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P Element-Mediated Transformation with the *rosy* Gene in *Drosophila melanogaster*

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We have used two kinds of P element constructs, Pc[(ry^+)B] and P[(ry^+)ΔSX9], for genetic transformation by microinjection of *D. melanogaster*. Pc[(ry^+)B] construct carrying the *rosy* gene within an autonomous P element was injected into a true M strain carring the ry^{506} mutation. The source of transposase for microinjection and transformation was provided by a P element helper plasmid designated p-Δ2-3hsπ, which was co-injected with nonautonomous P[(ry^+)ΔSX9] construct into same ry^{506} M strains. A dechorination method was adopted and 35 independent transformed lines were obtained from 1143 G_0 injected (35/1143). About 20% of the injected embryos eclosed as adults. Among G_0 eclosed flies, approximately 40% exhibited eye color that was similar to wild-type (ry^+), but about 60% of fertile G_0 transformed lines appeared to have no G_1 transformants. Therefore it is unlikely that G_0 expression requires integration of the *rosy* transposon into chromosomes. Pc[(ry^+)B] and P[(ry^+)ΔSX9] constructs were found to be nearly same in the frequency of P element-mediated transformation. On the basis of these results, nonautonomous P elements constructs could be used as same effective vectors in P element-mediated transformation for introducing and fixing genes in insect populations.

KEY WORDS: P Element, *rosy* Gene, Microinjection, Genetic Transformation

In *Drosophila melanogaster* more than fifty independent families of mobile elements have been identified. One of them, called the P element family, has been extensively studied for their usefulness in molecular genetic techniques and their significance in evolutionary biology (Rubin and Spradling, 1982; Kidwell, 1985; Engels, 1989). P elements are common in natural populations of *D. melanogaster*, but absent in many of the old laboratory stocks of this species. P elements can be divided into two size classes, complete elements and defective elements. An autonomous complete 2907-bp element that encodes a protein of 87 kDa, transposase, required for P element transposition has 31-bp perfect, inverted, terminal repeats (O'Hare and Rubin, 1983). Defective elements are generally smaller and nonautonomous and are derived from the 2.9-kb complete element by heterogeneous internal deletions. Some types of defective P elements can also be mobilized when supplied with transposase from a 2.9-kb element.

Transposition of P elements is regulated in two ways. First, the elements are mobile only in the germline cells. The tissue specificity of transposition is due to at least in part to inhibition of P element pre-mRNA splicing in somatic tissues (Laski *et al.*, 1986; Siebel and Rio, 1990; Chain *et al.*, 1991). The second level of regulation, transposition only arises from matings between P strain males carrying P elements and M strain females lacking P elements but not in the

reciprocal cross (Engels, 1989). A phenomenon of genetic syndrome, called 'hybrid dysgenesis', can be arisen in F_1 hybrid progenies from matings between P strain (♂) and M strain (♀) (Kidwell *at al.*, 1977). The characteristic features of the syndrome include temperature-dependent sterility (GD sterility), male recombination, chromosome rearrangements, segregation distortion and high rates of mutation.

P elements have become important tools for gene transfer, insertional mutagenesis, enhancer trapping, and gene cloning using transposon tagging. The use of transgenic technology for the genetic manipulation of insect genomes will be particularly highlighted. Recombinant DNA technology and transgenic techniques provide the means for the controlled genetic manipulation of vector genomes by the direct introduction of DNA into the germ line. Such manipulation of economically important insects therefore provides the opportunity to introduce and express foreign genes and/or disrupt existing gene functions so that the desirable modifications may be inherited by subsequent generations, removing the need for frequent mass releases. Therefore, one way to accomplish this might be the introduction of exogeneous genes by some type of drive mechanism (Kidwell and Ribeiro, 1992).

The P element of *D. melanogaster* may provide an ideal vehicle to determine whether mobile elements can be effectively used as a drive mechanism. P elements have proven to be invaluable as vectors for transformation of *D. melanogaster* (Spradling and Rubin, 1982; Rubin and Spradling, 1982). It is therefore plausible that mobile elements may be used as vectors not only for transformation, but also for dispersal, of engineered DNA.

In this paper we demonstrate that non-autonomous P element constructs could be used as same effective vectors comparing with autonomous P element in P element-mediated transformation in *D. melanogaster*. Autonomous Pc[(ry^+)B] construct (Karess and Rubin, 1984) and nonautonomous P[(ry^+)ΔSX9] construct with a P element helper plasmid designated p-Δ2-3hsπ were injected into a true M strain carring the ry^{506} mutation, respectively. Considerable success was achieved in a series of microinjection, in which transformed lines carrying the *rosy* gene within both autonomous and nonautonomous P construct were found to have wild-type eye color in G_1 progeny as in the similar transformation frequency. These results seem to augur well for the eventual success of P element-mediated transformation for introducing genes in this species.

Materials and Methods

Drosophila strains

All strains described are *D. melanogaster*, and the development of the embryos and all crossess were carried out at 23-25°C on standard cornmeal-molasses-agar medium. The *Drosophila* M strains ry^{506} were used as the microinjection recipient and the establishment of ry^+ transformants. This strain is a homozygous for a deletion that a portion of the *rosy* coding region (Lindsley and Zimm, 1992).

P element constructs

Two types of plasmids were constructed. The first construct was Pc[(ry^+)B] (Karess and Rubin, 1984), constructed from the P-*rosy* vector Carnegie 20 (Rubin and Spradling, 1982) and the 2907-bp autonomous P element from pπ25.1 plasmids (O'Hare and Rubin, 1983). The *rosy* (ry^+) gene sequence, contained on a 7.2-kb *Hind*III fragment, is positioned in a noncoding region of the element, downstream from the unique *Sal*I site at sequence position 2410 (Fig. 1, A). This element combines a marker gene and a source of P transposase in a single transposon construct. We made the second construct which consists of a nonautonomous derivative of Pc[(ry^+)B], designated P[(ry^+)ΔSX9] (Kim *et al.*, 1994). This construct was created by deleting approximately 1680-bp between the *Xho*I site at position 727 and the *Sal*I site at position 2410 (Fig. 2, B). The source of transposase for microinjection and transformation was provided by a P element helper plasmid designated pΔ2-3hsπ (a gift from Dr. Y. K. Kim, SUNY, Purchase, USA).

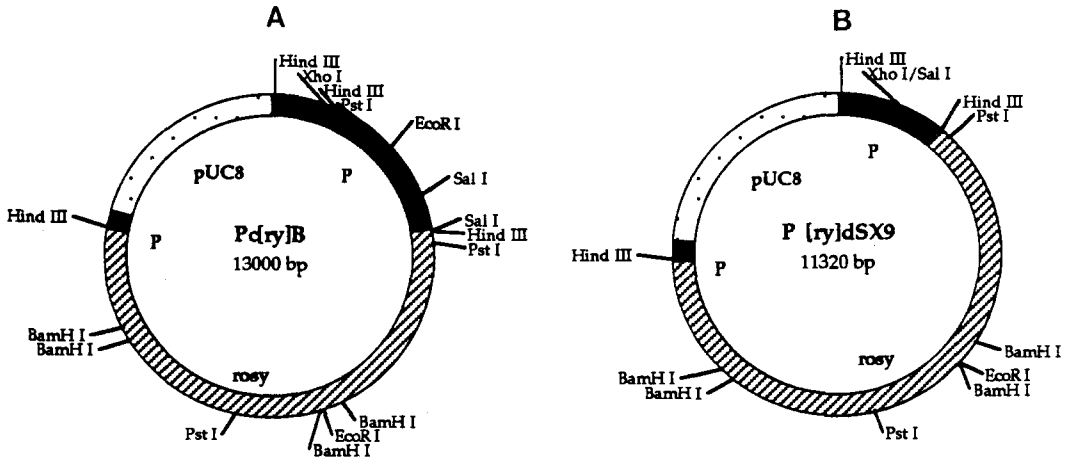

Fig. 1. Map of the constructs Pc[(ry^+)B] and P[(ry^+)ΔSX9]. P element sequences are represented by the filled-in shading, *rosy* sequences by the cross-hatched shading and the pUC8 plasmid sequences by the stippled shading.

Microinjection and transformation

We used a modified microinjection technique, dechorination method described by Spradling and Rubin (1982). Embryos were collected on an agar plate (2%) plus enough grape juice to color the solution deeply. Embryos collected from eggs laid within a 20 min period were rinsed and transferred on double-coated tape (20 × 30 mm) attached to a slide. Slides containing the embryos were chilled on ice for 5-10 min in order to make the embryos more rigid and easier to handle during the dechorination process. After chilling, we dechorinated the embryos by rolling them over the tape on the slide. The dechorinated embryos were then placed onto another strip of double-sided tape (2 × 20 mm) with the posterior ends of the embryos hanging off the tape. The embryos were lined up and desiccated in a box containing calcium chloride on ice for about 5 min and covered with water-saturated mineral oil. Injection followed immediately. After injection, the slides were placed into a humidity box containing wet towels to allow the development of the embryos on the tape under oil at 23-25°C. Within 15 hours of emergence, the larvae were transferred, together with the strip of culture medium, into vials containing regular food. Flies were mated singly with several virgin females or males to produce the G_1 generation. Details as to how transformants were initially selected are supplied in the text (Spradling, 1986).

Polymerase chain reaction (PCR)

The presence of P element sequences in the genomic DNA of individual transformed flies was first confirmed using PCR with oligonucleotide primers that specifically hybridize to the P element sequence. PCR was carried out using the entire transformed genomic DNA in a DNA Thermal Cycler (Perkin-Elmer Cetus) according to the following protocol: 5 μl Cetus 10x PCR buffer; 3 μl of 25 mM $MgCl_2$; 4 μl of dNTP; 2 μl (33 ng) of primer 1 (#829: 12→31 nucleotides, 5′-AACATAAGGTGGTCCCGTCG-3′), 2 μl (33 ng) of primer 2 (#830: 757←776 nucleotides, 5′-CGACTGGGCAAAGGAAATCC-3′); 1 μl of template DNA (100 ng/μl); 0.5 μl Ampli Taq DNA polymerase; sterile deionized water (32.5 μl) was added to the mixture to achieve a final volume of 50 μl; Mix and overlay with one drop of mineral oil in 0.5 ml Eppendorf reaction tubes. The amplification was performed for 30 cycles with 45 seconds denaturation at 95°C and 20 seconds annealing at 50°C, followed by a 1 minute + 1 second auto-extension at 72°C. The temperature was then dropped to 4°C for 15 minutes to overnight. The results of PCR were confirmed by electrophoresis using 5 μl on 1% agarose minigel.

In situ hybridization

The presence and cytological position of the P construct in each of the initial transformed line was also confirmed by *in situ* hybridization to polytene chromosome, using the plasmid pπ25.1 as probe. A non-radioactive method (Kim and Kidwell, 1994) was used for labeling and detection. DNA probes were labeled using PCR by incorporation of a nucleotide analog, digoxigenin-11-dUTP. Hybridization was detected by enzyme-linked immunoassay, using an antibody conjugate and subsequent enzyme-catalyzed color reaction.

Results and Discussion

The P element of *D. melanogaster* may provide an ideal vehicle to determine whether mobile elements can be effectively used as a drive mechanism. In this results, we also demonstrate that P elements can be used as suitable vectors for transformation of *D. melanogaster*. A summary of transformation results in this study is presented in Table 1. As shown in Table 1, about 20% of the injected embryos eclosed as adults. Among G_0 eclosed flies, approximately 40% exhibited eye color that was similar to wild-type (ry^+), presumably the result of transcription and translation of the *rosy* gene sequences in these constructs (Spradling, 1986). A total of 31 putative G_0 transformed lines were found to have no transformants (50 lines among 78 G_0 transformed lines were fertile in our experiment). Therefore it is unlikely that G_0 expression requires integration of the *rosy* transposon into chromosomes. Overall our transformation frequency was 3.1% of injected embryos (35/1143). The survival frequency of eclosed flies is similar to that of other reports, but the transformation frequency was somewhat higher. As seen in Table 1, Pc[(ry^+)B] and P[(ry^+)ΔSX9] constructs were found to be nearly same in the frequency of transformation. For example, about 24.4% (11/45) of the 45 fertile flies in using the Pc[(ry^+)B] construct appeared to be transformants. In case of P[(ry^+)ΔSX9] construct, the transformation frequency was found to be 23.5%

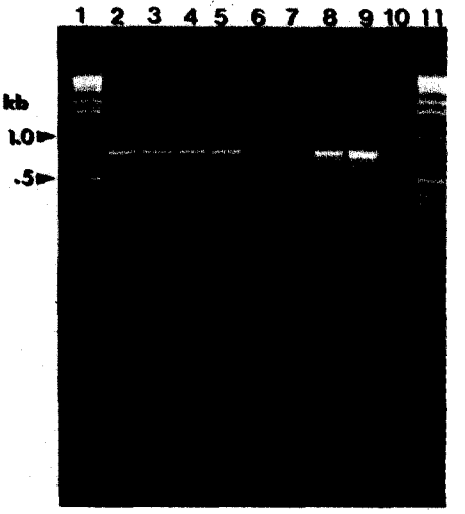

Fig. 2. The presence of P element sequences (Pc[(ry^+)B] construct) in the genomic DNA of individual transformed flies using PCR with P specific oligonucleotide primers. Lanes are as follows: (1 and 11) DNA size marker, kb ladder; (2-5) independent G_1 transformants; (6) G_0 transformants; (7) ry^{506}; (8) *Harwich*^{w}; (9) pπ25.1; (10) water blank.

Table 1. Results of microinjection experiments with Pc[(ry^+)B] and P[(ry^+)ΔSX9]

Construct	No. G_0 injected	No. (%) G_0 eclosed	No. (%) G_0 ry^+ flies	No. (%) fertile flies	No. G_1 ry^- flies	No. G_1 ry^+ flies	No. (%) independent transformants
Pc[(ry^+)B]	523	55	25	45	3,836	38	11 (24.4)
P[(ry^+)ΔSX9]	620	142	53	102	7,294	114	24 (23.5)
Total	1,143	197 (17.2)	78 (39.6)	147 (74.6)	11,130	152	35 (23.8)

(24/102) of the 102 fertile flies.

The presence of P element sequences in the genomic DNA of individual transformed flies was confirmed using PCR with P specific primers (Fig. 2). For example, four lines with independent G_1 transformants (lanes 2-5 in Fig. 2) obviously contain the Pc[(ry^+)B] construct in their genomes, and one G_0 transformed line (lane 6 in Fig. 2) also gave a positive result for the presence of the construct in the cytoplasm or genome. Further, the P element copy number and cytological map of the Pc[(ry^+)B] or P[(ry^+)ΔSX9] transposon insertions in the polytene chromosomes of the initial transformed lines was determined by *in situ* hybridization. Each of the initial transformed lines were found to carry only one insertion of the P element construct as shown, for example, in Fig. 3. Spradling (1986) reported that usually only a small fraction of the germline cells of a G_0 individual contain integrated rosy transposons and gave rise to ry^+ G_1 progeny. Our results are also consistent with his report.

The frequencies of G_1 ry^+ progeny (Fig. 4) produced by 35 transformed lines is summarized in Table 2. The results from Table 2 indicate that the frequency of G_1 progeny with ry^+ eye color was quite low from those G_0 individuals that produced transformed progeny. For example, in almost 50% of the 35 G_0 transformed lines ry^+ flies represented less than 3% of the total progeny recovered. We also performed the progeny tests by backcross between the transformed G_1 progeny and ry^{506} in order to establish a series of G_2 isolines. According to the results of progeny tests and *in situ* hybridization, seven independent lines among a total of 35 were found to carried X-linked insertions. One of these seven was also found to carry a recessive lethal mutation and a

Fig. 3. Photographs of the polytene chromosome squashes of individual larvae from the original *B1-8* (A panel) and *SX31-1* (B panel) transformed lines in which the Pc[(ry^+)B] and P[(ry^+)ΔSX9] transposons are inserted in chromosomes 3L(61F) and 2L(36B), respectively.

Fig. 4. Eye color mutation (ry^{506}: A panel) and wild-type eye color of G_1 transformed line (*SX31-1*: B panel) in the presence of P[(ry^+)ΔSX9] construct.

Table 2. The distribution of G_1 flies with *ry*$^+$ *eye* color from 35 G_0 transformed lines (see Table 1).

% *ry*$^+$ progeny in G_1	1–2	3–4	5–6	7–9	10–19	20–30
No. of G_0 transformed lines	16	7	5	4	2	1

Table 3. The distribution of P constructs on the polytene chromosome arms by using *in situ* hybridization and progeny tests from initial 35 independent transformed lines.

Construct	Chromosome arm						Total (lines)
	X	2L	2R	3L	3R	4th	
Pc[(ry$^+$)B]	2	1	3	3	2		11
P[(ry$^+$)ΔSX9]	5	5	4	3	7		24

second carried a recessive mutation affecting wing shape. These results indicate that P elements can cause to give rise to insertional mutations in genomes (Bingham *et al.*, 1981; Engels, 1989). Based on progeny tests and *in situ* hybridization experiments, none of the G_1 transformed lines contained more than a single insertion of the P element construct (Table 3). The P element insertion were found to occur at a wide variety of locations on all chromosomes, except 4th chromosome. It was known to be approximately uniform distribution of P element insertion sites in the genome (Spradling, 1986; Ronsseray and Anxolabehere, 1986). The P element insertions are also rarely found in the large heterochromatic regions. P elements are found to be preferentially inserted in the euchromatic arms of chromosomes (Engels, 1989).

On the basis of our results, nonautonomous P element constructs could be used as same effective vectors comparing with autonomous P element in P element-mediated transformation in *D. melanogaster*. These results seem to augur well for the eventual success by using both of autonomous and nonautonomus P constructs in P element-mediated transformation for introducing genes in insect populations. Therefore it is suggested that there may be a strong feasibility of using P transposable elements to drive engineering genes into insect populations, with a view towards the development of more efficient and permanent methods for the control of vector-borne diseases.

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*D. melanogaster*에 있어서 P Element를 이용한 *rosy* 유전자의 형질전환

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*D. melanogaster*를 대상으로 두 종류의 P element construct, 즉 Pc[(ry^+)B] 와 P[(ry^+)ΔSX9]을 사용한 형질전환 실험을 통하여 자율적 및 비자율적인 P element constructs 간의 vector 로써의 효율성을 분석했다. 자율적인 P element 내에 *rosy* 유전자를 포함하고 있는 Pc[(ry^+)B] construct를 진정 M 계통형인 ry^{506} 돌연변이(eye color) 계통에 주입시켰다. 또한 비자율적인 P element 내에 *rosy* 유전자를 포함하고 있는 P[(ry^+)ΔSX9] construct와 전이효소의 공급원으로써 p-Δ2-3hsπ helper plasmid를 역시 같은 계통의 ry^{506} 돌연변이 계통에 주입시켰다. Dechorination 방법에 의해 총 1143 embryo를 대상으로 microinjection 한 결과, 모두 35 계통의 형질전환된 정상형의 눈을 가진 계통을 얻었다. P element construct를 주입시킨 전체 1143 embryo 중에서 약 20% 정도가 성체로 부화되었으며, 부화된 개체(G_0) 가운데 약 40% 정도가 눈 색깔이 거의 정상형과 같은 G_0 transformant 로 나타났다. 그러나 생식력을 가진 G_0 transformant의 약 40%만이 G_1 transformant로 나타남으로써 모두 35 계통의 transformant를 얻었다. 따라서 G_0 단계에서 나타난 형질전환 현상에는 반드시 *rosy* transposon의 염색체 삽입에 의해서 만이 아니라 세포질내에서도 construct 내의 *rosy* 유전자의 발현이 가능한 것으로 분석되었다. 또한 형질전환율에 있어서는 Pc[(ry^+)B]와 P[(ry^+)ΔSX9] construct 간에 유의한 차가 없는 것으로 나타났다. 이상의 결과로 볼 때, 실험 목적에 따라 자율적인 P element 또는 helper DNA를 이용한 비자율적인 P element를 효과적인 vector 로 선택 이용함으로써 특정 유전자를 곤충집단내로 침투 및 고정시킬 수 있다고 판단된다.