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Germ line transformation of the olive fly *Bactrocera oleae* using a versatile transgenesis marker

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Abstract

The olive fruit fly (olive fly) *Bactrocera oleae* (Dacus), recently introduced in North America, is the most destructive pest of olives worldwide. The lack of an efficient gene transfer technology for olive fly has hampered molecular analysis, as well as development of genetic techniques for its control. We have developed a *Minos*-based transposon vector carrying a self-activating cassette which overexpresses the enhanced green fluorescent protein (EGFP). Efficient transposase-mediated integration of one to multiple copies of this vector was achieved in the germ line of *B. oleae* by coinjecting the vector along with *in vitro* synthesized *Minos* transposase mRNA into preblastoderm embryos. The self-activating gene construct combined with transposase mRNA present a system with potential for transgenesis of very diverse species.

Keywords: Olive fruit fly; *Bactrocera oleae*; germ line transformation; transgenesis marker.

Introduction

Arthropods are key pests of food and fibre crops as well as major disease vectors. Field resistance to pesticides presents an increasing challenge to pest control. Progress in molecular manipulation of insect species is an important way to further comprehension of the genetic and biochemical basis of insect biology. In addition, it will open the way for novel strategies to control insect pest populations, as, for example, through production of insects for sterile insect technique (SIT) programs.

The technologies for insect transgenesis developed so far rely on transposable elements. Manipulation of an insect genome with a transposable element was first achieved in *Drosophila* with the *P* element (Rubin & Spradling, 1982). However, the host range of *P* seems to be restricted to the drosophilids (Handler *et al*., 1993). The discovery of new elements with a broader host range has led to the development of vector systems for transformation of several non-drosophilid insect species, including the Mediterranean fruit fly *Ceratitis capitata* (Loukeris *et al*., 1995b; Handler *et al*., 1998; Michel *et al*., 2001), the mosquitoes *Aedes aegypti* (Jasinskiene *et al*., 1998; Coates *et al*., 1998), *Anopheles albimanus* (Perera *et al*., 2002), *Anopheles stephensi* (Catteruccia *et al*., 2000) and *Anopheles gambiae* (Grossman *et al*., 2001), the housefly *Musca domestica* (Hediger *et al*., 2001), the Australian sheep blowfly *Lucilia cuprina* (Heinrich *et al*., 2002), the silkmoth *Bombyx mori* (Tamura *et al*., 2000), the beetle *Tribolium castaneum* (Lorenzen *et al*., 2003; Pavlopoulos *et al*., 2004) and the New World screwworm *Cochliomyia hominivorax* (Allen *et al*., 2004).

Bactrocera oleae is the main pest of olives in the Mediterranean region and in California, where it has been introduced recently. Currently, control of *B. oleae* is based on the use of insecticides either in bait or in cover sprays, resulting in adverse effects on the environment and sometimes presence of unacceptably high levels of insecticides in olives and olive oil (Ferreira & Tainha, 1983). Development of olive fly control strategies leading to reduced or no use of insecticides has been attempted with mixed results, for example, 'lure and kill' (Haniotakis *et al*., 1991), localized bait spray (Pucci, 1990) and inundative or inoculative release of parasitoids (Tzanakakis, 1995). SIT has been used with limited success (Economopoulos *et al*., 1977), apparently because of low competitiveness of the mass-reared males compared to the wild males (Economopoulos

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& Zervas, 1982). Availability of a method for olive fly transgenesis is a prerequisite for genetic manipulation of this species for development of more effective control methods, such as release of insects carrying a dominant lethal allele (RIDL) strategies (Heinrich & Scott, 2000; Thomas *et al.*, 2000) and sensitization of insect populations to pro-insecticides (SIPP) (Markaki *et al.*, 2004).

The transposon *Minos* from *Drosophila hydei* belongs to the *Tc1*/Mariner family of transposable elements (Franz & Savakis, 1991) which, in addition to insects, has been shown to mediate transgenesis in human cells (Klinakis *et al.*, 2000a), in mouse somatic and germ cells (Zagoraiou *et al.*, 2001; Drabek *et al.*, 2003) and in the ascidian *Ciona intestinalis* (Sasakura *et al.*, 2003). Encouraged by this broad host range, we attempted germ-line transformation of *B. oleae* with a *Minos* vector carrying an enhanced green fluorescent protein (EGFP) gene. EGFP is a universal marker for insect transgenesis (Berghammer *et al.*, 1999).

In this report, we describe a self-stimulating transcription system for high levels of regulatable expression of marker genes in diverse species. Using this system in combination with a *Minos* transposon, we demonstrate stable and efficient transgenesis of *B. oleae*.

Results

A potentially universal EGFP-based genetic marker

To achieve high levels of EGFP expression, we used a self-activating and self-sustained positive feedback loop based on the tetracycline-sensitive transcriptional activator tTA (Gossen & Bujard, 1992), which is based on concomitant expression of EGFP and tTA from a bi-directional promoter driven by tTA. Two versions of the expression cassette were generated. The first version was derived from a commercially available cassette consisting of a 7-mer of the tetracycline operator (tetO) (Gossen & Bujard, 1992) flanked by two divergent copies of a cytomegalovirus (CMV) minimal promoter (the 'mammalian version'). The second was constructed for use in insects and consisted of a 14-mer of the tetO element flanked by two divergent copies of the minimal promoter of the *Drosophila melanogaster hsp70* gene (the 'insect version'). A 14-mer was used in the insect version in order to maximize expression levels (Fig. 1, top).

The insect cassette was tested in *D. melanogaster* and in the medfly *Ceratitis capitata* by *P*- and *Minos*-based germ line transformation, respectively. In *D. melanogaster*, most of the transgenic lines exhibited strong, ubiquitous and constitutive expression of EGFP throughout development (Fig. 1a–d), while only some lines showed tissue-specific expression patterns (not shown). Fluorescence was less intense in medfly transformants, which were characterized by specific patterns of expression during development (Fig. 1e–f).

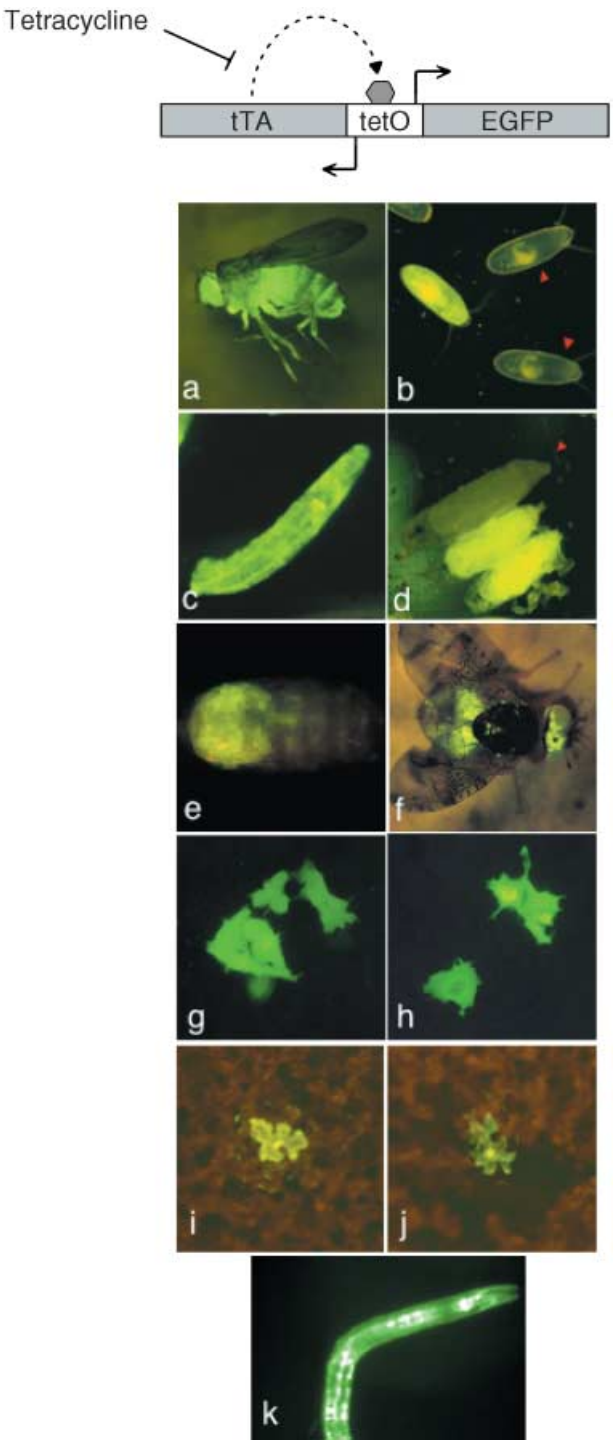

Figure 1. Top: the tTA/enhanced green fluorescent protein (EGFP) self-stimulating expression cassette. Bottom: EGFP expression. (a–d) transformed *Drosophila melanogaster* adults, embryos, third instar larva and pupae; red arrowheads point to non-transformed individuals. (e–f) Transformed medfly pupa and adult. (g–h) Human HeLa cells stably transfected with insect and mammalian cassette, respectively. (i–j) *Nicotiana tabacum* leaf transiently transfected with insect and mammalian cassette, respectively. (k) Transgenic *Caenorhabditis elegans* animal harbouring a plasmid vector carrying the insect cassette.

The versatility of the bidirectional loop was further explored in somatic cells of animals and plants. HeLa cells were transiently and stably transfected with both the 'mammalian' and the 'insect' construct; fluorescence was detectable 24 h post transfection (Fig. 1g–h) and peaked 48 h post-transfection. The same constructs were also introduced into tobacco leaves (*Nicotiana tabacum* and *N. bentamiana*) via gold particle 'bombardment'; examination 48 h post transfection showed strong EGFP expression in certain cells (Fig. 1i–j). The 'insect' vector was also introduced into the nematode *Caenorhabditis elegans*, leading to strong and uniform EGFP expression (Fig. 1k).

Experiments performed in *Drosophila* and in HeLa cells carrying stable, active integrations of the tTA-containing cassette showed that EGFP expression is reversibly suppressed by the teracycline analogue doxycycline at concentrations known to inhibit tTA binding to the promoter (data not shown). Furthermore, high levels of EGFP expression were dependent on the presence of the bidirectional loop because no fluorescence was obtained from control constructs containing the bidirectional promoter without the tTA gene (data not shown).

Olive fly transformation with a Minos *element*

Preblastoderm embryos of *the B. oleae* Demokritos strain were coinjected with the *Minos*-based 'insect' construct and *Minos* transposase mRNA, a method that has been previously shown to result in high transformation frequencies (Kapetanaki *et al*., 2002). Transient EGFP expression was observed in about 60% of the injected embryos examined (Fig. 2, panels A to C), indicating that the genetic marker is functional in the olive fly.

From the 3833 injected G0 embryos, 641 larvae hatched, from which 151 male and 174 female flies developed. The male G0 flies were mated in a single cage to 506 non-injected female flies, and the female G0 flies were mated in another cage to 250 wild-type male flies. The G1 progeny from the two crosses was screened for EGFP fluorescence at the third instar larval stage. A total of 8824 third instar larvae was screened; 67 of them exhibited green fluorescence in various tissues and were therefore considered transformant.

To establish transformed lines, 13 individual G1 flies were back-crossed to wild-type flies and the resulting G2

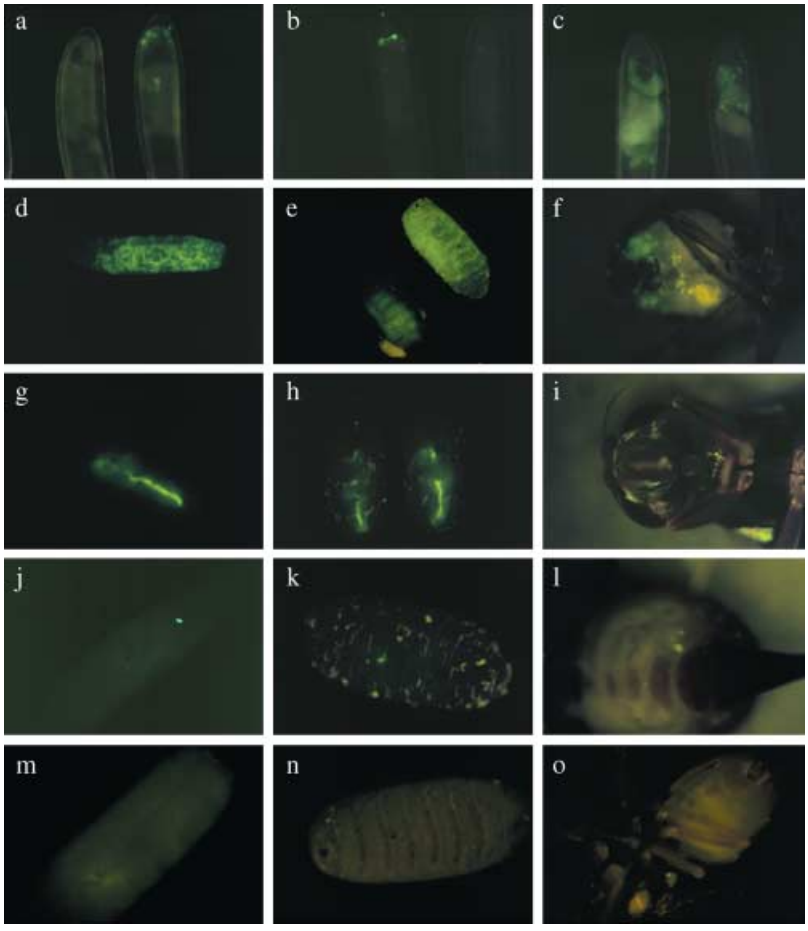

Figure 2. Patterns of enhanced green fluorescent protein (EGFP) expression in injected embryos and in transformed *Bactrocera oleae* individuals. (a–c) Injected eggs showing various patterns of transient EGFP expression. (d–f) EGFP expression in larva, pupa and adult G2 progeny from line 12. (g–i) EGFP expression in larva, pupa and adult G2 progeny from line 10. (j–l) EGFP expression in larva, pupa and adult G2 progeny from line 9. (m–o) Nontransformed larva, pupa and adult.

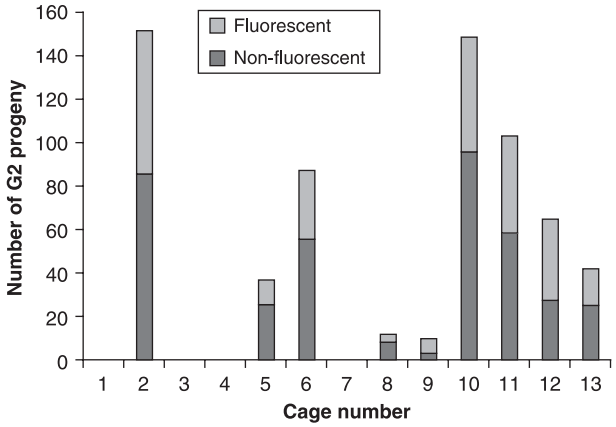

Figure 3. Frequencies of transformants among the olive fly G2 progeny. enhanced green fluorescent protein (EGFP)-expressing G1 progeny was individually backcrossed to wild-type flies. The G2 progeny was screened for EGFP expression. Bars indicate the total number of G2 flies from each G1 parent. The proportion of G2 progeny expressing the EGFP marker is also indicated. No G2 progeny were obtained from cage numbers 1, 3, 4 or 7.

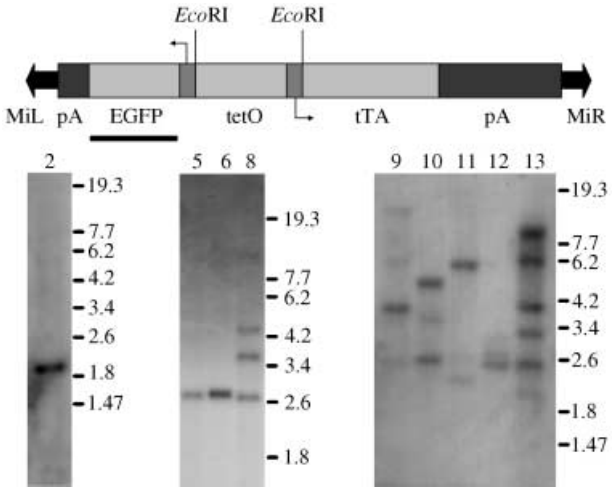

Figure 4. Southern blot analysis of transformed olive fly lines. Top: schematic of the 'insect version' transposon. The bar indicates the fragment used as probe for Southern blot analysis. Bottom: Southern blots of the *Eco*RI digested genomic DNA from individual enhanced green fluorescent protein (EGFP)-expressing G2 progeny. Line numbers are indicated at the top of the panel. Marker values are in kilobases.

third instar larvae were again screened for EGFP expression. Four of the crosses did not give any progeny. Where numbers allowed assessment, the proportion of progeny expressing the EGFP marker was consistent with segregation of a single allele (Fig. 3).

Clearly detectable EGFP fluorescence was observed in second and third instar larvae, pupae and adult flies in all transformed lines examined. Nine independent transformed lines were established (lines 2, 5, 6, 8, 9, 10, 11, 12 and 13). The pattern of EGFP expression differed considerably from line to line (Fig. 2). Furthermore, some lines showed variability of EGFP expression (variegation) between siblings, although the overall pattern was characteristic (lines 9, 11 and 13; data not shown). With one exception (line 9), patterns of expression did not change through the generations. At least two different expression patterns were detected and were segregating in line 9, one of which, highly localized, is shown in Fig. 2(j–l). Because line 9 contained multiple insertions, it is likely that two active insertions (i.e. insertions expressing EGFP) existed in this line, each with a distinct pattern of expression. Transmission of the EGFP marker has been maintained in all lines for at least eight generations, demonstrating that the marker is stably inherited, as expected in the absence of transposase.

Binding of the transcriptional activator tTA to its target is inhibited by tetracycline (Gossen & Bujard, 1992). Tetracycline-dependence of EGFP expression was tested in one of the lines (line 12), which is characterized by high levels of fluorescence in all stages. Fluorescence is not detectable in larvae of line 12 grown on medium containing 10 mg/l tetracycline (data not shown).

Molecular analysis of the transformed lines

Insertion of the *Minos* element into the *B. oleae* genome was verified by Southern blot analysis on *Eco*RI-digested genomic DNA with an EGFP probe. Any single, transposase-dependent insertion of the transposon into genomic DNA is expected to give a band of at least 1300 bp in size. Based on this criterion, the presence of a single insertion was detected in progeny of transgenic flies 2, 5 and 6, while progeny of transgenics 8, 9, 10, 11, 12 and 13 contained multiple insertions (Fig. 4). Of the nine cases analysed, only two, 5 and 6, were found to yield a band of the same size (Fig. 4). Additionally, these two showed the same pattern of EGFP expression, suggesting that they represent the same transformation event. The others were regarded as independent transformation events. Southern blot analysis performed in individual flies from lines 13 and 11 at subsequent generations showed that some of the bands were lost, clearly suggesting that individual bands represent independent, unlinked integrations of the element (data not shown).

To verify that the insertions are indeed transposase-mediated, integration sites were amplified by inverse PCR (Triglia *et al.*, 1988) from four of the lines. The PCR products were cloned and sequenced. As shown in Fig. 5, each of the four integration sites contains the *Minos* inverted terminal repeat followed by a TA dinucleotide and a sequence not present in the original plasmid. We therefore conclude that the *Minos* construct inserts through transposition and not by recombination.

The sequences adjacent to the insertion sites were subjected to BLAST analysis against GENBANK. In two of the

Line 9
Minos..**GGGGCTCG**TAATACTCTAGAATATCAAGATATAACTATCC*GATC*

Line 11
Minos..**GGGGCTCG**TATTCGGATTTGATTCG*GATC*

Line 12
Minos..**GGGGCTCG**TATGAGTGTATATTTGTTTAGGTGTTTGCTTATGTATGTATGTATGGCTGTTAAATATA
TTTGAGCATACATATTTATGATTAGTGGACTGTATATTCCAACATGTTTCTTATCTTAACATGTAAAATAATA
AACCTAAATTTATTGCTGCACGCCGTCTAAGTAGATAAT*GATC*

Line 10
Minos..**GGGGCTCG**TAGAAGCTGGCTAACGTGCTTGGCATAATTTATAGAAGCATTTGTGTAGGCTTGTCTTA
ATGGCGAAAAATTGAAAACATTTAGCATTTACAAGCACTTGGTACCGGCCTAGGAGCCA*GATC*

Figure 5. Sequences flanking *Minos* insertions. The end of the transposon sequence is in bold face. The *Mbo*I restriction site used in inverse PCR is underlined. The TA target dinucleotide is indicated.

sites analysed, the retrieved flanking sequences were too short to allow a meaningful BLAST analysis (Fig. 5). One of the two other sequences (line 10) showed a 91% identity (*E*-value: 2e-11) to a region within the large intron of the *corkscrew* gene of *Drosophila* (see Supplementary Material).

Discussion

We have constructed a versatile marker cassette, in which the EGFP gene is expressed from a bidirectional promoter controlled by the synthetic transcriptional activator tTA (Gossen & Bujard, 1992); the promoter also drives the expression of tTA. This results in self-sustaining expression of the tTA activator. This positive-feedback system should be constitutively active once transcription is initiated, for example from an enhancer near the site of insertion.

The marker cassette presents several interesting features. Firstly, strong EGFP expression in most stages of development, allowing easy selection of transformants. Secondly, the marker is active in diverse organisms, because it depends on the versatile tTA/tetO system rather than a host-derived promoter. Thirdly, transient expression of the marker in embryos injected with DNA, allowing early assessment of injection efficiency and functionality of the marker. This can be a highly desirable property, especially for transformation of species that have a long generation time or are difficult to breed. Fourthly, the EGFP expression patterns differ widely between different insertions in some species such as in medfly and in olive fly, a feature that can be used to distinguish easily between different transgenic lines. Additionally, the system can be used as a base for conditional expression of exogenous genes in various species. The results presented here suggest that the system may also be useful as a sensitive enhancer-trap (Bellen *et al*., 1989; Wilson *et al*., 1989) for identification of tissue- or stage-specific genes in diverse species.

It has been shown recently that a construct expressing tTA from a tetO promoter causes tetracycline-repressible lethality in transgenic medfly (Gong *et al*., 2005); toxicity of overexpressed tTA was presumably the cause of lethality. Such lethals would not have been recovered in our screen for transformants, which was performed in the absence of tetracycline and was based on EGFP expression. We observed, nevertheless, tetracycline-repressible lethality in a number of *Drosophila* transformants, only in the homozygous form. As we did not perform a systematic screen for dominant tet-repressible lethals, we cannot estimate the frequency of tTA-induced lethal transgenes carrying our bidirectional tTA/EGFP construct. It is also possible that the strong lethality observed by Gong *et al*. (2005) may be caused by higher levels of tTA, as a tTA variant was used which is optimized for expression in *D. melanogaster.*

Using the tTA/EGFP self-sustaining cassette, we have shown that the transposable element *Minos* from *D hydei* can mediate stable germ-line transformation of *B. oleae*, thus extending the number of species that have been transformed with this element. Transformation frequency, expressed as the fraction of G0 individuals producing transformed progeny, could not be estimated from these experiments due to the fact that G0 flies were bred in groups. However, the overall number of phenotypically detectable transformation events (67 EGFP expressing G1 progeny from 325 G0 adults) is sufficient to produce several independent transformants from a single experiment, as thousands of embryos can be injected and hundreds of G0 adults can be obtained within the productive 10-day period of egg laying.

Of the nine transformed lines analysed, three lines had a single copy of the transgene, line 11 carried two copies, lines 10 and 12 three and lines 8, 9 and 13 contained more than three copies, contrary to other *Minos*-based transformation experiments (Loukeris *et al*., 1995a,b) where most of the individuals analysed contained a single copy of the transgene. This difference may be a function of the species transformed. Alternatively, it may be the result of high levels of transposase in embryos injected with mRNA, rather than with a transposase-expressing plasmid (Kapetanaki *et al*., 2002). The presence of multiple *Minos* insertions in lines such as line 13, which show Mendelian inheritance of EGFP expression, suggests that all insertions except one are silent, that is, they do not express EGFP. This is supported by the observation that in subsequent generations

some of the insertions are lost without apparent changes in EGFP expression patterns. A possible explanation for the existence of silent insertions is discussed below.

Multiple integrations, although useful for enhanced transgene expression, may increase the potential for lethal or semilethal mutations, decreasing the viability of transgenic lines and furthermore, may complicate applied use of transgenic strains. This, however, could be avoided by using transposase-expressing helper plasmids or lower concentrations of transposase mRNA in embryo injections.

Patterns of EGFP fluorescence varied dramatically among different transgenic lines of olive fly (Fig. 2) and medfly (Fig. 1); in these species EGFP expression was characteristically tissue-specific and variegated. In contrast, the majority of *Drosophila* transformants carrying single insertions of the self-stimulating cassette exhibited strong and ubiquitous expression of EGFP (Fig. 1). Expression of the EGFP gene from the self-activating construct used in these experiments depends on initial activation (triggering) of the minimal *Hsp70* promoter. It is known that the complete *Drosophila Hsp70* promoter exhibits low activity in transgenic non-*Drosophila* species (Berger *et al.*, 1985; our own unpublished results from medfly). It is possible that the minimal *Drosophila Hsp70* promoter is also less active in medfly and olive fly, failing to 'trigger' the self-sustaining loop in these species, unless the transposon has inserted near an active enhancer. This could explain both tissue-specific, variegated expression from some insertions and lack of expression from others.

This report describes what appears to be a universal genetic marker for detection of transgenic organisms and its use for transgenesis of the major olive pest *B. oleae* by means of *Minos*-mediated germline transformation. With this system, the olive fly is now amenable to transgenesis. Availability of a transformation system for *B. oleae* should now make it possible to use genetic engineering for development of genetic strategies for control of this important insect pest.

Experimental procedures

Fly strains and rearing

The olive fruit flies used in this work originated from the Democritos Laboratories (Athens, Greece) *B. oleae* stock. Flies were bred locally at 25 °C, 45–50% relative humidity, under a 13 h light/11 h dark cycle and were fed on a diet consisting of 100 g yeast hydrolysate, 400 g sugar, 30 g egg yolk and 250 mg streptomycin. Newly hatched larvae were transferred on to the surface of larval medium consisting of 550 ml distilled water, 30 g soy hydrolysate, 0.5 g potassium sorbate, 2 g Nipagin, 20 g sugar, 75 g brewer's yeast, 30 ml concentrated HCl, 275 g cellulose powder, 20 ml olive oil and 7.5 ml Tween-80 (Tzanakakis, 1989). Larvae burrowed into the food and emerged again when ready to pupate. At this stage they were transferred into small plastic boxes for pupation. Pupae were kept at 25 °C until adult emergence.

Plasmids and helper RNA

Donor plasmid pMiBO14/GtTA2, containing the 'insect' self-sustaining marker expression cassette was constructed as follows: The EGFP cassette along with the *hsp70* minimal promoter and the SV40 polyadenylation sequence was isolated from p3XP3-EGFP (Horn & Wimmer, 2000) as a *Bst*BI (filled-in)/*Cla*I fragment and was cloned into the *Eco*RV and *Cla*I sites of pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) to generate pBS/hsTATA-EGFP. The tetracycline operator (tetO) sequence was obtained as an *Alu*I fragment from PBI-L (Clontech, Mountain View, CA, USA). Two copies of this fragment were cloned into the *Sma*I site of pBluescript II SK (+) to give a tetO-14mer (pBS/14tetO). The *hsp70* minimal promoter, the tTA gene, the *hsp70* polyadenylation sequence and the tetO 14mer (tetO-14) were then cloned into an *Eco*RI/*Not*I-digested pBS/hsTATA-EGFP in a five-fragment ligation giving pBO14/GtTA. The *hsp70* minimal promoter was on a *Bam*HI/*Kpn*I fragment from plasmid p3XP3-EGFP (Horn & Wimmer, 2000), hsp70pA was on a *Xba*I/*Not*I fragment from plasmid pHSS6hs/LMi2 (Loukeris *et al.*, 1995b) and tetO-14 was on a *Bam*HI/*Not*I fragment from pBS/14tetO. The tTA gene was a *Kpn*I/*Nhe*I fragment from plasmid pBI/GtT. Plasmid pBI/GtT was constructed as follows: the EGFP gene was cloned on a *Pst*I/*Xba*I fragment from plasmid pEGFP-N1 pBI/GtT (Clontech) into plasmid pPBI-l (Clontech), replacing the luciferase gene, resulting in plasmid pPB1-g. The tTA gene derived from pUHD15-1 plasmid (Gossen & Bujard, 1992) on a *Eco*RI/*Bam*HI fragment was then cloned blunt into the *Pvu*II site of pPB1-g, resulting in plasmid pBI/GtT. The *Xho*I site of pBO14/GtTA was destroyed by fill in to give pBO14/GtTA2. An *Xho*I/*Xba*I fragment of this vector containing the whole cassette except for the tTA gene and the hsp70pA was cloned into pMiLRtetR (Klinakis *et al.*, 2000b) to give pMiBO14-G. The rest of the cassette from pBO14/GtTA2 was subsequently moved as an *Xba*I/*Not*I fragment into pMiBO14-G to reconstitute the tTA gene, resulting in pMiBO14/GtTA2. The donor plasmid pMiBO7/GtTA was constructed in an analogous manner as pMiBO14/GtTA2. *Minos* transposase mRNA was synthesized using the linearized plasmid pBlue(SK)MimRNA as a template (Pavlopoulos *et al.*, 2004), according to the manufacturer's instructions (Ambion mMessage mMachine T7 kit Austin, TX, USA).

Expression from the tTA/EGFP cassette in animal and plant cells

Germ line transformation of *D. melanogaster* and *Ceratitis capitata* was performed as described previously (Loukeris *et al.*, 1995a,b). HeLa cells were cultured and transfected with super-coiled plasmid DNA by Ca^{++} coprecipitation as described previously (Klinakis *et al.*, 2000b) and examined for EGFP fluorescence 12 h post-transfection. Transfection of *Nicotiana tabacum* leaf cells was carried out with a PDS-1000/Helios Bio-Rad gene-gun (Bio-Rad, Hercules, CA, USA). DNA was loaded on 1 μm gold particles. Bombardment was performed under vacuum using 1100 psi rupture discs and the leaf-disc-containing Petri dish was placed on the top shelf of the apparatus. Leaves were cultivated for two days in Murashige and Skoog (SM) medium (Murashige & Skoog, 1962) and were subsequently examined for EGFP fluorescence. Standard procedures were followed for *Caenorhabditis elegans* strain maintenance, crosses and other genetic manipulations (Brenner, 1974). The nematode rearing temperature was kept at 20 °C. Supercoiled plasmid DNA was injected into the gonads of *Caenorhabditis elegans* N2 animals, together with plasmid pRF4 carrying the dominant transformation marker *rol-6(su1006)* as

described (Mello *et al*., 1992). Transgenic lines were obtained by screening the F_2 generation progeny of injected hermaphrodites for roller animals. Individual transgenic rollers were examined for EGFP fluorescence.

Olive fly embryo collection and microinjection

Adult females were allowed to oviposit on to ceresin wax cones for 30 min. Deposited eggs were collected by rinsing with deionized water and dechorionated for 1 min in 2.5% sodium hypochlorite, followed by extensive washes with deionized water. Eggs were aligned on 24 × 50 mm cover slips using double-sided tape, the posterior pole of each egg facing the outer edge of the tape, and covered with halocarbon oil (Sigma, St Louis, MO, USA). Transposon plasmid pMiBO14/GtTA2 (400 ng/μl) and helper RNA (100 ng/μl) (Kapetanaki *et al*., 2002) in injection buffer (1) were coinjected into the posterior pole of eggs, 45–120 min after oviposition. Following injection, G0 embryos were kept for 2–3 days in a humidified Petri dish at 25 °C. Hatchlings were placed on larval food and grown as described above. G0 individuals were back-crossed to the parental strain. G0 males were group-mated to virgin female flies in a standard *Bactrocera oleae* 30 × 30 × 30 cm cage at a 1 : 4 (male : female) ratio; G0 females were group-mated to males at a 1 : 1 ratio. G1 eggs were collected daily until no more viable eggs were produced. Eggs were washed with dH_2O and incubated at 25 °C for 48 h in 0.3% propionic acid for hatching. First instar larvae were transferred to 90 cm Petri dishes containing larval food. G1 individuals were allowed to develop to third larval instar and were assayed for EGFP expression.

Mating and selection strategy

EGFP positive G1 individuals were transferred into small plastic boxes and left to pupate as described above. Single G1s were backcrossed to wild-type flies in small cages (5 × 5 × 10 cm). G1 males were kept at a ratio of 1 : 6 with females, G1 females at a ratio 1 : 1 with males. Thin domes of ceresin wax were introduced into each cage as oviposition surfaces on the third day of adult life. Eggs were collected from the fifth day of adult life and subsequent collections occurred every other day until the 10th collection. Third instar G2 larvae were screened for EGFP expression and the EGFP-positive larvae selected and backcrossed to the parental strain as above. Three generations of backcrosses of EGFP-positive adults to the parental strain were performed. Subsequently, transformed lines were maintained by selection for EGFP positive progeny for further breeding.

Southern blot analysis

DNA from single adult flies was purified essentially as described previously (Laird *et al*., 1991). Flies were homogenized in 0.25 ml 50 mM Tris-HCl pH 8.0, 100 mM EDTA, 1% SDS, 100 mM NaCl, 330 μg/ml proteinase K and incubated at 55 °C overnight. Samples were treated with a DNAse-free RNAse solution (200 μg/ml) at 37 °C for 1 h. This incubation was followed by two phenol/chloroform extractions and one chloroform extraction. The supernatant was transferred to a fresh tube, DNA was isopropanol precipitated, washed with 70% ethanol and resuspended in 20 μl sterile ddH_2O. DNA concentrations were determined by spectrophotometry at 260 nm. Five micrograms of genomic DNA were digested with *Eco*RI, and the resulting fragments separated on a 0.8% agarose gel. After transfer to nylon membranes, blots were hybridized with a ^{32}P-labelled GFP probe. The probe was a 720 bp *Bam*HI/*Xba*I fragment containing most of the GFP gene.

Inverse PCR

Genomic DNA (5 μg) of transformants was digested with iso*Mbo*I (Minotech Heraklion, Greece) and restriction fragments were circularized by overnight ligation at 16 °C at a final concentration of 200 ng/ml. PCR was performed on the circularized fragments using primers Imio1 (5′-AAGAGAATAAAATTCTCTTTGAGACG-3′) and Imii1 (5′-CAAAAATATGAGTAATTTATTCAAACGG-3′), followed by a second round of nested PCR with primers Imio2 (5′-GATAATATAGTGTGTTAAACATTGCGC3′) and Imii2 (5′-GCTTAAGAGATAAGAAAAAAGTGACC3′) (Klinakis *et al*., 2000a). PCR conditions were in both cases: 94 °C for 30 s; 58 °C for 30 s; 72 °C for 2 min; 30 cycles. PCR fragments were separated on a 2% agarose gel. Purified fragments were cloned into vector pGEM-T easy (Promega, Madison, WI, USA) and sequenced using T7 and SP6 primers.

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References

Allen, M.L., Handler, A.M., Berkebile, D.R. and Skoda, S.R. (2004) PiggyBac transformation of the New World screwworm, *Cochliomyia hominivorax*, produces multiple distinct mutant strains. *Med Vet Entomol* **18**: 1–9.

Bellen, H.J., O'Kane, C.J., Wilson, C., Grossniklaus, U., Pearson, R.K. and Gehring, W.J. (1989) P-element-mediated enhancer detection: a versatile method to study development in Drosophila. *Genes Dev* **3**: 1288–1300.

Berger, E.M., Marino, G. and Torrey, D. (1985) Expression of *Drosophila* hsp70-CAT hybrid gene in *Aedes* cells induced by heat shock. *Som Cell Molec Gen* **11**: 371–377.

Berghammer, A.J., Klingler, M. and Wimmer, E.A. (1999) A universal marker for transgenic insects. *Nature* **402**: 370–371.

Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.

Catteruccia, F., Nolan, T., Loukeris, T.G., Blass, C., Savakis, C., Kafatos, F.C. and Crisanti, A. (2000) Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature* **405**: 959–962.

Coates, C.J., Jasinskiene, N., Miyashiro, L. and James, A.A. (1998) Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci USA* **95**: 3748–3751.

Drabek, D., Zagoraiou, L., deWit, T., Langeveld, A., Roumbaki, C., Mamalaki, C., Savakis, C. and Grosveld, F. (2003) Transposition of the *Drosophila hydei Minos* transposon in the mouse germ line. *Genomics* **81**: 108–111.

Economopoulos, A.P. and Zervas, G.A. (1982) The quality problem in olive flies produced for SIT experiments. Invited international symposium on the SIT and the use of radiation in genetic insect control. *IAEA SIT/PUB* **592**: 357–368.

Economopoulos, A.P., Artis, N., Zervas, G., Tsitsipis, G., Haniotakis, G., Tsiropoulos, G. and Mamoukas, A. (1977) Control of the olive fly, *Dacus oleae* (Gmelin), by the combined effect of insecticides and release of gamma sterilized insects. *Z Angew Ent* **83**: 201–215.

Ferreira, J.R. and Tainha, A.M. (1983) Organophosphorus insecticide residues in olives and olive oil. *Pestic Sci* **14**: 167–172.

Franz, G. and Savakis, C. (1991) *Minos*, a new transposable element from *Drosophila hydei*, is a member of the Tc-1 like family of transposons. *Nucleic Acids Res* **19**: 6646.

Gong, P., Epton, M.J., Fu, G., Scaife, S., Hiscox, A., Condon, K.C., Condon, G.C., Morrison, N.I., Kelly, D.W., Dafa'alla, T., Coleman, P.G. and Alphey, L. (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nature Biotechnol* **23**: 453–456.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* **89**: 5547–5551.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., Stevens, T.K., Mukabayire, O. and Benedict, M.Q. (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. *Insect Mol Biol* **10**: 597–604.

Handler, A.M., Gomez, S.P. and O'Brochta, D.A. (1993) A functional analysis of the P-element gene-transfer vector in insects. *Arch Insect Biochem Physiol* **22**: 373–384.

Handler, A.M., McCombs, S.D., Fraser, M.J. and Saul, S.H. (1998) The lepidopteran transposon vector, piggyBac, mediates germline transformation in the Mediterranean fruitfly. *Proc Natl Acad Sci USA* **95**: 7520–7525.

Haniotakis, G., Koryzakis, M., Fitsakis, T. and Antonidaki, A. (1991) An effective mass-trapping method for the control of *Dacus oleae* (Diptera: Tephritidae). *J Econ Entomol* **84**: 564–569.

Hediger, M., Niessen, M., Wimmer, E.A., Dubendorfer, A. and Bopp, D. (2001) Genetic transformation of the housefly *Musca domestica* with the lepidopteran derived transposon piggyBac. *Insect Mol Biol* **10**: 113–119.

Heinrich, J.C. and Scott, M. (2000) A repressible female-specific lethal genetic system for making transgenic insect strains suitable for a sterile-release program. *Proc Natl Acad Sci USA* **97**: 8229–8232.

Heinrich, J.C., Li, X., Henry, R.A., Haack, N., Stringfellow, L., Heath, A.C.G. and Scott, M.J. (2002) Germ-line transformation of the Australian sheep blowfly *Lucilia cuprina*. *Insect Mol Biol* **11**: 1–10.

Horn, C. and Wimmer, E.A. (2000) A versatile vector set for animal transgenesis. *Dev Genes Evol* **210**: 630–637.

Jasinskiene, N., Coates, C.J., Benedict, M.Q., Cornel, A.J., Rafferty, C.S., James, A.A. and Collins, F.H. (1998) Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. *Proc Natl Acad Sci USA* **95**: 3743–3747.

Kapetanaki, M.G., Loukeris, T.G., Livadaras, I. and Savakis, C. (2002) High frequencies of *Minos* transposon mobilization are obtained in insects by using *in vitro* synthesized mRNA as a source of transposase. *Nucleic Acids Res* **30**: 3333–3340.

Klinakis, A.G., Loukeris, T.G., Pavlopoulos, A. and Savakis, C. (2000a) Mobility assays confirm the broad host-range activity of the *Minos* transposable element and validate new transformation tools. *Insect Mol Biol* **9**: 269–275.

Klinakis, A.G., Zagoraiou, L., Vassilatis, D.K. and Savakis, C. (2000b) Genome-wide insertional mutagenesis in human cells by the *Drosophila* moile element *Minos*. *EMBO Reports* **1**: 416–421.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R. and Berns, A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* **19**: 4293.

Lorenzen, M.D., Berghammer, A.J., Brown, S.J., Denell, R.E., Klinger, M. and Beeman, R.W. (2003) *piggyBac*-mediated germline transformation in the beetle *Tribolium castaneum*. *Insect Mol Biol* **12**: 433–440.

Loukeris, T.G., Arca, B., Livadaras, I., Dialedtaki, G. and Savakis, C. (1995a) Introduction of the transposable element *Minos* into the germ line of *Drosophila melanogaster*. *Proc Natl Acad Sci USA* **92**: 9485–9489.

Loukeris, T.G., Livadaras, I., Arca, B., Zabalou, S. and Savakis, C. (1995b) Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. *Science* **270**: 2002–2005.

Markaki, M., Craig, R.K. and Savakis, C. (2004) Insect population control using female specific pro-drug activation. *Insect Biochem Mol Biol* **34**: 131–137.

Mello, C.C., Kramer, J.M., Stinchcomb, D. and Ambros, V. (1992) Efficient gene transfer in *C. elegans*: extrachromosomal maintenance integration transforming sequences. *EMBO Journal* **10**: 3959–3970.

Michel, K., Stamenova, A., Pinkerton, A.C., Franz, G., Robinson, A.S., Gariou-Papalexiou, A., Zacharopoulou, A., O'Brochta, D.A. and Atkinson, P.W. (2001) *Hermes*-mediated germ-line transformation of the Mediterranean fruit fly *Ceratitis capitata*. *Insect Mol Biol* **10**: 155–162.

Murashige, T. and Skoog, F. (1962) Revised medium for rapid growth and bioessays with tobacco tissue cultures. *Physiol Plant* **15**: 425–433.

Pavlopoulos, A., Berghammer, A.J., Averof, M. and Klingler, M. (2004) Efficient transformation of the beetle *Tribolium castaneum* using the *Minos* transposable element: quantitative and qualitative analysis of genomic integration events. *Genetics* **167**: 737–746.

Perera, O.P., Harrell, R.A. and Handler, A.M. (2002) Germ-line transformation of the South American malaria vector, *Anopheles albimanus*, with *piggyBac*/EGFP transposon vector is routine and highly efficient. *Insect Mol Biol* **11**: 291–297.

Pucci, C. (1990) Valutazione dell'efficacia delle esche proteiche avvelenate per il controllo del Dacus oleae (Gmel): sperimentazione condotta nel triennio 1988–90 nell'alto lazio. *Frustula Entomologica, N S* **XIII**(XXVI): 173–198.

Rubin, G.M. and Spradling, A.C. (1982) Genetic transformation of Drosophila with transposable element vectors. *Science* **218**: 348–353.

Sasakura, Y., Awazu, S., Chiba, S. and Satoh, N. (2003) Germ-line transgenesis of the Tc1/*mariner* superfamily transposon *Minos* in *Ciona intestinalis*. *Proc Natl Acad Sci USA* **100**: 7726–7730.

Tamura, T., Thibert, C., Royer, C., Kanda, T., Abraham, E., Kamba, M., Komoto, N., Thomas, J.L., Mauchamp, B., Chavancy, G., Shirk, P., Fraser, M., Prudhomme, J.C., Couble, P., Toshiki, T., Chantal, T., Corinne, R., Toshio, K., Eappen, A., Mari, K., Natuo, K., Jean-Luc, T., Bernard, M., Gerard, C., Paul, S., Malcolm, F., Jean-Claude, P. and Couble, C. (2000) Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nature Biotechnol* **18**: 81–84.

Thomas, D., Donnelly, C., Wood, R. and Alphey, L. (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* **287**: 2474–2476.

Triglia, T., Peterson, M.G. and Kemp, D.J. (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. *Nucleic Acids Res* **16**: 8186.

Tzanakakis, M.E. (1989) Small-scale rearing: *Dacus oleae*. In *Fruit Flies their Biology, Natural Enemies and Control*. Vol. 3B. (Robinson, A.S. and Hooper, G., eds), pp. 105–118, Elsevier, Amsterdam.

Tzanakakis, M.E. (1995) *Entomology*, pp. 365–377. University Studio Press, Thessaloniki, Greece. (in Greek)

Wilson, C., Pearson, R.K., Bellen, H.J., O'Kane, C.J., Grossniklaus, U. and Gehring, W.J. (1989) *P*-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Dev* **3**: 1301–1313.

Zagoraiou, L., Drabek, D., Alexaki, S., Guy, J.A., Klinakis, A.G., Langerveld, A., Skavdis, G., Mamalaki, C. and Grosveld, F. (2001) In vivo transposition of *Minos*, a *Drosophila* mobile element, in mammalian tissues. *Proc Natl Acad Sci USA* **98**: 11474–11478.