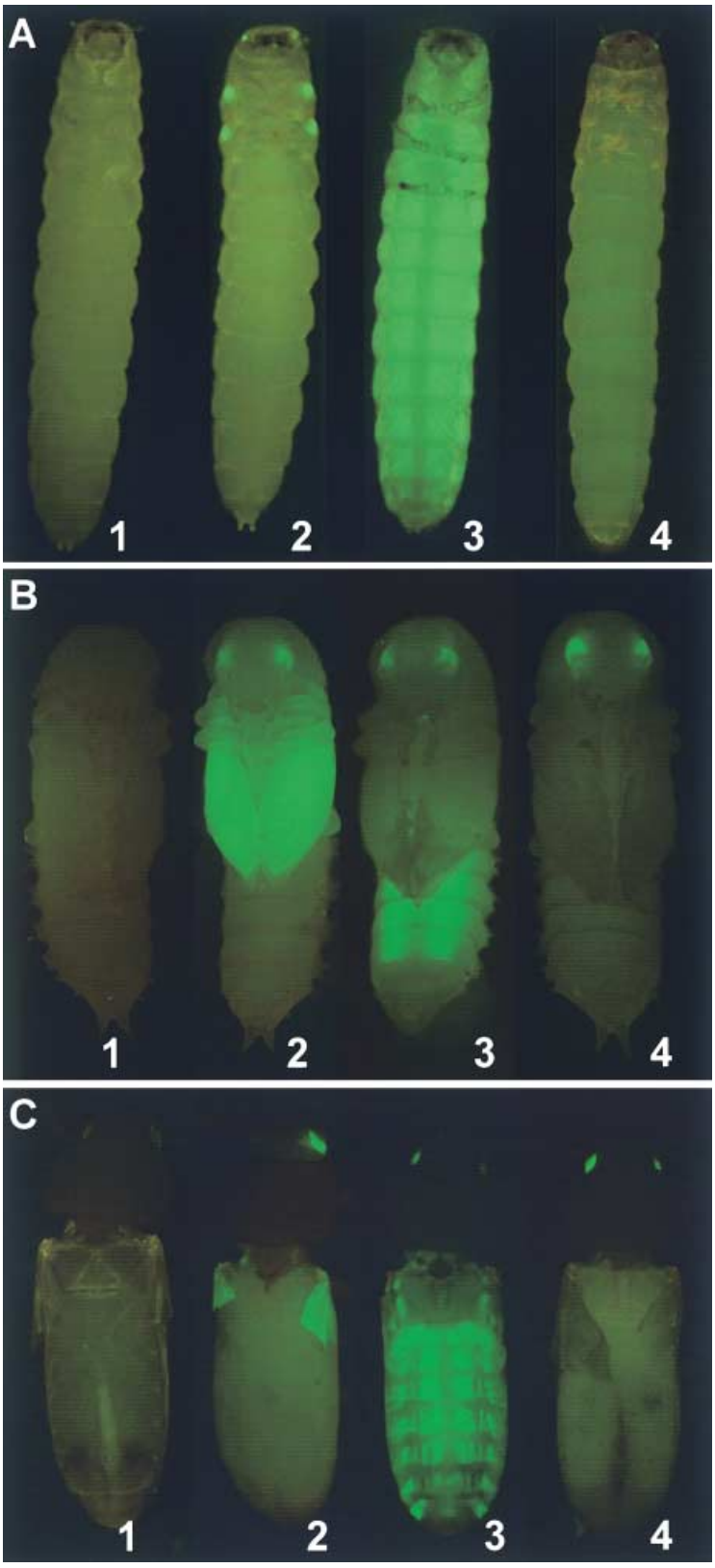

Figure 1. Expression patterns of the 3xP3-EGFP reporter in transgenic *T. castaneum* strains. Larvae (A), pupae (B) and adults (C) of nontransformed and transformed strains illustrating enhancer-trap expression patterns. Beetle strains shown include nontransformed *pearl* (1), enhancer-trap lines Pig-23 (2) and Pig-19 (3), and a typical non-enhancer-trap strain Rem-5 (4). Rem-5 was derived by remobilization of the Pig-19 insertion. To reveal adult abdomens, elytra were removed and wings were partially clipped (except in C3, where the wings and elytra were completely removed). Note the novel expression patterns in the Pig-23 line (larval imaginal wing and elytral discs, and pupal and adult wings and elytra, A2, B2 and C2) and in the Pig-19 line (intersegmental muscles, A3, B3 and C3). Note also the loss of muscle expression in the Rem-5 line.

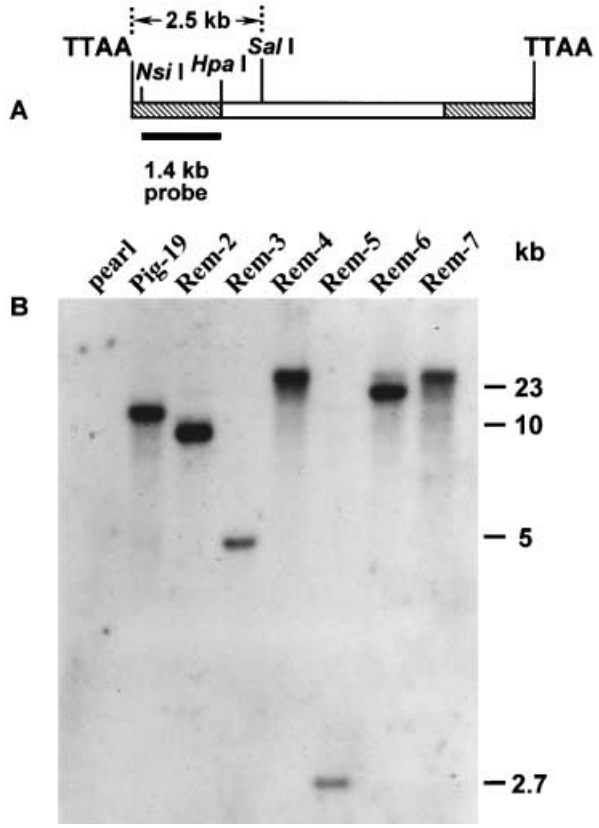

Figure 2. Southern hybridization analysis of remobilized Pig-19 insertions of *T. castaneum*. (A) Map (not to scale) of the pBac{3xP3-EGFPaf} vector showing the location of the *Nsi*I, *Hpa*I and *Sal*I restriction sites. The bar indicates the fragment used as hybridization probe. (B) Autoradiogram of Southern blot containing genomic DNA from various strains digested with *Sal*I and hybridized with the probe shown in (A). For each insert, only a single junction fragment (> 2.5 kb) should hybridize to the probe. Note the absence of native, *piggyBac*-related sequences in the nontransformed parental strain *p*, and the presence of single, unique insertions in the donor strain Pig-19 and in all six remobilized strains.

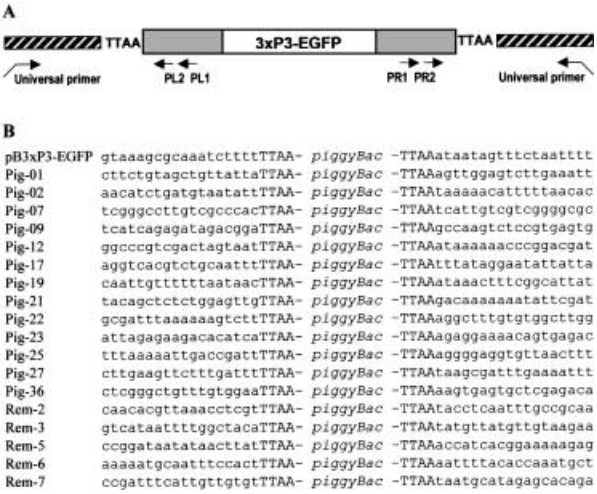

Figure 3. Isolation and sequence analysis of *piggyBac* insertion sites from transformed lines. (A) Schematic (not to scale) of the 3xP3-EGFP marked *piggyBac* element showing the primers used for universal PCR. PL1 and PL2 indicate nested *piggyBac*-specific primers complementary to a region near the left *piggyBac* terminus. PR1 and PR2 indicate nested *piggyBac*-specific primers complementary to a region near the right *piggyBac* terminus. Solid grey indicates the *piggyBac* element flanked by the duplicated TTAA insertion site, while white indicates the location of 3xP3-EGFP within the element. Hatched lines depict the chromosomal location of the insert site. (B) Sequence of the pB3xP3-EGFP-af plasmid vector, and insertion site sequences from 18 transformed lines. DNA sequences have been submitted to GenBank with the following accession numbers, AY225194-AY225214.

events. The results indicated unique fragment sizes for five of the six remobilized lines (Rem-4 and Rem-7 could not be distinguished), and confirmed that none of the remobilized lines carried the original Pig-19 insertion. Sequence data (see below) established that Rem-4 and Rem-7 also are unique insertions. The *piggyBac* probe did not hybridize to DNA isolated from nontransformed *pearl* (*p*) beetles, indicating that this strain is devoid of endogenous *piggyBac* elements. From these results we conclude that the *piggyBac* element was mobilized from its original position (the Pig-19 insertion site) and reinserted into various sites within the beetle genome.

Insertion junctions

To determine the degree of precision of *piggyBac* transposition in *T. castaneum* and to directly confirm insertion into *T. castaneum* chromosomes, we examined the sequences of the junctions between the inserted *piggyBac* element and the host *T. castaneum* genome. Forty-five independent transformants were outcrossed to *p* beetles, then inbred with EGFP selection for more than 10 generations. Insertion junctions for 18 of these were subsequently isolated by universal PCR (Fig. 3A) and the preinsertion target sequences determined (Fig. 3B). These included the Pig-19 muscle-enhancer line, six other primary insertion lines harbouring the construct A donor element, five lines derived by remobilization of the Pig-19 insertion, and six harbouring larger donor elements. Analysis of these sequences reveals that all insertions terminate correctly with normal *piggyBac* inverted repeats and that all are flanked by the duplicated *piggyBac* target sequence (TTAA). Sequence logo analysis (Schneider & Stephens, 1990) revealed no other conserved sequence motifs flanking the target sequence (data not shown). These results indicate that *piggyBac* inserts by a transposase-mediated mechanism, and suggest that the four-base target sequence is necessary and sufficient for integration in *T. castaneum*.

Transposon-tag mutagenesis is a potential application of this research. Thus, we wanted to assess whether any of the *piggyBac* insertions occurred in recognizable genes. BlastX analysis of 21 insertion sites revealed five significant matches to dipteran proteins (Table 2). This could be an underestimate of the gene disruptions represented in the sample, because BlastX would detect only highly

Table 2. *piggyBac* junctions and best BLASTX match

Insertion	Best match	Description	Organism	AA identity (%)	AA similarity (%)	BLASTX *e*-value
Pig-05 (L)	EAA05281	DISCO IP2 (Dip2)	*A. gambiae*	75 (46/61)	81 (50/61)	$4e^{-22}$
Pig-09 (F)	EAA13531	Putative reverse transcriptase	*A. gambiae*	43 (25/58)	63 (37/58)	$1e^{-06}$
Pig-12 (F)	EAA03266	Putative transposase	*A. gambiae*	47 (32/67)	67 (45/67)	$1e^{-10}$
Pig-30 (R)	AAG37332	NONA-like protein	*D. virilis*	65 (21/32)	90 (29/32)	$6e^{-08}$
Rem-6 (F)	AAM75050	Carboxyesterase	*D. melanogaster*	41 (36/86)	59 (51/86)	$5e^{-11}$

BLASTX analysis was performed against a non-redundant sequence database (GENBANK, nr) using the Blosum80 matrix with default values. L, genomic sequence flanking left piggyBac terminus; R, genomic sequence flanking right *piggyBac* terminus; F, genomic sequence flanking both *piggyBac* termini.

conserved domains, but would miss poorly conserved coding regions, untranslated regions, introns and regulatory regions. It has not been determined whether any of the five insertions are homozygous lethal.

Genetic mapping of insertions

To determine the distribution of insertion targets, and to differentiate between local hops and long-distance jumps in remobilized lines, empty (preinsertion) sites were genetically mapped by single-strand conformation polymorphism (SSCP) analysis. Map positions of 10 *piggyBac* inserts, including five primary and five remobilized, are shown in Table 3. The 10 appear to be randomly distributed over six of the 10 linkage groups. The Pig-19 insertion maps to position 1.7 on linkage group 3 (LG3), while the five remobilized insertions, Rem-2 and Rem-4–7, map to scattered locations on LGA, LGC and LG7. It is clear from these data that the remobilized Pig-19 insertion can reinsert at diverse locations throughout the genome.

The assignment of the Pig-01 insertion to LG3 was independently confirmed using an LG3 balancer chromosome, *3.2 Bamp* (R. W. Beeman, USDA-ARS-GMPRC, Manhattan, KS, USA, unpublished data). There were no recombinants among 135 progeny from a two-point linkage test between Pig-01-associated EGFP fluorescence and the *3.2 Bamp* balancer.

Table 3. Map positions of *piggyBac* insertions in *Tribolium castaneum*

Insertion	Donor element*	Linkage group	Map position	Origin
Pig-01	A	3	47.8	Primary
Pig-17	A	5	44.5	Primary
Pig-22	F	4	48.4	Primary
Pig-33	F	5	54.0	Primary
Pig-19	A	3	1.7	Primary
Rem-2	A	C	16.5	Pig-19
Rem-4	A	7	0.0	Pig-19
Rem-5	A	A	50.9	Pig-19
Rem-6	A	7	17.4	Pig-19
Rem-7	A	7	47.3	Pig-19

Map position refers to the distance in centiMorgans from position zero at one (arbitrary) end of the recombination map of each linkage group.
*A = transformation vector pBac{3xP3-EGFPaf} (Horn & Wimmer, 2000). *F* = GAL4 driver line (M. Klingler, Zoologisches Institut, Universität München, Germany, unpublished data).

Discussion

We have shown that the *piggyBac* element catalyses remarkably efficient transformation of the germline in *Tribolium castaneum*, that it tolerates large insertions, that this element shows potential as an enhancer-trap vector in this species, that primary insertions can be remobilized, and that insertions occur by a proper, transposase-mediated mechanism into random locations in the *T. castaneum* genome.

We demonstrated that secondary insertions, remobilized from the muscle-enhancer donor site on LG3, integrate at scattered locations on several non-homologous chromosomes. Southern hybridization analysis indicated that only a single insertion was present in each remobilized line. In particular, the remobilized lines appear to lack silent insertions into heterochromatin, which would not be detected by segregation analysis, but would interfere with efforts to clone genes after transposon tagging. These observations bode well for the use of *piggyBac* in transposon-tag mutagenesis. Efforts are underway to develop transgenic donor and helper lines that could be interbred to activate transposition, obviating the need for tedious embryo injections. In developing this strategy it will be important to identify promoters that ensure expression of transposase in the germline, but do not produce deleterious effects as a result of excessive transposition in somatic tissue. Endogenous *piggyBac*-related sequences have been detected in lepidoptera and diptera, but not in other taxa (Handler, 2002), and appear to be absent in *T. castaneum*. If this conclusion is borne out, there will be little reason to expect spontaneous or unregulated remobilization in transgenic beetle lines.

This report also provides evidence that *piggyBac* could be an effective enhancer-trap vector. We found three enhancer-trap phenotypes among approximately 45 transgenic lines using the EGFP reporter. This is probably a conservative estimate, as we would not have detected subtle or tightly regulated expression patterns or those not visible through the pigmented cuticle in whole adults. Also, embryonic and larval stages were not systematically examined. So far there is little indication that *piggyBac* insertions show consistent preference or nonpreference for sequences beyond the TTAA target. Interplasmid transposition assays

in *Drosophila melanogaster* embryos, and germline insertions in *D. melanogaster* chromosomes both suggest a bias for certain residues flanking the TTAA target (Li *et al.*, 2001), but inspection of our insertion junctions in *T. castaneum* reveals no such preference. Our evidence that *piggyBac* seems able to target coding regions as well as gene regulatory regions bolsters hope that this element will be useful for both transposon-tag mutagenesis and enhancer trapping.

Experimental procedures

T. castaneum *strains*

The white-eyed *T. castaneum* strain used in this work was homozygous for the recessive eye-colour mutation *pearl* (*p*) (Park, 1937). This strain lacks eye pigments, thereby improving the detectability of eye-specific fluorescence. For genetic mapping we used two near-homozygous inbred strains, namely GA-2 (Lorenzen *et al.*, 2000b) and the previously undescribed strain ab-in20. The latter is a highly inbred derivative (Scott Thomson, personal communication) of a strain originally collected near Bogotá, Colombia (Vasquez & del Castillo, 1985). For linkage analysis we used a third linkage group balancer chromosome *3.2 Bamp* (R. W. Beeman, USDA-ARS-GMPRC, Manhattan, KS, USA, unpublished data) and the visible mutations *aureate* (*au*) (Hoy *et al.*, 1966) and *aureate*^{14} (*au*^{14}) (J. V. Stuart, Purdue University, West Lafayette, IN, USA, unpublished data). Beetles were reared in yeast-fortified wheat flour under standard conditions (Beeman & Stuart, 1990).

Plasmids

The *piggyBac* helper plasmid, phspBac (Handler & Harrell, 1999), which encodes *piggyBac* transposase driven by hsp70, was used in combination with any of six donor plasmids carrying non-autonomous *piggyBac* elements ranging in size from 3.78 kb to 12.4 kb. The donor constructs are as follows. Construct A is transformation vector pBac{3xP3-EGFPaf} described by Horn & Wimmer (2000). Constructs B–F are derived from A by insertion of DNA segments of varying lengths. The only additional coding sequence present in these larger constructs derives from either lacZ or GAL4 (driven by various promoters), which are unlikely to affect the viability of transgenic lines. Differences in transformation efficiency are therefore most likely due to transposon size.

Microinjection

T. castaneum embryos were collected from *p* beetles within three hours of oviposition (24 °C). Embryos were washed with 2% bleach and thoroughly rinsed with water at room temperature. Embryos were injected (~5% egg volume) through the chorion with a mixture of helper (~400 ng/μl) and donor (~500 ng/μl) plasmid DNA in injection buffer (fourfold dilution of phenol red solution, Sigma cat. no. P0290). Injections were completed within six hours of egg collection. Following injection, G_0 embryos were held for two days in a humidified chamber at 32 °C. While humidity is crucial to early development, eggs must be dried prior to hatch. Therefore, two days after injection (approximately 1 day before hatch) embryos were allowed to dry. Hatchlings were placed on flour with 5% yeast. G_0 beetles surviving to adulthood were outcrossed to the *p* strain in single-pairs, and the G_1 progeny assayed for EGFP expression. In experiment A, however, only eight G_0 adults were processed in this manner, of which six gave rise to EGFP-expressing offspring. The remaining 138 fertile G_0 adults were self-crossed in single pairs. Of these 69 crosses, 55 gave rise to EGFP-positive offspring. The quotient 55/69 = 80% is an overestimation of the transformation rate, however, because both parents had a chance to produce transformed offspring. If the true transformation rate in this experiment was *p*, we expect that the proportion of crosses between injected animals not giving rise to EGFP-offspring would be $(1 - p)^2$. It follows for experiment A that $(1 - p)^2 = 14/69$. From this equation, *p* is calculated as 55%. Together with the eight outcrossed G_0 beetles, a combined value of 56% is obtained (Table 1).

Remobilization of an integrated element

Preblastoderm embryos were collected from Pig-19 beetles and injected with the phspBac transposase helper alone. Helper concentration was 600 ng/μl, or about 50% greater than that used during coinjections with donor plasmids. Injected embryos were handled as described above. G_0 adults were outcrossed to *p* beetles and G_1 progeny were assayed for loss of muscle-enhancer pattern with retention of eye-specific EGFP expression. The Pig-19 insertion had not been rendered homozygous at the time of this experiment, so not all the injected G_0 eggs carried the insertion. These were excluded from subsequent calculation of remobilization rate.

EGFP analysis

EGFP expression was observed using a Leica MZ FLIII fluorescence stereomicroscope (Leica Microsystems Inc.) equipped with a GFP Plus filter set (excitation filter: 480/40 nm, barrier filter: 510 nm). Photography was performed with a MagnaFire digital camera (Optronics).

Southern hybridization analysis

Genomic DNA was extracted (Lorenzen *et al.*, 2002b) from transgenic lines after approximately 15 generations of inbreeding the original G_1 lines. The DNA from transformed beetles or from non-transformed parental *p* beetles was digested with *Sal*I. Digested genomic DNA (~2 μg per lane) was separated on a 0.8% agarose gel by field inversion gel electrophoresis and transferred on to a GeneScreen membrane (NEN Life Sciences). A 1394 bp, ^{32}P-labelled *Nsi*I/*Hpa*I fragment consisting of the left *piggyBac* terminus was hybridized to the membrane overnight using standard procedures. Membranes were washed at 65 °C in 2× SSC, 0.1% SDS.

Insertion junction sequences

Genomic DNA (~8 ng) from transformed lines served as template for a first round of universal PCR using a universal primer and a *piggyBac*-specific primer. One microlitre of the primary PCR reaction was used as template for a second round of PCR with a nested *piggyBac*-specific primer and the appropriate linker primer. The *piggyBac*-specific primers for the left terminus were PL1, 5′-ATCAGTGACACTTACCGCATTGACA-3′ (first round); and PL2, 5′-TGCAAACAGCGACGGATTC-3′ (second round). Those for the right terminus were PR1, 5′-CGATAAAAACACATGCGTC-3′ and PR2, 5′-TACGCATGATTATCTTTAACGTA-3′. PCR conditions and a partial list of universal primers used are given in Beeman & Stauth (1997). Another group of universal primers contained various restriction endonuclease recognition sequences at the 3′ end, followed by eight fully degenerate bases and a 5′ linker with the

sequence CGTCAGCTTGATTAAGTCAACGATC (e.g. Pst-uni, 5′-CGTCAGCTTGATTAAGTCAACGATCNNNNNNNNCTGCAG-3′). Resulting PCR products were ligated into pCR4-TOPO (Invitrogen) and sequenced. The preinsertion junction region from the nontransformed host strain was amplified (via universal PCR) using a universal primer and insertion site-specific primers. Junctions were confirmed via PCR using template DNAs isolated from the appropriate transgenic lines.

Genetic mapping of insertions

We used single-strand conformational polymorphism analysis to identify dimorphisms between two highly inbred *T. castaneum* strains, GA-2 and ab-in20, using primer pairs specific for each preinsertion region. PCR products were analysed on Novex® Pre-cast 4–20% polyacrylamide TBE gels (Invitrogen). Dimorphic markers were scored, and recombination frequencies calculated. The insertion sites were mapped on to a whole-genome recombination map at an average resolution of approximately 1.5 cM, using a backcross family that consisted of 179 siblings, and a marker set totaling more than 400 unique DNA sequences derived from BACs, cDNAs and other sources. Details of this mapping project will be published elsewhere.

We used linkage analysis to confirm the map location of the Pig-01 insertion. Single-pair crosses were set up between Pig-01 males and *3.2 Bamp*/*au*^{14} females. *Bamp* male F_1 progeny were screened for EGFP expression, and *trans*-heterozygotes thus identified were outcrossed in single-pair crosses to *au* virgin females. Outcross progeny were sorted by phenotype and linkage calculated.

Sequence analysis

DNA templates were sequenced using an ABI 3700 DNA sequencer (Sequencing and Genotyping Facility, Plant Pathology, Kansas State University). Data analysis was performed using the Vector NTI® sequence analysis program (InforMax, Inc., Bethesda, MD, USA). Insertion-site sequences (GenBank accession numbers AY225194–AY225214) were analysed for similarity to known or predicted proteins by BlastX analysis (Altschul *et al.*, 1997) against a nonredundant sequence database (GenBank, nr) using the Blosum80 matrix (*e*-value cutoff = e^{-6}) with default values. In cases where the best BlastX match was to an unnamed protein, Psi-Blast analysis was performed.

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