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 G0 eggs surviving to the adult stage, transformation rates (percentages of fertile G1 beetles producing at least one G2 transformant)
Table 1. Efficiency of piggyBac-mediated germline transformation in T. castaneum

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

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 G0 crosses giving rise to EGFP-expressing offspring/total no. of fertile G0 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 G0 beetles giving rise to G1 transformants, the proportion of EGFP-expressing G0 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 transposed clusters, probably due to multiple transposon insertions in the offspring of transformed animals.

*Because G0 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 G0 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 G1 families) ranged from < 1–90% (Table 1). Segregation analysis of construct E transfectants (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 transfectants indicated 2–4 insertions per line (data not shown). However, because this set was derived from G0 self-crosses, this represents an overestimate of the actual number of independent insertions per G0.

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, phpSBac (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 G1 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 SalI and hybridized to an NsiI/HpaI 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.
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 TTAAT insertion site, while white indicates the location of the 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.
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 insertions are homozygous lethal.

**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

*Table 2. piggyBac junctions and best BLASTX match*

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Best match</th>
<th>Description</th>
<th>Organism</th>
<th>AA identity (%)</th>
<th>AA similarity (%)</th>
<th>BLASTX e-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig-05 (L)</td>
<td>EAA05281</td>
<td>DISCO IP2 (Dip2)</td>
<td>A. gambiae</td>
<td>75 (46/61)</td>
<td>81 (50/61)</td>
<td>4e-22</td>
</tr>
<tr>
<td>Pig-09 (F)</td>
<td>EAA13531</td>
<td>Putative reverse transcriptase</td>
<td>A. gambiae</td>
<td>43 (25/58)</td>
<td>63 (37/58)</td>
<td>1e-06</td>
</tr>
<tr>
<td>Pig-12 (F)</td>
<td>EAA03266</td>
<td>Putative transposase</td>
<td>A. gambiae</td>
<td>47 (32/67)</td>
<td>67 (45/67)</td>
<td>1e-10</td>
</tr>
<tr>
<td>Pig-30 (R)</td>
<td>AAG37332</td>
<td>NONA-like protein</td>
<td>D. virilis</td>
<td>65 (21/32)</td>
<td>90 (29/32)</td>
<td>6e-08</td>
</tr>
<tr>
<td>Rem-6 (F)</td>
<td>AM75050</td>
<td>Carboxyesterase</td>
<td>D. melanogaster</td>
<td>41 (36/86)</td>
<td>59 (51/86)</td>
<td>5e-11</td>
</tr>
</tbody>
</table>

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.

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

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

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).
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 3B *Bamp* (R. W. Beeman, USDA-ARS-GMPRC, Manhattan, KS, USA, unpublished data) and the visible mutations *au14* (Hoy *et al.*, 2001) and *au3* (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[7xP3-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. P02920). Injections were completed within six hours of egg collection. Following injection, *G0* 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. *G0* beetles surviving to adulthood were outcrossed to the piggyBac 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. *G1* adults were outcrossed to *p* beetles and *G2* 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 *G0* eggs carried the insertion. These were excluded from subsequent calculation of remobilization rate.

**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. *G0* adults were outcrossed to *p* beetles and *G1* 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 *G0* eggs carried the insertion. These were excluded from subsequent calculation of remobilization rate.

**EGFP analysis**

EGFP expression was observed using a Leica M2 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 *G0* 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, 32P-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′-ATCAGTGACACTTGGCATTGACA-3′ (first round); and *PL2*, 5′-TGCAAACAGCGACGGATTC-3′ (second round). Those for the right terminus were *PR1*, 5′-CGATAAAAACACATGCGTC-3′ and *PR2*, 5′-TACGATGATTTTATACGTA-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 CGTCAGCTTGAATTACGATC (e.g. Pst-un1, 5'-CGTCAGCTTGAATTACGATC-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 Nover® 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 recombinant BAC library (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, S.F., Madden, T.L., Schaeffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402).

**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^-2) with default values. In cases where the best BLASTX match was to an unnamed protein, PSI-BLAST analysis was performed.

**Acknowledgements**

We thank E. Wimmer for discussion of transgenic strategies and M. Fraser, A. Handler and E. W. for the gift of plasmids. This work was supported by grants from the National Science Foundation (MCB-9630179), the National Institutes of Health (R01-HD29594), the Human Frontier Science Program Organization, the Deutsche Forschungsgemeinschaft and was further supported by the Agricultural Research Service. This article is contribution no. 03–194-J from the Kansas Agricultural Experiment Station. All programs and services of the U.S. Department of Agriculture are offered on a nondiscriminatory basis, without regard to race, colour, national origin, religion, sex, age, marital status or handicap.

**References**


