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Germline transformation of the butterfly *Bicyclus anynana*

Jeffrey M. Marcus†, Diane M. Ramos and Antónia Monteiro*

Department of Biological Sciences, University at Buffalo, Buffalo, NY 14260, USA
** Author for correspondence (monteiro@buffalo.edu).*

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Ecological and evolutionary theory has frequently been inspired by the diversity of colour patterns on the wings of butterflies. More recently, these varied patterns have also become model systems for studying the evolution of developmental mechanisms. A technique that will facilitate our understanding of butterfly colour-pattern development is germline transformation. Germline transformation permits functional tests of candidate gene products and of *cis*-regulatory regions, and provides a means of generating new colour-pattern mutants by insertional mutagenesis. We report the successful transformation of the African satyrid butterfly *Bicyclus anynana* with two different transposable element vectors, *Hermes* and *piggyBac*, each carrying *EGFP* coding sequences driven by the *3XP3* synthetic enhancer that drives gene expression in the eyes. Candidate lines identified by screening for *EGFP* in adult eyes were later confirmed by PCR amplification of a fragment of the *EGFP* coding sequence from genomic DNA. Flanking DNA surrounding the insertions was amplified by inverse PCR and sequenced. Transformation rates were 5% for *piggyBac* and 10.2% for *Hermes*. Ultimately, the new data generated by these techniques may permit an integrated understanding of the developmental genetics of colour-pattern formation and of the ecological and evolutionary processes in which these patterns play a role.

Keywords: germline transformation; butterfly; genetics; transposons; evolution and development; *Bicyclus anynana*

1. INTRODUCTION

Biological hypotheses ranging from the ecological role of aposematic coloration (Wallace 1881; Nishida 2002) and the evolution of mimicry (Bates 1863; Kapan 2001), to the basis of phenotypic plasticity (Merrifield 1892; Brakefield *et al.* 1998) have historically been inspired by the eye-catching colour patterns on the wings of butterflies. More recently, butterfly colour patterns have also become an important model system for understanding the relationship between development and evolution because they are highly variable, consist of clearly defined subunits, exist in two dimensions and yet are structurally very simple, making them very amenable for study and manipulation (Nijhout 1991; Beldade & Brakefield 2002; McMillan *et al.* 2002).

Unfortunately, progress in understanding the developmental genetic processes underlying butterfly colour-pattern formation has been limited, for two reasons. First, researchers have not been able to characterize any of the mutations that alter colour patterns (Weatherbee *et al.* 1999; Monteiro *et al.* 2003), so there is little mechanistic understanding of how mutant colour-pattern phenotypes are produced. Second, while gene expression patterns that resemble adult colour patterns are suggestive (Carroll *et al.* 1994; Brunetti *et al.* 2001), there are very few data available showing that these gene products have a functional role in colour-pattern formation. Germline transformation is one genetic technology that has been used for both *in vivo* tests of gene function (Brand & Perrimon 1993) and for the production of mutations that are easy to characterize at the molecular level (Cooley *et al.* 1988).

Inspired by the recent studies that identify several transposable elements capable of insertion into multiple arthropod genomes (Berghammer *et al.* 1999), including two species of moths (Peloquin *et al.* 2000; Tamura *et al.* 2000), we tested whether two transposons, *Hermes* and *piggyBac*, are capable of inserting into the genome of the African satyrid butterfly *Bicyclus anynana* (figure 1*a*). The success of these experiments represents the first demonstration, to our knowledge, of germline transformation in a butterfly.

2. MATERIAL AND METHODS

We tested whether the *piggyBac* construct *pBac[3xP3-EGFP]* and the *Hermes* construct *Her[3xP3-EGFP]* (generously provided by Ernst Wimmer), carrying the marker gene for enhanced green fluorescent protein (EGFP), could insert into the germline of the butterfly *B. anynana*. Eggs were collected from maize leaves following 1 h oviposition bouts, and placed into glass Petri dishes on thin strips of double-sided adhesive tape. Eggs were injected with equal concentrations (500 ng μl^{-1}) of one of the plasmids mentioned above, and a helper plasmid containing the coding sequence of either the piggyBac (plasmid construct *pHsp82Pbac*) or Hermes transposase (plasmid construct *pKhsp82Hermes*) driven by the *Drosophila* heat-shock promoter (Horn *et al.* 2000), using a pulled glass needle attached to a Picospritzer III microinjection apparatus. After injection, embryos were placed in an incubator at 27 °C and 80% relative humidity until the larvae hatched 4–6 days later, and were transferred to food plants with a small paintbrush. Adults reared from injected eggs were mated in individual cages with 3–5 virgin individuals of the opposite sex to establish families. Eggs were collected from each family, and the larvae were screened for the presence of EGFP in their six larval stemmata, and again in the adult compound eye with a Nikon SMZ1500 fluorescent microscope.

All EGFP-positive F_1 individuals were crossed separately with wild-type, whereas in all subsequent generations, matings were conducted within families to generate homozygous lines. We confirmed the presence of the *EGFP* gene in all of these families by PCR (forward primer EGFPFlas CGT GAC CAC CCT GAC CTAC, reverse primer EGFPRlas TGA TCG CGC TTC TCG TT, PCR conditions: 1 × 94 °C, 2 min; 40 × (94 °C, 30 s; 58.2 °C, 30 s; 72 °C, 1 min); 1 × 72 °C, 6 min). The transposon insertion sites in transformed families were amplified by inverse PCR (genomic DNA digested by HaeIII, MspI, Sau3a or TaqI restriction enzymes, circularized by T4 ligase and amplified with primer pairs PLF and PLR, PRF and PRR, HLF and HLR, and HRF and HRR (Horn & Wimmer 2000), PCR conditions: 1 × 95 °C, 5 min; 30 × (95 °C, 30 s; 65 °C, 1 min; 68 °C, 2 min); 1 × 72 °C, 10 min; some PCR products were reamplified with the same primers and conditions to increase product concentration) and sequenced. Flanking sequences were then examined to determine whether the sequences contained the expected target-site duplications for each transposon.

† Present address: Department of Biology, Western Kentucky University, Bowling Green, KY 42101, USA.

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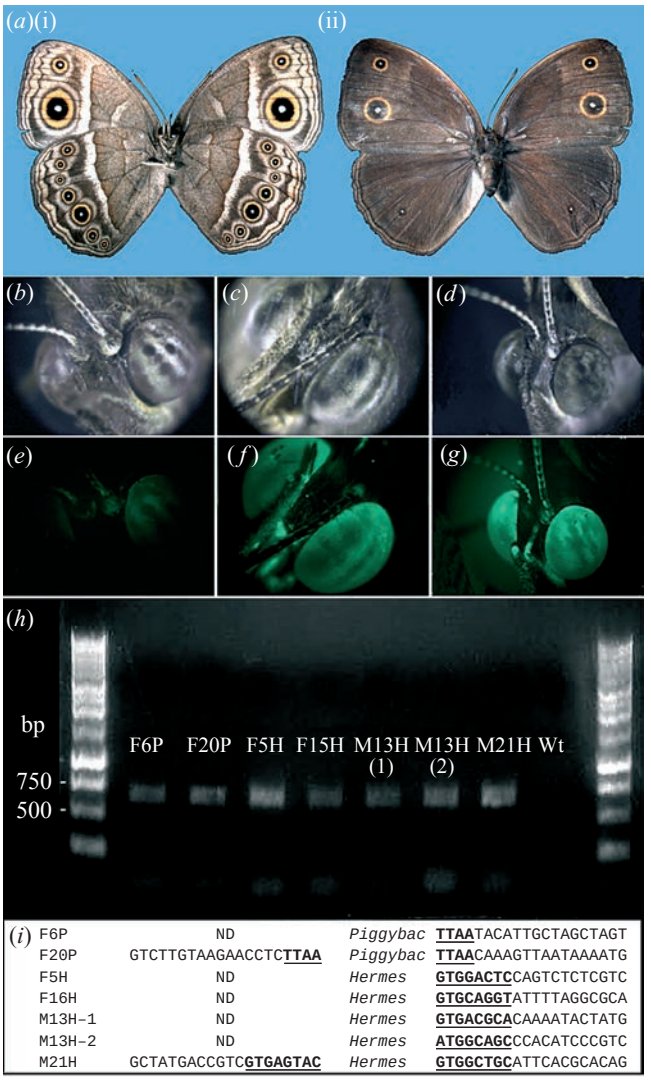

Figure 1. Germline transformation of the butterfly *Bicyclus anynana*. (*a*) (i) Ventral and (ii) dorsal views of wild-type *B. anynana*. (*b–d*) Adult *B. anynana* eyes under bright-field illumination. (*e–g*) The same butterflies under epifluorescent illumination. (*b*,*e*) Wild-type *B. anynana*. (*c*,*f*) *Bicyclus anynana* transformed with *piggyBac* [*3XP3-EGFP*]. (*d*,*g*) *Bicyclus anynana* transformed with *Hermes* [*3XP3-EGFP*]. (*h*) PCR amplification of *EGFP* inserts in transformed lines of *B. anynana*. (*i*) Partial flanking sequences surrounding transposon insertion sites. Proposed piggyBac and Hermes target site duplications are shown in bold. In one case, M13H, different flanking sequences were obtained from different sublines descended from a single injected individual. Both sublines test positive for EGFP expression both by visual inspection and by *EGFP* PCR amplification (see (*h*)). We suggest that the original injected individual that founded this subline had more than one *Hermes* insertion, most probably on different chromosomes, and these insertions were then separated from one another by genetic segregation when mated to wild-type animals in the F_1 generation.

3. RESULTS

In total, 3357 eggs were injected with the *piggyBac* construct and 6741 eggs were injected with the *Hermes* construct. Approximately 95% of the injected embryos died before hatching, presumably as a result of dehydration via the punctured hole in the chorion. Control (uninjected) eggs that were otherwise treated in the same way as injected eggs have a hatch rate of *ca*. 80%. Out of these injected eggs, 40 animals derived from eggs injected with *piggyBac* and 39 animals derived from eggs injected with *Hermes* survived to adulthood and produced offspring when mated to wild-type uninjected individuals. From the families derived from the injected individuals, we identified two *piggyBac* families (5.0%) and four *Hermes* families (10.2%) with one or more offspring expressing EGFP (figure 1*b–g*). Offspring from these EGFP-expressing individuals backcrossed to wild-type, produced PCR amplification products when amplified with *EGFP* primers (figure 1*h*). Finally, we used inverse PCR to sequence flanking regions of each insertion. We were not able to sequence both ends of every insertion, but all of the sequences that we were able to obtain included the expected target-site duplications (figure 1*i*). We continued rearing and inbreeding three lines of transgenic butterflies (one *piggyBac* and two *Hermes*). The presence of the *EGFP* gene was again confirmed by PCR in individuals of the fifth generation.

4. DISCUSSION

Our findings demonstrate that transposon-based germline transformation in butterflies is possible and takes place at efficiencies comparable to those reported in moth transformation experiments (Peloquin *et al.* 2000; Tamura *et al.* 2000). Although the transformation rate among injected individuals that survived injection is reasonably good, improvements in the injection protocol to increase survivorship are needed to increase the efficiency of experiments such as those described here, and this is a subject that is currently under investigation. The detection of EGFP-positive individuals was often difficult owing to the faint EGFP expression in most of the transformed individuals. This may have been caused by position effects on gene expression or the presence of the normal eye pigments that could block the EGFP signal. Detection of EGFP-positive individuals can be facilitated by digital photographic techniques, and our ability to detect transformants with weak EGFP expression might have been greater had we used digital imaging as part of the screening process. Our transformation rates should therefore be viewed as minimum estimates. Finally, it appears that male *B. anynana* have slightly darker eyes than females, so we recommend screening the sexes separately.

With slight modifications to the injected constructs, the technique of germline transformation can be used for enhancer trapping and insertional mutagenesis screens for mutations that alter colour-pattern phenotypes. Further, misexpression constructs of candidate genes or reporter constructs with candidate *cis*-regulatory regions can be included within a transposon vector and introduced into the butterfly germline so that their role in colour-pattern formation can be tested *in vivo*. This ability to manipulate a butterfly genome will greatly advance our understanding of how butterfly colour patterns develop and allow us to integrate fully the development of these patterns with their ecology and evolution.

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