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Efficient Transformation of the Beetle *Tribolium castaneum* Using the *Minos* Transposable Element: Quantitative and Qualitative Analysis of Genomic Integration Events

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ABSTRACT

Genetic transformation in insects holds great promise as a tool for genetic manipulation in species of particular scientific, economic, or medical interest. A number of transposable elements have been tested recently as potential vectors for transformation in a range of insects. *Minos* is one of the most promising elements because it appears to be active in diverse species and has the capacity to carry large inserts. We report here the use of the *Minos* element as a transformation vector in the red flour beetle *Tribolium castaneum* (Coleoptera), an important species for comparative developmental and pest management studies. Transgenic G_1 beetles were recovered from 32.4% of fertile G_0's injected with a plasmid carrying a *3xP3-EGFP*-marked transposon and *in vitro* synthesized mRNA encoding the *Minos* transposase. This transformation efficiency is 2.8-fold higher than that observed when using a plasmid helper. Molecular and genetic analyses show that several independent insertions can be recovered from a single injected parent, but that the majority of transformed individuals carry single *Minos* insertions. These results establish *Minos* as one of the most efficient vectors for genetic transformation in insects. In combination with *piggyBac*-based transgenesis, our work allows the introduction of sophisticated multicomponent genetic tools in Tribolium.

THE use of transposable elements for genetic transformation in insects has attracted wide interest as a valuable tool for developmental studies, for biotechnological purposes, and for designing strategies to control important disease vectors and agricultural pests (Adams and Sekelsky 2002; Ito *et al.* 2002; Tomita *et al.* 2003). In 1982, the landmark transformation of *Drosophila melanogaster* using the *P* element (Rubin and Spradling 1982) raised the false expectation that genetic manipulation of other insects was close at hand. However, the *P* element proved to be inactive in non-drosophilid insects (Handler *et al.* 1993), and it took more than a decade to realize that other insect transposons have a broader host range. Using such transposons, it has been possible during the last 8 years to expand transgenic technology to several other insect taxa. Representatives of four insect orders (Diptera, Lepidoptera, Coleoptera, and Hymenoptera) have been genetically transformed by a handful of type II transposable elements: *Hermes*, *mariner*, *piggyBac*, and *Minos* (reviewed in Atkinson *et al.* 2001; Handler 2001; see also Sumitani *et al.* 2003).

Minos was originally identified in *D. hydei* and belongs to the *Tc1/mariner* superfamily of transposable elements (Franz and Savakis 1991). It is 1.8 kb long, with two 255-bp inverted terminal repeats flanking a two-exon transposase gene (Franz *et al.* 1994). Recombinant purified transposases encoded by members of this superfamily are able to catalyze transposition *in vitro* (reviewed in Plasterk *et al.* 1999). This independence from species-specific factors has been proposed to account for their widespread occurrence among metazoa and for their usefulness as DNA delivery vectors (Vos *et al.* 1996). In agreement with this, *Minos*-based vectors have been used for the genetic transformation of the dipteran species *D. melanogaster* (Loukeris *et al.* 1995a), *Ceratitis capitata* (Loukeris *et al.* 1995b), and *Anopheles stephensi* (Catteruccia *et al.* 2000) and most recently for the germline transformation of the ascidian *Ciona intestinalis* (Sasakura *et al.* 2003). Furthermore, *Minos* activity has been demonstrated in divergent groups of insects (Shimizu *et al.* 2000; Zhang *et al.* 2002) and in mammalian tissues and cell lines (Klinakis *et al.* 2000b; Zagoraiou *et al.* 2001; Drabek *et al.* 2003). In this article we report the transformation of the red flour beetle *Tribolium castaneum* using the *Minos* element as a vector.

The spectrum of insect species amenable to transformation has also been significantly broadened by the development of new marker genes that allow the straightforward identification of transgenic animals. Earlier selection systems were based on the rescue of mutants affecting eye pigmentation (reviewed in Ashburner *et al.* 1998). These genetic markers were easy to score, but required a serious investment of time when applied to new species,

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since an eye-color gene had to be molecularly characterized and a corresponding loss-of-function mutant isolated each time. This laborious procedure is circumvented by the use of fluorescent proteins as dominant markers, which enable scoring of transgenic progeny in wild-type backgrounds (Tsien 1998). Until recently, however, the use of these markers was restricted by the lack of functional regulatory sequences able to drive their expression in diverse species. A breakthrough toward general transformation markers active in multiple species was achieved by the utilization of artificial promoters responsive to well-conserved transcription factors. An artificial Pax6-dependent promoter, known as *3xP3* (Sheng *et al.* 1997), combined with an enhanced version of the green fluorescent protein (*EGFP*), was first used successfully in Tribolium and Drosophila (Berghammer *et al.* 1999b). This marker has subsequently allowed the rapid identification of transgenic individuals, at various developmental stages, in at least three different orders of insects (reviewed in Horn *et al.* 2002).

A further level of improvement can be achieved by engineering new sources of transposase that are not dependent on the existence of characterized active promoters in the particular species of interest. During the transformation procedure, eggs are usually injected with two plasmids, one carrying the marked transposon vector (referred to as the donor) and the other expressing the transposase (referred to as the helper). The transposase is usually under the control of a Drosophila promoter (*e.g.*, *hsp70*). However, these promoters may not be sufficiently active in all species (Zhang *et al.* 2002). Substitution of the helper plasmid by *in vitro* synthesized capped mRNA alleviates the need for testing already established promoters or characterizing new ones to drive expression of the transposase gene in the targeted species. In the case of *Minos*-mediated transgenesis, this "ready-to-use" transposase has been shown to increase transformation rates significantly in both *D. melanogaster* and *C. capitata* (Kapetanaki *et al.* 2002). Here we show that this improvement is more generally applicable and extends to *T. castaneum*.

We report here an effective method for the stable genetic transformation of the red flour beetle *T. castaneum*, using the *Minos* transposable element. We present a molecular analysis of *Minos* integration events into the host genome and provide quantitative measures for several parameters that determine the usefulness of this element as an effective tool for genetic manipulation in Tribolium. Given the increasing attention that Tribolium receives as an experimental organism for developmental studies, evolutionary comparisons, and pest control, the availability of a second highly active transformation vector, in addition to *piggyBac* (Berghammer *et al.* 1999b; Lorenzen *et al.* 2003), is an important step toward the introduction of sophisticated genetic techniques (insertional mutagenesis, enhancer trapping, and gene trapping) in this organism.

MATERIALS AND METHODS

***T. castaneum* rearing and micro-injections:** A nonisogenized strain of *T. castaneum*, homozygous for the recessive eye-color mutation *pearl* (*p*; Lorenzen *et al.* 2002), was used throughout these experiments. The lack of eye pigments in this strain facilitates the detection of fluorescence in the eyes of transformed individuals.

Beetles were reared under standard laboratory conditions and processed as previously described (Berghammer *et al.* 1999a; Lorenzen *et al.* 2003; and http://www.zi.biologie.uni-muenchen.de/science/tribolium/klingler/frames.html). For injections, beetles were allowed to lay eggs for 3 hr at 24°. Eggs were treated with 0.2% bleach for 1 min; transferred to coverslips; and injected with a mixture containing 500 ng/μl of the donor plasmid pMi{3xP3-EGFP}, 375 ng/μl of the helper plasmid pHSS6hsILMi20 or 375 ng/μl of transposase-capped mRNA, and 0.05% of the inert dye phenol red (Sigma, St. Louis) in water. Coverslips with injected embryos were then transferred to apple juice agar plates at 33°, within sealed plastic containers, to avoid desiccation. Male and female survivors (G_0's) were backcrossed individually to three female or two male *pearl* beetles, respectively. A detailed protocol is available on request.

Plasmids: Throughout these experiments we used the pMi{3xP3-EGFP} donor plasmid (kindly provided by A. Klinakis, A. Babaratsas, and C. Savakis), containing a *Minos* transposon with the *EGFP* coding sequence under control of the *3xP3* promoter (Horn and Wimmer 2000; Figure 1C). The *3xP3-EGFP* gene cassette produces green fluorescence in the eyes of individuals carrying this construct (Berghammer *et al.* 1999b).

The helper plasmid pHSS6hsILMi20, containing the *Minos* transposase-coding sequence under the control of the Drosophila *hsp70* promoter, has been described previously (Klinakis *et al.* 2000a). The plasmid pBlueSKMimRNA, used for the *in vitro* synthesis of *Minos* transposase mRNA, is a derivative of pNB40ILTMi (Kapetanaki *et al.* 2002). A *Pst*I(blunt)/*Not*I fragment of pNB40ILTMi, containing the *Minos* transposase transcription unit, was cloned into *Kpn*I(blunt)/*Not*I-cut pBlue-ScriptSK II+ (Stratagene, La Jolla, CA), placing the *Minos* transposase under the T7 promoter.

***In vitro* synthesis of *Minos* transposase mRNA:** Capped *Minos* transposase mRNA was prepared from the pBlueSKMimRNA vector (linearized with *Not*I), using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). The transcription reaction was carried out using T7 RNA polymerase, according to the manufacturer's instructions, followed by phenol-chloroform extraction and isopropanol precipitation of the mRNA. Small aliquots of the mRNA were stored in isopropanol at −20°. Before micro-injection, the mRNA was precipitated, washed with ethanol, resuspended in water, and quantitated using a spectrophotometer.

Southern blot analysis and inverse PCR: Genomic DNA was prepared from pools of adult beetles using the Puregene DNA isolation kit (Gentra Systems, Research Triangle Park, NC). About 2 μg of genomic DNA was digested with either *Pvu*II or *Alu*I, size separated by agarose gel electrophoresis, and blotted onto PROTRAN nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Radiolabeled probes and hybridizations were carried out using standard techniques (Sambrook *et al.* 1989). Inverse PCR was carried out with *Alu*I-digested genomic DNA and *Minos*-specific primers, as described previously (Klinakis *et al.* 2000b). Amplified DNA fragments were cycle sequenced and subjected to BLAST analysis in the EMBL/GenBank databases.

Epifluorescence microscopy: EGFP fluorescence was observed using a Leica MZ12 fluorescence stereomicroscope, equipped with Plan apo 1.6× objective, a 100-W Hg lamp,

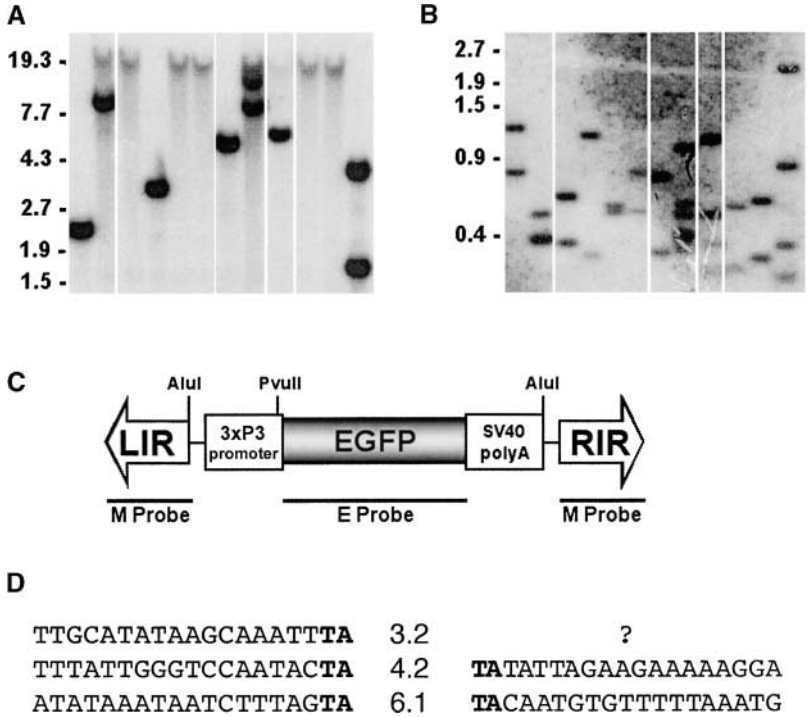

FIGURE 1.—Molecular analysis of *Minos* insertions. (A) Southern analysis of *Pvu*II-digested genomic DNA probed with sequences corresponding to the *EGFP* coding region (E probe). Each lane represents genomic DNA isolated from a pool of 12 transgenic beetles that derive from a single transformed parent. A single band is expected per *Minos* insertion. (B) Southern analysis of *Alu*I-digested genomic DNA from the same pools of beetles as in A, probed with sequences corresponding to the *Minos* inverted repeats (M probe). Two bands per *Minos* insertion are expected. (C) Map of the *Mi{3xP3-EGFP}* transposon construct used in this work (not to scale). LIR and RIR correspond to the left and right inverted repeats of *Minos*, respectively. The *EGFP* coding sequence is placed under the control of an artificial promoter containing three Pax-6 binding sites and the Drosophila *hsp70* basal promoter. (D) Flanking sequences of three independent Minos insertions in the Tribolium genome (from RNA lines 3.2, 4.2, and 6.1), recovered by inverse PCR. The characteristic duplicated TA dinucleotide (in boldface type) is found on either side of the integration site.

and a GFP filter set (excitation filter 480/40 nm, emission filter 510 nm). Photography was performed using a Wild MPS 51S camera or a ProgRes C14 digital camera.

RESULTS

High-efficiency of transformation using DNA or mRNA helpers: The ability of the *Minos* element to transpose into the germline of *T. castaneum* was tested by co-injecting a plasmid carrying the *Mi{3xP3-EGFP}* transposon (Figure 1C) with either of two sources of *Minos* transposase: a helper plasmid or capped mRNA encoding the *Minos* transposase (see MATERIALS AND METHODS). Approximately 600 preblastoderm embryos of a white-eyed *pearl* strain were injected in each case. The vast majority of surviving G_0 individuals were fertile and their offspring (G_1's) were scored for fluorescence in their eyes to identify transgenic animals.

Among the 167 fertile G_0's injected with the helper plasmid, 19 produced transgenic offspring ("DNA lines"), while 67 of 207 fertile G_0's injected with the helper mRNA produced transgenic offspring ("RNA lines"). Thus, the deduced transformation efficiencies are 11.4% for the DNA helper and 32.4% for the RNA helper (Table 1). Chi-square analysis shows that this 2.8-fold difference in transformation rates is highly significant ($P < 0.001$). No difference in transformation efficiency was observed between male and female G_0's.

Transposition of *Minos* into the host genome: Integration of the *Minos* element into the host genome was confirmed by Southern hybridization. Genomic DNA was prepared from pools, each composed of ~12 fluorescent sibling G_1 beetles (derived from the same injected G_0). Twelve such samples were prepared from G_0's injected with the helper plasmid and 24 from G_0's injected with helper mRNA. Each pool was tested for the number of integrated copies of *Minos*, using two different restriction enzyme/probe combinations. *Pvu*II-digested DNA was probed for the EGFP coding sequence (E probe), resulting in one band per insertion (Figure 1, A and C), while *Alu*I-digested DNA was probed for the *Minos* inverted repeats (M probe), resulting in two bands per insertion (Figure 1, B and C). The M probe used did not hybridize to genomic DNA from untransformed *pearl* beetles (nor do specific primers for the *Minos* terminal repeats amplify any product), suggesting that no *Minos*-related transposable elements were already present in the coleopteran genome.

Sixty-three insertions were detected in total among the transformed DNA and RNA lines subjected to Southern analysis (Tables 2 and 3). All detected insertions are characterized by bands >1.45 kb with the E probe and bands >0.26 and 0.38 kb with the M probe (these are the minimum sizes expected for integral copies of the *Mi{3xP3-EGFP}* element), suggesting that these represent insertions of the entire *Mi{3xP3-EGFP}* element into the host genome.

Insertions carrying sequences of the donor plasmid (external to the transposon) are expected to produce a 1.58-kb band with the E probe and 0.27- and 0.4-kb bands with the M probe, due to the presence of additional *Pvu*II and *Alu*I sites in the donor plasmid. These fragment sizes were detected on just three occasions, exclusively in lines carrying multiple *Minos* insertions (Tables 2 and 3). The presence of external sequences

TABLE 1

Summary of transformation experiments

Donor	Helper	No. of embryos injected	No. of surviving G_0's	No. of fertile G_0's	No. of transformants producing G_0's	% transformation efficiency[a]
pMi{3xP3-EGFP}	DNA	~600	171	167	19	11.4
pMi{3xP3-EGFP}	RNA	~600	210	207	67	32.4

[a] Percentage of fertile G_0's producing transformed G_1 progeny.

was confirmed by probing with the plasmid backbone of pMi{3xP3-EGFP} (data not shown). The vast majority of insertions did not show these bands and are thus thought to derive from genuine transposition events of the *Minos* element into the Tribolium genome.

To confirm this, we carried out inverse PCR and sequenced the DNA flanking the *Minos* element in three independent RNA lines. Like other members of the *Tc1/mariner* superfamily, *Minos* is known to insert in a TA dinucleotide, which is duplicated upon insertion (Arca *et al.* 1997). In the three lines that we sequenced, the inverted terminal repeats of the *Mi{3xP3-EGFP}* element were flanked by this characteristic TA dinucleotide, followed by sequences that were unrelated to those of the donor plasmid (Figure 1D). Database searches revealed that in one of these lines (line 4.2), the *Minos* element was inserted within a previously identified 360-bp satellite DNA element present in the Tribolium genome (Ugarkovic *et al.* 1996).

Number of transformed progeny per G_0 (cluster size): The percentage of fluorescent progeny (transformed G_1's) obtained per G_0, referred to as cluster size, was determined for beetles injected with the DNA and RNA helpers, respectively (Figure 2). The distributions of these values are broad (Figure 2), with the mean cluster size per transformant-producing G_0 being 10.3% for the DNA helper (ranging between 1 and 35%) and 15.2% for the RNA helper (ranging between 1 and 96%). Thus,

TABLE 2

DNA lines subjected to Southern and segregation analysis

G_0 cross	Gender	Fluorescent-eyed G_1 progeny	Total G_1 progeny	Cluster size[a] (%)	G_0 insertions[b]	G_1 cross[c]	Segregation analysis[d]	G_1 insertions[e]	Nature of insertions[f]
1	M	33	228	14.47	1	1.1	1		
2	M	5	352	1.42	1	2.1	1		
3	M	23	344	6.69	1	3.1	1		
4	M	60	297	20.20	2	4.1	1		
5	M	13	297	4.38	1	5.1	1		
6	M	13	283	4.59	1	6.1	1		
						6.2	1		
7	M	39	310	12.58	1	7.1	1		
8	M	118	337	35.01	3	8.1	2	3	Two linked,
						8.2	1	2	plasmid sequence
9	F	15	231	6.49	1	9.1	1		
10	F	78	283	27.56	3	10.1	3	3	
						10.2	1		
						10.3	1	1	
11	F	8	213	3.76	1	11.1	1		
12	F	20	184	10.87	1	12.1	1		

[a] Cluster size is the percentage of transformed G_1's produced by individual G_0's backcrossed to *pearl* beetles.

[b] Number of *Minos* insertions in the germline of each G_0, as inferred from transformed G_1 progeny subjected to Southern analysis.

[c] Fluorescent G_1 siblings backcrossed individually to *pearl* beetles.

[d] Number of *Minos* insertions in the germline of each G_1, as inferred from the segregation ratios of the *3xP3-EGFP* marker in the G_2 generation.

[e] Number of *Minos* insertions in the germline of each G_1, as inferred from transformed G_2 progeny subjected to Southern analysis.

[f] The majority of integration events are single unlinked *Minos* insertions. Only lines displaying either linked insertions (deduced from a difference between the number of actual and independently segregating insertions) or insertions carrying plasmid sequences (deduced by Southern analysis) are shown.

TABLE 3

RNA lines subjected to Southern and segregation analysis

G_0 cross	Gender	Fluorescent-eyed G_1 progeny	Total G_1 progeny	Cluster size[a] (%)	G_0 insertions[b]	G_1 cross[c]	Segregation analysis[d]	G_1 insertions[e]	Nature of insertions[f]
1	M	93	311	29.90	1	1.1	1	1	
						1.2	1	1	
2	M	74	249	29.72	2	2.1	2	2	
						2.2	1	1	
3	M	33	273	12.09	1	3.1	1	1	
						3.2	1	1	
4	M	69	322	21.43	2	4.1	1	1	
						4.2	1	1	
5	M	62	204	30.39	2	5.1	1	1	
6	M	108	264	40.91	1	6.1	1	1	
						6.2	1	1	
7	M	261	272	95.96	≥4	7.1	3	4	Two linked
						7.2	1	2	
						7.3	2	3	
						7.4	1	1	
8	M	12	222	5.41	2	8.1	2	2	
						8.2	1	1	
9	M	14	175	8.00	2	9.1	1	1	
10	M	89	191	46.60	≥3	10.1	1	1	Two linked,
						10.2	1	2	plasmid sequence
11	M	6	330	1.82	1				
12	F	14	167	8.38	2				
13	F	11	256	4.30	2				
14	F	44	190	23.16	1				
15	F	44	197	22.34	2				
16	F	66	207	31.88	2				
17	F	11	267	4.12	1				
18	M	9	283	3.18	3				
19	M	103	292	35.27	2				
20	F	66	207	31.88	3				Plasmid sequence
21	F	11	268	4.10	1				
22	F	64	157	40.76	2				
23	M	8	316	2.53	3				
24	M	5	334	1.50	1				

[a] Cluster size is the percentage of transformed G_1's produced by individual G_0's backcrossed to *pearl* beetles.

[b] Number of *Minos* insertions in the germline of each G_0, as inferred from transformed G_1 progeny subjected to Southern analysis.

[c] Fluorescent G_1 siblings backcrossed individually to *pearl* beetles.

[d] Number of *Minos* insertions in the germline of each G_1, as inferred from the segregation ratios of the *3xP3-EGFP* marker in the G_2 generation.

[e] Number of *Minos* insertions in the germline of each G_1, as inferred from transformed G_2 progeny subjected to Southern analysis.

[f] The majority of integration events are single unlinked *Minos* insertions. Only lines displaying either linked insertions (deduced from a difference between the number of actual and independently segregating insertions) or insertions carrying plasmid sequences (deduced by Southern analysis) are shown.

while the RNA helper almost triples the transformation frequency (see above), cluster size increases by only ~50%. Consequently, the number of G_1 progeny that need to be screened per G_0 to detect a transformant is similar for RNA and DNA helpers.

Number of independent insertions obtained per G_0: We used Southern analysis in pools of 12 transgenic G_1 progeny per G_0 (described above) to measure the number of independent *Minos* insertions that can be obtained from the germline of a single injected G_0; *i.e.*, sampling 12 transformed gametes from each germline. Pilot experiments indicated that the hybridization conditions used were sensitive enough to detect insertion(s) present even in only 1 of the 12 beetles constituting each sample (data not shown).

Among the 12 DNA lines subjected to this analysis, 9 lines carried a single insertion, 1 carried two insertions, and 2 carried three insertions (Table 2). Among the 24

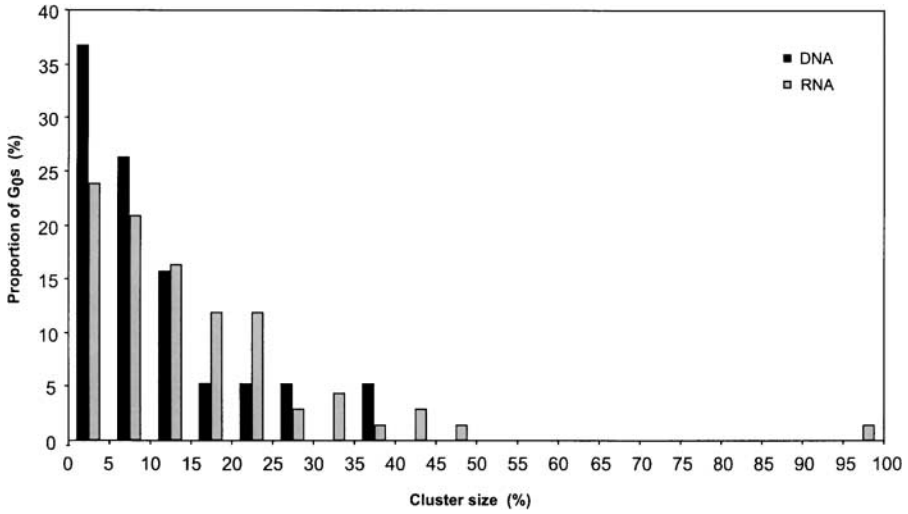

FIGURE 2.—Distribution of cluster sizes (percentage of transformed G_1's obtained per G_0) in DNA *vs.* RNA lines. The number of G_0's with a given cluster size is expressed as a fraction (%) of the total number of transformant-producing G_0's. DNA and RNA lines produced similar numbers of offspring.

RNA lines that were analyzed, 8 carried a single insertion, 11 carried two insertions, and 5 carried three or more insertions (Table 3). It is evident that the RNA helper is capable not only of transforming a higher proportion of injected animals, but also of generating a larger number of independent insertions per germline, in comparison to the DNA helper (multiple insertions in 67% *vs.* 25% of the lines tested).

To address whether there is a direct relation between the number of insertions per G_0 germline and the proportion of transformed G_1 progeny produced per G_0 (cluster size), we examined whether there is a correlation between these values for individual DNA and RNA lines. The correlation coefficient is high for the DNA lines (Figure 3A, $r = 0.92$), supporting the expectation that these values should be directly related to each other and to the level of activity of the transposase in individual injected G_0's. The correlation coefficient for the RNA lines, however, was found to be significantly lower (Figure 3B, $r = 0.50$). This is illustrated by RNA-injected G_0's carrying multiple insertions but giving rise to <5% transformed progeny and, conversely, by G_0's carrying a single insertion but giving rise to >20% transformed progeny. The latter may be explained if the RNA helper can drive transposition at earlier stages, giving rise to larger clones of germ cells marked by the same insertion.

Most transformed (G_1) individuals carry single *Minos* insertions: We have shown that individual G_0 beetles can carry multiple independent insertions of a *Minos* element in their germline. Depending on whether these insertions have hit the same or independent germ cells, these can be recovered as multiple insertions in a single G_1 or as single insertions in several different G_1 progeny. To resolve this, we performed Southern analysis in pools of 12 G_2 transformants produced by individual G_1 beetles (backcrossed to *pearl* beetles), to deduce the number of insertions present in the genome of these G_1's. We also used the segregation ratio of transformed to non-transformed progeny of individual G_1 beetles, to estimate the number of unlinked insertions present in these G_1's. In most cases, the number of insertions determined by Southern hybridization was consistent with that deduced from segregation ratios, with three exceptions, which presumably correspond to linked insertions (Tables 2 and 3).

As expected, for G_0's giving rise to single inserts, all G_1's were found to contain the same single insertion of the transposon. However, also in the case of G_0's giving rise to multiple insertions, a significant proportion of their G_1 offspring were found to each carry only a single copy of the transposon (Tables 2 and 3), suggesting that in many cases different germ cells had been targeted in one G_0 animal. Overall, the majority of transformed G_1's are inferred to carry single insertions of the transposon.

Eye fluorescence phenotypes: It is well known that the expression of transformation markers can be influenced by the particular locus where a mobile element is inserted (LOUKERIS *et al.* 1995a; HORN *et al.* 2000). In our experiments, significant differences were observed in the expression of the *3xP3-EGFP* marker among different lines of transformants, ranging from strong expression in the entire eye to weak expression in a small number of ommatidia. A significant number of DNA and RNA lines (about one-quarter of the lines examined) produced G_1's with distinct eye fluorescence phenotypes. The majority of these lines contained different *Minos* insertions. Similarly, distinct eye phenotypes were observed segregating in the progeny of G_1's carrying more than one *Minos* insertion. This behavior is similar to that observed with other transformation markers (*e.g.*, *white* in Drosophila) and may help to distinguish individuals that carry different *Minos* insertions.

Enhancer trapping by *Mi{3xP3-EGFP}*: The *3xP3-EGFP* marker used in these experiments is regulated by a minimal promoter and a number of upstream Pax-6 binding sites that activate expression specifically in the eyes and in parts of the central nervous system in

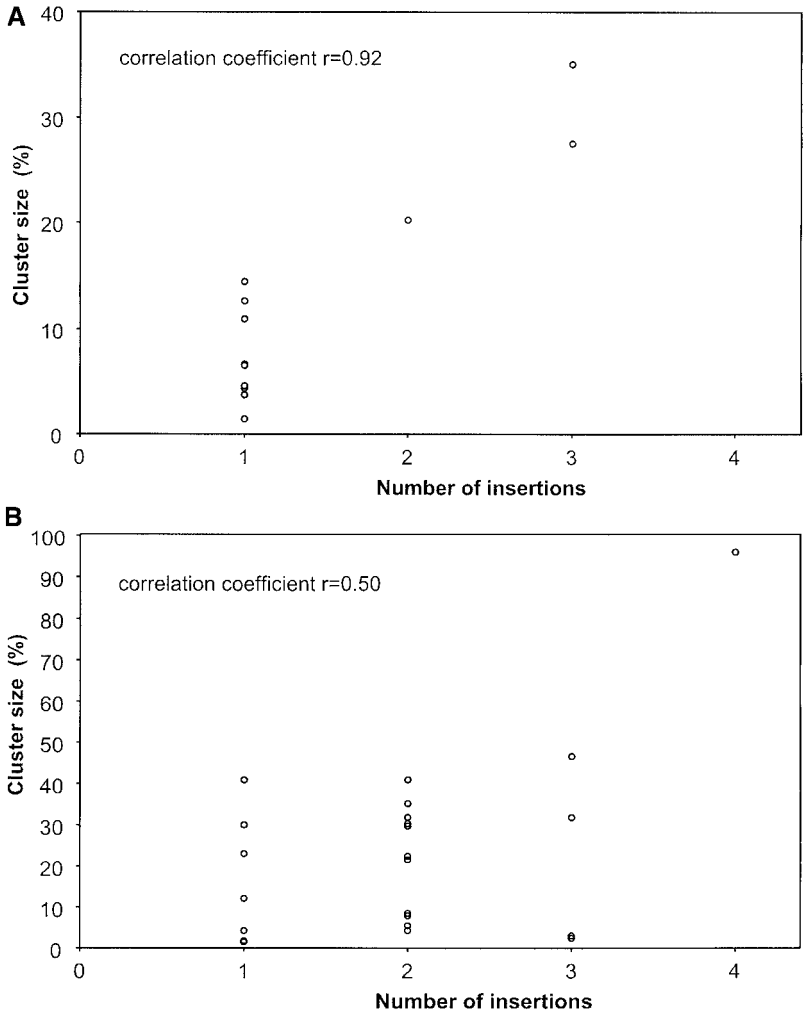

FIGURE 3.—Correlation between the number of insertions recovered per G_0 germline and the proportion of transformed G_1 progeny produced per G_0 (cluster size). (A) Plot of cluster size *vs.* number of insertions for DNA lines, showing a high positive correlation between these values ($r = 0.92$). (B) Plot of cluster size *vs.* number of insertions for RNA lines, showing a lower correlation between these values ($r = 0.50$). Note difference in scale in the *y*-axis for DNA *vs.* RNA lines.

Tribolium (BERGHAMMER *et al.* 1999b; LORENZEN *et al.* 2003). Depending on the site of integration of the transposon in the host genome, it is conceivable that *EGFP* expression could also be activated by nearby enhancers, an effect that is widely known as "enhancer trapping" (O'KANE and GEHRING 1987; BELLEN *et al.* 1989; BIER *et al.* 1989). Enhancer trapping has been reported recently, using the *piggyBac* element in Drosophila and Tribolium (HORN *et al.* 2003; LORENZEN *et al.* 2003). To determine whether this occurs at an appreciable frequency with the *Mi{3xP3-EGFP}* element, we looked at the patterns of EGFP fluorescence in larvae, pupae, and adults of all 86 transformed lines. Enhancer traps were identified as novel EGFP expression patterns in at least 10 lines (Figure 4). This suggests that enhancer trap screens could be carried out in Tribolium using *Minos*-based vectors.

DISCUSSION

High-efficiency transformation of Tribolium using *Minos*: We have tested the ability of the *Minos* element to transpose in the beetle *T. castaneum* and examined a number of quantitative and qualitative parameters that bear on its utility as a tool for genetic manipulations in this species.

First, we determined the frequency at which the surviving G_0 (injected) beetles give rise to transformed progeny. Transformation frequencies were 11.4% using plasmid DNA as a helper and 32.4% using mRNA as helper. These frequencies are sufficiently high for routine transgenic experiments and, in the case of the RNA helper, the transformation rate is among the highest reported in insects (ATKINSON *et al.* 2001; HANDLER 2001). Equally impressive transformation rates in Tribolium have been achieved using *piggyBac*-based vectors as well (BERGHAMMER *et al.* 1999b; LORENZEN *et al.* 2003).

The second parameter we examined is the frequency at which transformed G_1 progeny are recovered from transformant-producing G_0's, the so-called cluster size. This gives an estimate of the number of G_1 progeny that need to be screened per G_0 to recover a transformation event. Cluster sizes have a broad distribution (Figure 2) with mean values of 10.3% for the DNA helper and 15.2% for the RNA helper. Using the RNA helper, it seems possible to recover ~55% of transformed lines

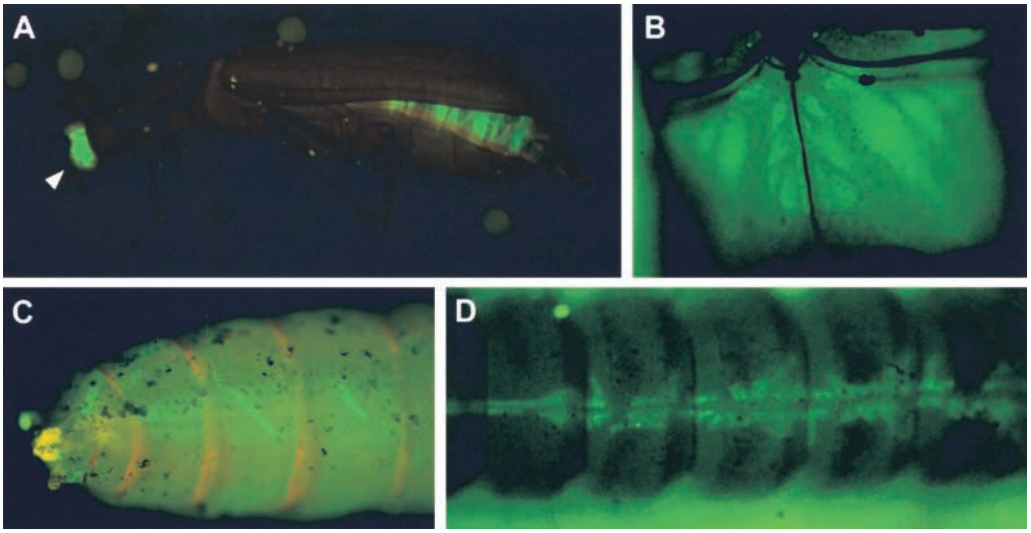

FIGURE 4.—Enhancer trap lines recovered from *Mi{3xP3-EGFP}* insertions. Lines show new patterns of EGFP fluorescence, in addition to those driven by the *3xP3* element. (A) Expression in adult abdominal muscles (arrowhead marks the expected *3xP3*-driven expression in the eye), (B) expression in adult thoracic muscles (ventral view), (C) expression in ventro-lateral stripes in the larva, (D) expression in the dorsal hemolymph vessel of the larva.

by screening just 10 individuals (G_1's) per G_0 and ~75% of transformed lines by screening ~20 individuals per G_0. These numbers are sufficiently high to allow for comfortable screening of large numbers of injected beetles and are comparable to those obtained with the *piggyBac* element (LORENZEN *et al.* 2003).

A third parameter of interest is the number of independent *Minos* insertions that can be recovered per injected individual. Multiple insertions recovered per G_0 may be an advantage in screens where each independent insertion has the potential to reveal new information (*e.g.*, enhancer trap screens), but they may be a disadvantage in screens where single insertional events need to be recovered (*e.g.*, insertional mutagenesis screens). The most useful situation is when independent insertions can be recovered in different G_1 progeny arising from a single G_0. In our experiments, 25% of the DNA lines and 67% of the RNA lines tested contained more than one *Minos* insertion, but in a significant proportion of these cases single insertions were recovered in individual G_1 beetles. These could often be recognized by different eye fluorescence phenotypes. Overall, the majority of transformed G_1's that were recovered carried single *Minos* insertions within their genome.

Other important parameters that influence the use of transposon vectors are the stability of insertions, the tendency of the element to insert as single or tandem copies within the genome, and the specificity of transposition events, *i.e.*, whether the mobile element is cleanly excised from its flanking sequences and whether it has a preference for particular target sequences. In all studies that have been carried out to date *Minos* insertions appear to be extremely stable in the absence of a source of transposase (LOUKERIS *et al.* 1995a; KAPETANAKI *et al.* 2002). In our experiments this was seen most clearly in the Southern analysis, where we consistently recovered the same bands in G_1 individuals and in their G_2 progeny (data not shown). We have also confirmed that the great majority of *Minos* insertions are single unlinked insertions, that most of them do not carry the flanking plasmid sequences, and that the only obvious preference for target sites is the presence of the TA dinucleotide at the site of insertion (ARCA *et al.* 1997). Interestingly, two of the three lines carrying linked insertions correspond to cases where plasmid sequences were also found incorporated into the genome. A two-step mechanism, involving the integration of a second *Mi{3xP3-EGFP}* transposon into the donor plasmid, followed by transposition of the resulting compound transposon into the genome, could explain these results (LOUKERIS *et al.* 1995a).

Finally, an important parameter for many transformation experiments is the ability of the vector to carry large inserts. While we have not tested the effect of insert size on transformation efficiency in Tribolium (all our experiments were carried out with the 2-kb *Mi{3xP3-EGFP}* element), a number of relevant observations are available from Drosophila: using the same RNA helper, transformation frequencies of 32% were obtained with a 5.8-kb transposon, 12% with a 7-kb transposon, and 25% with a 9.1-kb transposon (A. METAXAKIS and C. SAVAKIS, personal communication; A. PAVLOPOULOS, unpublished observations). These results suggest that the transposition activity of *Minos* in Drosophila is not severely affected by large insert sizes. Given that *Minos* activity is unlikely to depend on host-specific factors (as deduced from the broad host specificity of this element), it is likely that these vectors will be able to carry relatively large inserts also in Tribolium and in other species of interest. A similar ability to carry large insert sizes (up to 9.5 kb) has also been demonstrated for *piggyBac*-based transposons (LORENZEN *et al.* 2003).

Overall, these results demonstrate that *Minos* has the ability to transpose very efficiently in *T. castaneum* and can be used as a vector for routine transformation experiments in this species. Although particular quantitative

parameters may vary in different experiments (depending on variations in the injection protocol, amount and quality of the helper, overall size of the transposable element, etc.), the performance measured in these pilot experiments suggests that *Minos* will be an excellent tool for most applications (see below).

DNA *vs.* RNA helper: Comparing the results obtained using DNA and RNA helpers suggests that the RNA helper is a more efficient source of transposase: it gives an almost three-fold increase in the frequency of transformation (32.4% *vs.* 11.4%), increases somewhat the cluster size (15.2% *vs.* 10.3%), and increases the number of independent insertions obtained per G_0. All this is achieved without lowering the survival or fertility rates of injected G_0's (Table 1), indicating that the RNA helper could be more useful for generating transformants in large-scale projects. The main drawbacks of using the RNA helper are the additional steps required to synthesize the mRNA, the extra precautions that must be taken to prevent RNA degradation, and in some cases the need to deal with multiple insertions that are obtained in a larger proportion of the G_1's.

Prospects for genetic manipulation in Tribolium: Efficient transformation in Tribolium allows powerful new tools and techniques to be used in this species. These include the use of insertional mutagenesis, enhancer trapping, and gene trapping for the identification of new genes (O'Kane and Gehring 1987; Cooley *et al.* 1988; Bellen *et al.* 1989; Bier *et al.* 1989; Spradling *et al.* 1995); the use of the *UAS*/GAL4 and FLP/*FRT* systems for targeted misexpression and mosaic analysis (Golic and Lindquist 1989; Brand and Perrimon 1993); and the use of reporter constructs for studying *cis*-regulatory elements (Ludwig *et al.* 2000), techniques that have so far been available only in model organisms like Drosophila, *Caenorhabditis elegans*, and the mouse.

Besides *Minos*, another transposable element, *piggyBac*, has been shown to mediate transformation and enhancer detection with high efficiency in Tribolium (Berghammer *et al.* 1999b; Lorenzen *et al.* 2003). Having two different vectors for transformation offers several advantages and greatly expands the possibilities for genetic manipulation in a species: it helps overcome problems of insertional biases of individual elements (Spradling *et al.* 1999), allows efficient dual transposon systems to be used for insertional screens (with separate helper and mobile/mutator elements integrated into the genome; Cooley *et al.* 1988; Horn *et al.* 2003), allows the use of compound transposons to facilitate the detection of excision events and to generate chromosomal deletions (Huet *et al.* 2002), and allows the generation of new insertions in genetic backgrounds where other transposable elements remain stable (Hacker *et al.* 2003). Thus, powerful genetics and reverse-genetic technologies that were once available only in Drosophila can now be applied to Tribolium.

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