TÜBİTAK

Turkish Journal of Biology

http://journals.tubitak.gov.tr/biology/

Research Article

Turk J Biol
(2016) 40: 845-855
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doi:10.3906/biy-1510-55

Germline transformation of the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), with a *piggyBac* transposon vector

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Received: 21.10.2015 • **Accepted/Published Online:** 07.12.2015 • **Final Version:** 21.06.2016

Abstract: The olive fruit fly, *Bactrocera oleae*, is a highly significant pest in olive growing countries, and controlling it may be enhanced by using genetically modified strains, especially for sterile insect technique programs. To improve and expand this technology, *piggyBac*-mediated germline transformation was achieved in a laboratory-adapted wild olive fruit fly strain. A *piggyBac* vector was used that is marked with both green (EGFP) and red (DsRed) fluorescent protein genes, with a duplicate *piggyBac* 5' terminal inverted repeat sequence inserted between the marker genes for subsequent immobilization of vectors integrated into the host genome. Five transformant G1 adults were selected based on marker gene expression, yielding an estimated minimum germline transformation frequency of approximately 1.8% per G0 adult. All transgenic lines expressed DsRed and EGFP, although DsRed was more visible and robust compared to EGFP expression, which remained stable for more than 20 generations. Marker expression and PCR analysis, including an insertion site sequence, was consistent with stable genomic insertions. This is the first study of *B. oleae* transformant lines to assess life fitness parameters, including egg hatching, larval survival, larval-to-pupal survival, pupal-to-adult survival, and fertility. In three transgenic lines, survival at all biological stages was similar; overall fitness was significantly lower compared to wild-type olive flies, but similar to fitness levels previously reported for transgenic Mexican fruit flies. The studies presented here demonstrate the development of marked strains for olive fly using *polyubiquitin*-regulated fluorescent proteins in transformation vectors that can be stabilized for strain stability and ecological safety. This is the first successful effort to establish transgenic strains for an important agricultural pest in Turkey.

Key words: *Bactrocera oleae*, olive fruit fly, transformation, *piggyBac*, transposon vector

1. Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the most important pest affecting olive orchards in Mediterranean countries, including Turkey (Economopoulos, 2002; Daane and Johnson, 2010; Genç, 2012). It is also distributed in South and Central Africa, the Canary Islands, Central America, California, the Near and Middle East, and China. Thus far, the only region from which olive fly infestations have not been reported is Australia (Nardi et al., 2005). The olive fly is a monophagous pest (Weens and Nation, 2003) and females may lay multiple eggs in a single olive fruit (Genc and Nation, 2008a), where larvae complete their development. This causes severe economic damage due to quantitative losses and reduced quality of both olives and olive oil.

Olive flies are reared in the laboratory on olive fruits (Genc and Nation, 2008b), and artificial diets have also been developed (Tsitsipis and Kontos, 1983; Tzanakakis, 1989; Genc, 2008). However, continuous rearing of wild flies on artificial diets affects their physiology, behavior, reproductive biology, and allele frequencies for the polymorphic alcohol dehydrogenase locus (Economopolos, 1980; Loukas et al., 1985; Cosmidis et al., 2002). Traditionally, the control of olive fly in Turkey and many other olive-growing countries has been based on chemical insecticides. However, intensive use of insecticides (cover spray or baits) has led to the positive selection of two acetylcholinesterase mutations, resulting in insecticide resistance (Vontas et al., 2002, 2011).

The sterile insect technique (SIT) is the most encouraging biologically based technique for fruit fly integrated control (Enkerlin and Mumford, 1997; Hendrichs et al., 2002). It is a species-specific control method dependent upon the release of large numbers

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of sterile male insects (Knipling, 1955; Koyoma et al., 2004; Dyck et al., 2005) and has been successfully used against several pest species (Handler and O'Brochta, 2012) including several tephritid flies. Evaluation of SITs for olive fly faced difficulties including poor laboratory adaptation for mass rearing and less than optimal artificial diets, and programs for olive fly SITs were abandoned (Economopoulos et al., 1977; Zervas and Economopoulos, 1982; Economopoulos, 2001). The success of SITs for olive fly is dependent upon larval rearing; larvae should have features similar to the wild larvae, including comparable vigor and behavior (Genc and Nation, 2008b; Genç, 2012).

The *piggyBac* transposable element originated from the cabbage looper moth, *Trichoplusia ni* (Fraser et al., 1983; Cary et al., 1989), and it has been used in effective gene-transfer vectors in tephritids such as Mediterranean fruit fly *Ceratitis capitata* (Handler et al., 1998; Gong et al., 2005; Dafa'alla et al., 2006; Scolari et al., 2008; Schetelig et al., 2009a, 2009b), Mexican fruit fly *Anastrepha ludens* (Condon et al., 2007; Meza et al., 2011), Caribbean fruit fly *A. suspensa* (Handler and Harrell, 2001a, 2001b; Zimowska et al., 2009; Schetelig and Handler, 2013a, 2013b), oriental fruit fly *Bactrocera dorsalis* (Handler and McCombs, 2000), olive fly *B. oleae* (Ant et al., 2012), and Queensland fruit fly *B. tryoni* (Raphael et al., 2011). The *piggyBac* vector system is also widely used in other insects within five different orders (Handler and Schetelig, 2014).

Different marker systems have been used to distinguish transformed flies from wild flies, such as eye pigmentation markers (Handler et al., 1998), chemical resistance markers (Steller and Pirrotta, 1985), and fluorescent protein gene markers (Lee et al., 1988; Handler and Harrell, 2001a; Schetelig and Handler, 2013b). Fluorescent protein markers can also be used for field detection of transgenic flies in traps and have been shown to be stable for up to 2–3 weeks in dead flies under dry conditions (Handler and Harrell, 2001b) or in liquid traps (Nirmala et al., 2011). To further study the genetic modification of the olive fly, *B. oleae*, we report here the germline transformation of an olive fly strain native to Turkey using a *piggyBac* vector marked with two fluorescent protein genes, which can be immobilized after integration for the enhanced environmental safety of released flies.

2. Materials and methods

2.1. Insect strains and rearing

The wild-type strain of olive fruit fly was collected from infested fruits in Çanakkale Province, Turkey, and reared in the Department of Agricultural Biotechnology at Çanakkale Onsekiz Mart University for 2 years for adaption to laboratory conditions. The colony was reared at 26 ± 1 °C, 60%–65% RH, under an 18-h light/6-h dark cycle. Wild and transformant larvae were maintained on a diet consisting of 825 mL of distilled water, 45 g of soy hydrolysate, 112 g of unhydrolyzed brewer's yeast, 30 g of sugar, 30 mL of olive oil, 11.25 mL of Tween 20, 3 g of nipagin, 0.75 g of potassium sorbate, 45 mL of HCl, and 462 g of cellulose powder (Tsitsipis and Kontos, 1983; Tzanakakis, 1989). Mature third instars were placed on moistened vermiculite for pupation until adult emergence. Adult flies were reared on a 3:1 mixture of sugar and yeast hydrolysate and water (Tzanakakis, 1989), and paraffin domes were used as oviposition substrates to obtain eggs (Tzanakakis, 1989).

2.2. Plasmids

The *piggyBac* vector, pB[L1-EGFP-L2-DsRed-R1], is a previously described stabilization vector (Handler et al., 2004; Meza et al., 2011) with an internal *piggyBac* 5' (left arm; L2) terminal sequence added between the PUb-nls-EGFP and PUb-DsRed.T3 marker sequences and in direct tandem orientation with the external 5' terminal (L1) sequence, as previously described (Figure 1). Briefly, the vector plasmid was designed by digesting pB[PUb-DsRed1] (Handler and Harrell, 2001a) upstream to its *piggyBac* 5' terminal sequence with addition to the PUb-nls-EGFP marker and linked *piggyBac* 5' terminal sequence from pB[PUb-nls-EGFP] (Handler and Harrell, 2001b), making an L1-PUb-nls-EGFP-L2-PUb-DsRed1-R1 configuration for the vector. Along with the helper plasmid, this vector integrates using the L1 and R1 ends with transformant individuals marked with both EGFP and DsRed or the subvector L2 and R1 ends, which have only the DsRed marker. The helper plasmid, *phsp-pBac*, designed as the *piggyBac* transposase gene under *hsp*70 regulation with a deletion of the 5' terminal sequence, was reported previously (Handler and Harrell, 1999).

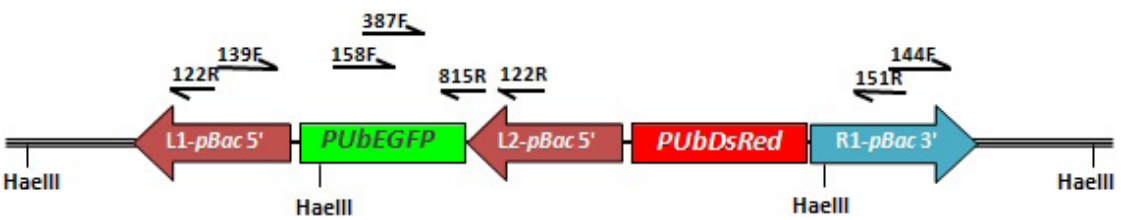

Figure 1. Schematic of the pB[L1-EGFP-L2-DsRed-R1] vector (not to scale). The relative positions of *Hae*III restriction sites and primers are shown. See Table 1 for primer sequences; primer 158F represents EGFP-158F primer and 815R represents EGFP-815R.

2.3. Embryo preparation and microinjections

Olive fly eggs were collected in paraffin domes by washing with 0.05% propionic acid and were transferred to sterile, moistened white filter paper. Embryo microinjection was modified from the *Drosophila* (Rubin and Spradling, 1992) and Mediterranean fruit fly (Handler et al., 1998; Handler, 2000) procedures. Embryos were dechorionated for 1 min in 1.6% hypochlorite solution then washed several times with 0.02% Triton-X100. Dechorionated embryos were placed on coverslips with double-stick tape with the posterior pole towards the edge of the tape, desiccated at room temperature for 8–10 min, and then covered with halocarbon oil (Series 700, Sigma). Preblastoderm embryos were microinjected with a 600 µg/mL vector and 400 µg/mL *phsp-pBac* helper DNA mixture in the injection buffer (5 mM KCl; 0.1 M sodium phosphate, pH 6.8) in the posterior ends. Microinjected eggs were placed in an oxygenated and humidified tissue culture chamber at 25 °C, and they were heat-shocked at 37 °C for 1 h at 16–20 h after microinjection. Emerged G0 adult flies were backcrossed individually to wild olive flies with resulting G1 adult progeny screened under epifluorescence microscopy for EGFP and DsRed expression using a Leica MZ FLIII stereomicroscope with GFP1 or Texas Red (TxRed) filters, respectively.

2.4. PCR analysis

Transformant flies identified by marker fluorescence were tested for genomic vector insertions by direct PCR using primers to the *piggyBac* 5' terminal inverted sequence (TIR) and the PUb-EGFP marker gene (*EGFP*) (Table 1; Figure 1). Inverse PCR was performed as described previously (Meza et al., 2011) in order to determine the genomic insertion sites at the 5' and 3' termini of the integrated pB[L1-EGFP-L2-DsRed-R1] vector. Then 1–3 µg of transformant genomic DNA samples was isolated with the Blood & Tissue Kit (QIAGEN) and digested with *Hae*III, and fragments were circularized by ligation overnight at 16 °C. PCR was accomplished with ligated DNA using primers 122R and 139F for the 5' *piggyBac* (*pBac*) end and 144F and 151R for the 3' *pBac* end, with Expand Long Template DNA Polymerase (Roche Applied Science). PCR cycling conditions were as follows: denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 2 min, with an extension at 68 °C for 10 min. PCR amplicons were cloned into the TOPO TA vector pCR 2.1 (Invitrogen). BigDye terminator (Applied Biosystems) was used for sequencing with vector primers in order to detect the genomic sequences at the insertion site. PCR amplicons were determined on a 1% agarose gel and removed and extracted for subcloning and then sequencing.

2.5. Phenotype analysis of DsRed and EGFP

Phenotypic expression of marked transgenic individuals was observed under a Leica MZ FLIII Stereozoom microscope using a mercury lamp and a long pass wavelength FLUOIII filter system (Leica). To detect EGFP and DsRed, fluorescence filter sets GFP1 (ex: 425/60; em: 480 LP) and TxRed (ex: 560/40; em: 610 LP) were used, respectively. A Leica DFC320 camera was used for documentation.

Table 1. PCR primers used for internal direct PCR and inverse PCR for genomic insertion site sequence analysis of pB[L1-EGFP-L2-DsRed-R1] transformant olive flies.

Primer	Sequences: 5'-3' orientation
122R	ATCAGTGACACTTACCGCATTGACA
139F	CCAGAGCGATACAGAAGAAGC
144F	CCTCGATATACAGACCGATAAAACAC
151R	CTAAAATAAGGCGAAAGGCAA
387F	GCAGAAGAACGGCATCAAGGTG
EGFP-158F	GCGACGTAAACGGCCACAAGTT
EGFP-815R	TACTTGTACAGCTCGTCCATGCCG

2.6. Biological attributes of transgenic olive fruit flies

Fresh eggs were collected for 2–3 h in wax domes from each transgenic colony (G3) containing 100 males and 100 females and a wild-type laboratory colony of olive flies. One hundred eggs per replicate were transferred to petri dishes containing moistened black filter paper (n = 4) and incubated at 26 ± 1 °C for 4 days to assess the rate of larval hatching. One hundred newly hatched first instars were gently collected under the Olympus SZX9 Stereozoom microscope and transferred to petri dishes containing a larval diet (Tsitsipis and Kontos, 1983). From additional eggs, 100 first instars were collected and transferred to petri dishes containing a larval diet (Tsitsipis and Kontos, 1983). Survival to subsequent biological stages was recorded to calculate overall fitness (McCombs et al., 1993; Meza et al., 2011).

3. Results

3.1. Transformation experiments

Germline transformation of *B. oleae* was tested in a wild strain of olive fruit fly by microinjection of the *piggyBac* vector, pB[L1-EGFP-L2-DsRed-R1], and a *hsp70*-regulated transposase helper into 3714 preblastoderm embryos in a total of four independent experiments. For the first experiment, 512 embryos were injected, from which 15 larvae hatched with 10 emerging as adults (Table 2). The 169 G0 adults were backcrossed to wild flies in small groups (5♀:3♂). Three of the four transformation experiments yielded viable G1 progeny; from these, 2153 adults were screened for DsRed and GFP expression as adults (Table 2). A total of three putative transformant G1 adults from

Table 2. Transformation experiments with the vector pB[L1-EGFP-L2-DsRed-R1].

Experiments	No. of embryos injected	No. of G_0 larvae (% hatch)	No. of G_0 eclosed adults	No. of G_0 lines	G_1 eclosed adults	No. of G_1 transformants
1	512	15 (2.9)	10	3	42	0
2	873	11 (1.2)	7	2	0	0
3	1249	103 (8.2)	83	17	1458	2
4	1080	91 (8.4)	69	21	653	3
Total	3714	220 (5.9)	169	43	2153	5

two independent crosses (lines 24 and 10) were detected, and three transgenic mating groups (lines 24-M2, 24-F1, and 10-F2) were established by backcrossing (Table 3). Line 24-M2 yielded 10 transformant adults (G2) with red and green body color phenotypes, line 24-F1 yielded three transformant adults, and line 10-F2 two transformant adults (Table 2). In all three crosses, the G2 ratio of female to male progeny was approximately 1:1 (Table 3), coinciding with a single integration event of the transgene, although insertion site sequence analysis of 24-F1 suggested that two independent integrations occurred in that line. If the segregation analysis is correct, this indicates that the two integrations occurred in the same linkage group (chromosome). Since we could not distinguish between integrations in the sibling 24-F1 and 24-M2 lines, we must assume they arose from the same G0 transformation, while insertion site sequencing revealed two independent integrations in 10-F2. Therefore, the estimated minimum transformation frequency is 1.8% (3 events/169 G0s). We could not estimate the actual frequency based on fertile G0s due to group matings; however, previous tephritid transformations typically yielded approximately 50% G0 fertility, which would increase the transformation to as high as 3.6%. This is comparable to the ~4% estimated transformation frequency for the previous *piggyBac* transformation of *B. oleae* (Ant et al., 2012).

3.2. Fluorescent protein marker expression

Olive fruit fly individuals were observed under bright-field and epifluorescent microscopy with the filter sets TxRed and GFP1 (Figure 2). Adults were observed laterally under bright-field (Figure 2A), DsRed, and EGFP expressions (Figures 2B and 2C). They were shown dorsally under bright-field (Figure 2D), DsRed, and EGFP expressions (Figures 2E and 2F). Adults were also observed ventrally under bright-field (Figure 2G), DsRed, and EGFP expressions (Figures 2H and 2I). All transgenic flies expressed DsRed and EGFP consistent with the genomic integration of the entire vector. However, DsRed expression was brighter, stronger, and more clearly visible in the thorax muscles, legs, and abdomen (Figures 2B, 2E, and 2H) compared to EGFP, which was less visible except for autofluorescence, especially in the eyes of both wild-type and transformed adults (Figures 2C, 2F, and 2I). EGFP detection may be masked or quenched due to cuticle melanization in adult flies, which has been observed for other tephritid species, or by coexpression by the second marker. Moreover, expression of DsRed was also clearly visible throughout the body in mature larvae (Figures 3A and 3B) and pupae (Figures 3C and 3D), while EGFP was not (data not shown).

Homozygous lines were established by setting up single-pair matings (1♀:1♂) for each of the three lines and screening the progeny for fluorescence. After 8 generations of pair mating fluorescent adults, the 24F-1 line was considered putatively homozygous, producing only DsRed-marked transgenic progeny. This line was maintained for 20 generations and screened for fluorescence every generation, showing that the DsRed marker is stable in the germline.

3.3. PCR analysis and insertion site sequences

Direct PCR on the genomic DNA of the three transgenic olive fly lines using the 387F-122R and EGFP158F-

Table 3. Number and phenotype of G_2 progeny from G_1 heterozygous parental lines backcrossed to wild type.

Transgenic lines (G_1)	Total no. of G_2 larvae	Total no. of G_2 adults	No. of G_2 adults expressing DsRed and EGFP
24-M2	39	17	10
24-F1	14	8	3
10-F2	9	4	2

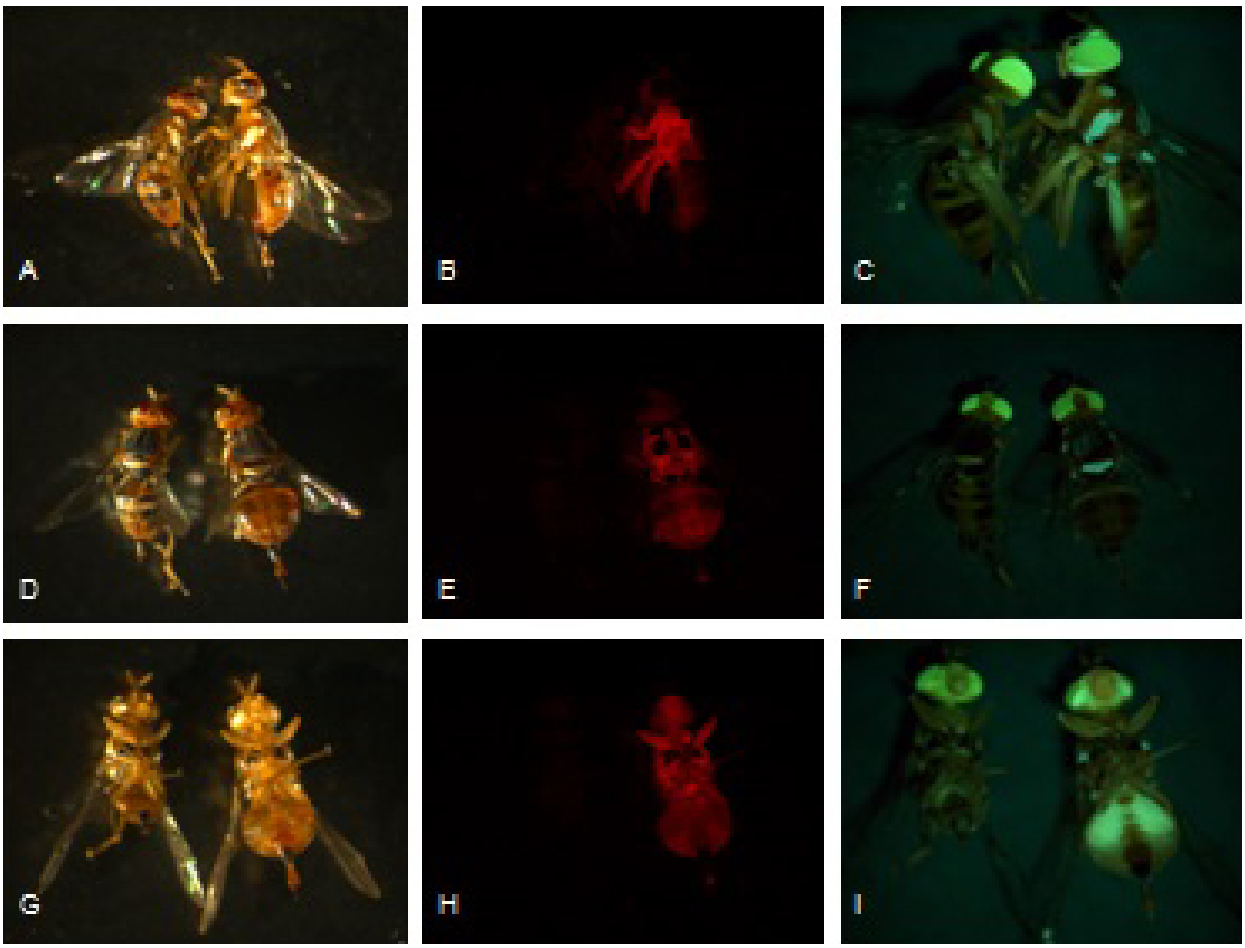

Figure 2. Images of adult transgenic *B. oleae* line 24-F1 with red (DsRed) and green (EGFP) fluorescent markers. Adults were observed under bright-field and epifluorescent microscopy with the filter sets TxRed and GFP1. Images compare nontransformant flies on the left and transformed flies on the right in each panel. (A) Adult lateral view under bright-field, (B) TxRed filter, (C) GFP1; (D) dorsal view under bright-field, (E) TxRed filter, (F) GFP1; (G) ventral view under bright-field, (H) TxRed filter, and (I) GFP1.

EGFP815R primer sets generated the expected sequence lengths of 839 bp and 657 bp for internal vector sequences, respectively (Figure 4). Both amplicons are consistent with intact genomic vector integration, and the 387F-122R amplicon, spanning EGFP and the internal 5' (L2) TIR, was indicative of entire vector integration.

3.4. Genomic insertion sites

In order to confirm that *piggyBac*-mediated genomic integrations occurred, insertion sites were isolated by inverse PCR, subcloned, and then sequenced. The 122R-139F primer set was used to isolate the 5' vector junctions, and the 151R-144F primer set was used for the 3' junctions (Figure 5). For successful PCR reactions, each subcloned sequence was verified for linkage to the expected *piggyBac* 5' or 3' TIR at a TTAA target site, with the genomic sequence terminating at a GG/CC *Hae*III restriction site used to digest the genomic DNA before circularization. The genomic sequence was then used to search the NCBI database using the BLASTn algorithm to determine if the vector integrated into a known orthologous sequence (Figure 5; Supplementary Figure 1). Insertion site sequences were isolated for the 5' junction in line 10-F2, and two distinct 3' junction sequences were isolated for line 24-F1, indicating that at least two independent integrations occurred in this line. The line 10-F2 sequence shows the greatest identity with a *D. melanogaster* small nuclear RNA U6 sequence (Das et al., 1987), and one of the 24-F1 integration sequences shows the greatest identity with the *nesprin-1* nuclear envelope genes (Morel et al., 2014) found in two other tephritid species, *B. dorsalis* and *C. capitata*. No significant similarities were observed for the second 24-F1 integration.

3.5. Life fitness parameters

Biological attributes of the transgenic lines were compared to laboratory-adapted olive flies, and survival at all biological stages was very similar (Table 4). Fertility was

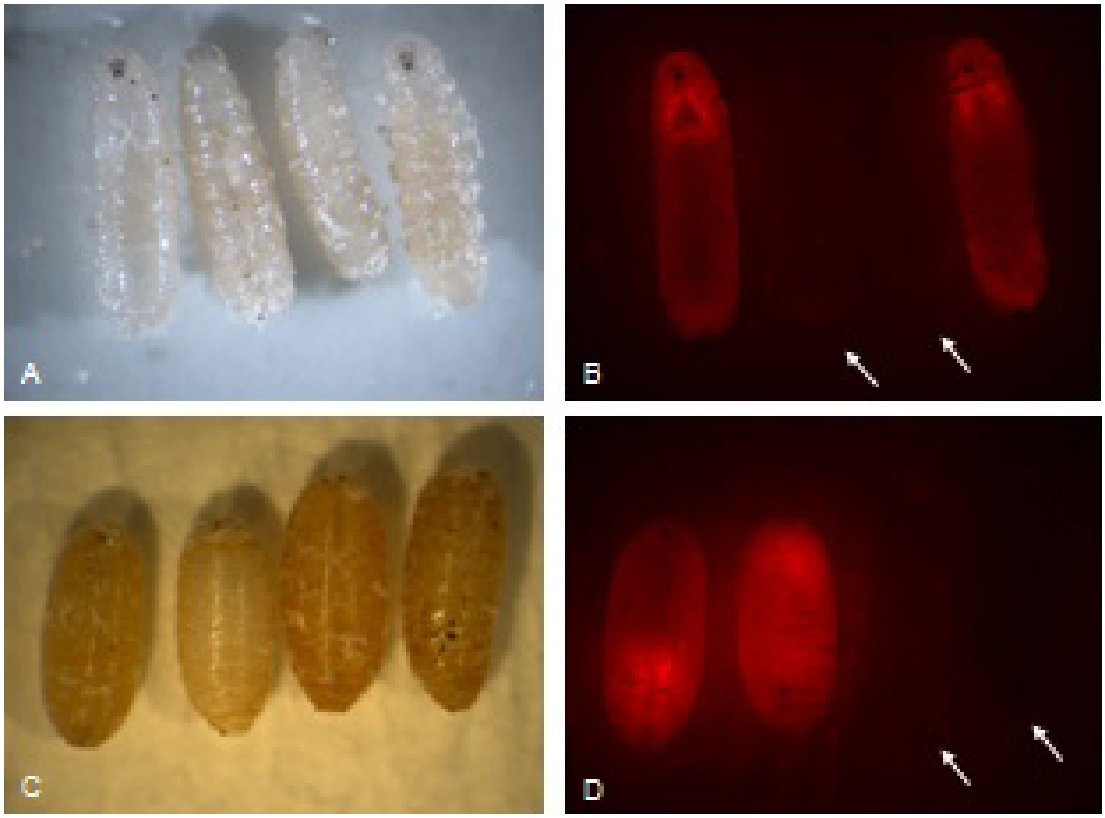

Figure 3. Images of *B. oleae* nontransformed and transgenic line 24-F1 larvae (top panels) and pupae (bottom panels). Larvae were observed under bright-field and epifluorescent microscopy with the TxRed filter set. Arrows indicate nontransformed individuals. (A) Larvae under bright-field, (B) larvae under TxRed filter, (C) pupae under bright-field, and (D) pupae under TxRed filter.

compared in all three transgenic lines and ranged from 89% in 10-F2, 85% in 24-F1, and 88% in 24-M2 compared to 94% in the wild-type control. Larval viability ranged from 82% to 86% in the transgenic lines compared to 90% in the control. Pupal survival ranged from 94% to 97% in the transgenic lines compared to 96% in the control and was not significantly different (Table 4). Pupal-to-adult survival rates ranged from 85% to 89% in transgenic lines compared to 93% in the control, and overall fitness was, more importantly, lower in the transgenic lines than in control flies, with line 24-M2 showing the highest fitness level.

4. Discussion

Here we report the transformation of the olive fruit fly, *B. oleae*, with the *piggyBac* transposon vector, pB[L1-EGFP-L2-DsRed-R1]. Unlike previous attempts to transform this species, *D. melanogaster polyubiquitin* (PUb)-regulated fluorescent protein markers were used to identify transformants, and a *D. melanogaster hsp70*-regulated transposase helper plasmid mediated the transformation (Koukidou et al., 2006; Ant et al., 2012). The goal of this study was also to create transgenic olive fruit flies using a host wild-type strain initially collected from Çanakkale Province in Turkey and adapted to laboratory conditions for 2 years. At least three transformation events verified by insertion-site sequencing from 169 G0 adults resulted in an estimated transformation frequency of ~1.8%, which could be as high as ~3.6% when considering significant infertility or flies failing to mate, which was not apparent in group G0 matings. For the three transformed lines analyzed, segregation analysis was consistent with either a single transgene vector copy in each genome or multiple copies integrated into the same chromosome.

DsRed expression in each of the lines was more visible and robust compared to EGFP expression, unlike the phenotypes described in the Mexican fruit fly, *Anastrepha ludens*, which were transformed with the same vector (Meza et al., 2011). This may be the result of differing insertion site position effects, or it may be a species-specific effect consistent with the initial olive fly transformation using the *Minos* vector marked by EGFP regulated by the tetracycline operator (Koukidou et al., 2006). The transgenic EGFP fluorescence pattern is also difficult

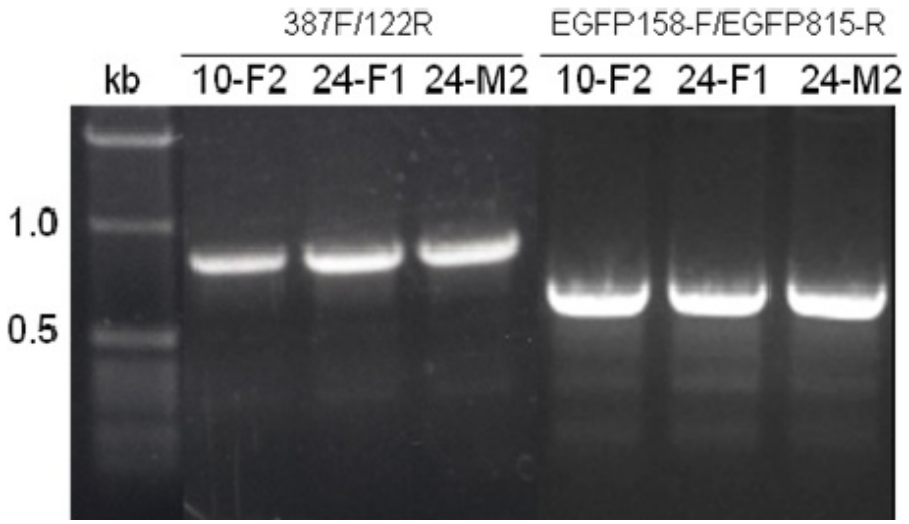

Figure 4. A composite agarose gel showing PCR products from indicated transgenic olive fly line genomic DNA using the 387F-122R or EGFP158F-EGFP815R primers that have expected sequence lengths of 839 bp and 657 bp, respectively.

1) Line 10-F2 pBac 5'sequence
Blastn: *D. melanogaster* small nuclear RNA U6 (locus 3R-96A) GenBank acc: NR_002081; 2e-21; 100% identity

GTTTTTGTACGTAGGTAAATGGATATTTAA <<**pBac 5'-CCCTAGAAAGATAGTCTGCG**

2) Line 24-F1 pBac 3' sequence
Blastn: No significant similarity found

ATATGATTATCTTTCTAGGG-pBac 3'>>
TTAATATACGGTAAGCATTTTAAATAAATA

3) Line 24-F1 pBac 3' sequence
Blastn: *nesprin*-1 gene *B. dorsalis* GenBank acc: XM_011212259; 2e-34; 94% identity/*C. capitata* GenBank acc: XM_012299958; 8e-23; 87% identity

ATATGATTATCTTTCTAGGG-pBac 3'>>
TTAAATATTATAAATTTTAAAAATAATATA

Figure 5. The 5' and 3' proximal insertion site sequences (red) for vector insertions in lines 10-F2 and 24-F1 and adjacent *piggyBac* TIR sequences (bold). Sequences were isolated by inverse PCR using primer sets 122R-139F for the 5' junction and 151R-144F for the 3' junction. Adjacent genomic insertion site sequences were subjected to BLASTn analysis with the lowest e-value matches given with their description, GenBank accession number, e-value, and identity percentages. Full insertion site sequences from the vector *piggyBac* termini to the proximal *Hae*III genomic restriction site (GG/CC) are shown in Supplementary Figure 1.

Table 4. Percentages of developmental stages of transgenic olive fly (G_{10}) (mean ± SD, Fisher test, LSD) (n = 4).

Transgenic lines	Egg hatching (%)	Larval viability (%)	Larval-to-pupal survival (%)	Pupal-to-adult survival (%)	*Overall fitness
10-F2	88.93 ± 5.11 b	81.58 ± 7.01 d	96.21 ± 2.21 a	84.76 ± 5.67 b	0.66 ± 0.08 d
24-F1	85.46 ± 3.46 d	83.76 ± 6.84 c	93.85 ± 4.96 b	88.12 ± 6.43 a	0.69 ± 0.08 c
24-M2	87.69 ± 4.37 c	85.66 ± 7.26 b	96.67 ± 3.78 a	88.63 ± 4.89 a	0.73 ± 0.09 b
Control	94.02 ± 3.09 a	90.08 ± 3.88 a	95.43 ± 4.62 a	92.91 ± 6.98 a	0.79 ± 0.07 a

*Overall fitness = (larval viability/100) (larval-to-pupal survival/100) (pupal-to-adult survival/100) (McCombs et al., 1993).
Values in columns with different superscript letters are significantly different.

to detect in adult olive flies due to the highly melanized cuticle. Molecular analysis of transformants was limited to PCR of the internal fluorescent protein genes and the vector TIRs consistent with intact genomic sequences and sequencing of the *piggyBac* vector 5' and 3' genomic insertion sites for the 10-F1 and 24-F1 lines, respectively. The sequences are consistent with *piggyBac*-mediated events into TTAA target sites, and a BLASTn search indicated strong sequence identity to a highly conserved *D. melanogaster* small nuclear U6 RNA gene (Das et al., 1987) and a *nesprin-1* nuclear envelope gene found in other tephritid species. The discovery of two independent 3' insertion site sequences in line 24-F1 indicates that at least two chromosomally linked integrations occurred in this line. Since only single integration was in an identifiable sequence (*nesprin-1*), a consistent syntenic relationship (i.e. with *Drosophila*) cannot be established for the two insertion site loci. Beyond a determination of *piggyBac*-mediated transformations, this insertion site sequence analysis demonstrates the possibility of using *piggyBac* insertional mutagenesis as a genomic analysis tool in *B. oleae* and its potential negative effect when developing strains for application (e.g., sexing and sterility strains for SIT), which could be reflected in the fitness costs discussed below.

Three transgenic lines were tested for several life fitness parameters, including egg hatching, larval survival, larval-to-pupal survival, pupal-to-adult survival, and fertility. Survival of the strains at all biological stages was similar, although overall fitness was more importantly lower in the transgenic lines compared to wild-type control flies. This could be due to vector insertions occurring within biologically important sequences within the host genomes. Nevertheless, this first evaluation of life fitness for transgenic olive fly strains indicated overall fitness levels, ranging from 0.66 to 0.73, to be nearly identical to the 0.64 to 0.74 range found in three lines in the Mexican fruit fly (*Anastrepha ludens*) transformed with the same vector (Meza et al., 2011). The olive fly lines were also found to be stable in terms of expected transgene marker expression, which remained unchanged for more than 20 generations.

The transformation vector used for this study is notable in that it has the potential to become immobilized with respect to newly introduced *piggyBac* transposase. This could be achieved by eliminating the single *piggyBac* 3' TIR along with the internal 5' TIR by transposase helper injection. Once the internal vector cassette is deleted, the remaining 5' TIR and EGFP marker gene is expected to be stabilized with respect to the subsequent presence of transposase, which could be introduced by an invasive or symbiotic organism. This stabilization process would enhance its stability as a marker strain for SIT as well as its ecological safety in terms of potential interspecies movement of the transgenes. Thus, a goal for future studies will be the postintegration stabilization of the *piggyBac* vector in these strains, as was originally demonstrated in *D. melanogaster* (Handler et al., 2004) and then *A. ludens* (Meza et al., 2011).

The SIT method is typically based on the use of gamma-irradiation to cause dominant lethal mutations and rearrangements of chromosomes in male sperm, which results in infertile matings with females in the field. In Turkey, SIT has been used to control the Mediterranean fruit fly, though radiation used for sterility has been of some concern for years. While highly effective, male irradiation results in diminished fitness and mating competitiveness requiring the need to release 100-fold or greater overflooding ratios of sterile males to males in the field. This results in programmatic inefficiencies and high costs that could be lessened by new olive fly strains that incorporate systems for female lethality for sexing, male sterility, and whole-body and sperm-specific marking, which have all been demonstrated for other tephritid fruit fly pests (Handler and Schetelig, 2014).

The studies presented here demonstrate the development of marker strains for olive fly using *polyubiquitin*-regulated fluorescent proteins employing transformation vectors that can be stabilized for strain stability and ecological safety. This first successful effort to

establish transgenic strains for an important agricultural pest in Turkey should also be a model for studies of other agricultural (*Lobesia botrana*, *Ostrinia nubilalis*, etc.) and disease vector (*Anopheles* spp.) pests.

Acknowledgments
This research was supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK; Project Grant No. 105 O 558; HG), the Turkish Academy of Sciences (Award No. TÜBA-GEBIP 2009; HG), the USDA-NIFA-Biotechnology Risk Assessment Grants (BRAG) program (Award No. 2011-39211-30769; AMH), the Emmy Noether Program SCHE 1833-1/1 of the German Research Foundation (MFS), and the LOEWE Center for Insect Biotechnology & Bioresources of the MFS.

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1) *B. oleae* line 10-F2 pBac 5' TIR *D. melanogaster* small nuclear RNA U6 (locus3R-96A) GenBank acc: NR_002081

GGCCATGCTAATCTTCTCTGTATCGTTCCAATTTTAGTATATGTTCTGCCGAAGCAAGAACAAATGTAAT
TTGGAAGTTACGGTACTTATATCATTCGCTTACCGAAAATGCAACTGCGAATTATAATTCTCATAGGCAA
CGAACGAAAAATGTGTAAGCAAAAATGCAAAATGTATAACGGTAAGAGGAAGAAAAGTGTGTGAGAGAGA
GAGTGAGCGGGGCGGAACATTTTGTGATCACGTTTTTGTACGTAGGTAAATGGATATTTAAGTTTTTGTA
CGTAGGTAAATGGATATTTAA<<**pBac5'CCCTAGAAAGATAGTCTGCGTAAAATTGACGCATGCATTCT**
TGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGANATCTCAGTCNCNCT
NGGAGCTCCCGTGAGGCGTGCTTGNCAATGCGGTAAGTGTCACTGATNA

2) *B. oleae* line 24-F1 pBac3' TIR *nesprin-1* gene *B. dorsalis* GenBank acc: XM_011212259/*C. capitata* GenBank acc: XM_012299958

CCCTCACTAAAGGGGACTAGTCCTGGCAGGGTTTAAACGAATTCGCCCTTCCTCGATATACAGACCGATA
AAACACAAGCGTCAATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTAGGG-
pBac 3'>>
TTAAATATTATAAATTTTAAAAATAATATAATAATAATTTAAATTTTTTAAAAATCTTAAATTATGGCAT
TTGTTTATAAATATCTTCAATCTCTATTCCCACACAGATTTGCCAGGATAATTTGAACTCGCAAACGGAT
CGTTTGTCTGAGCTGCGCGATATCGTTACGAAAATAGCCGCCGATATTGGTTTGGATGCCTCCGGCC

3) *B. oleae* line 24-F1 pBac 3' TIR No Hit

CGCCAGCTNAGANTNNNNCCCTCACTAAAGGGGACTTAGTCCTAGGCAGAGGTTTAAACGAATTTCGCCC
TTTCCTCGATATACAGACCGATAAAACACATAGCGTCAATTTTTACGCATGATTATCTTTAACGTACGTC
ACAATATGATTATCTTTCTAGGG-pBac3'>>
TTAATATACGGTAAGCATTTTAAATAAATACAGACGCAAAGGCAAACAGGGTTTATGTATATATACGAGT
ATATATTTATATATTATATACATGCCTATATACATAAAACCTCACATAATGCTTGAAAGCGCGTAAAATT
GCAAAAGGTTTATAAATTTGGATATGTATGTACAAGTATATTGTCGTAAGTGCGGACTATTTTTTAGAGC
ATGTTAAAACTTTTAAATCCGATATAATAGCAAAAAATATATTTTTATTGCAATTTTTTTGTTTAGTGTT
TGTTTTAGCTAGTGAAGGAAAAGCAACAGAATGCACTCTTCTCTTACATAAAGTATAATAAGCTCGTTTA
CACCGAAAAGTACTTAAAAAAAATGTATTTTTTGGTCATTTGGTTTTCAAGAAGAAATAAAACGTACTAC
ATAATTTAATATTATGGCC

Supplementary Figure 1. Full-length insertion site sequences from the vector *piggyBac* termini to the proximal *Hae*III genomic restriction site (GG/CC) for transgenic lines described in Figure 1.