THE *PiggyBac*-BASED TRANSGENIC *Drosophila* EXPRESS TETRA-CYCLINE-DEPENDENT TRANSACTIVATOR

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The Tetracycline regulatory system is based on the Tet repressor protein (TetR) of the *Escherichia coli* Tn10 tetracycline (Tc) resistance operon and its operator (tetO) (Hillen and Berens, 1994). TetR blocks transcription of these genes by binding to the tetO operator sequences in the absence of Tc. Two gene expression systems, TetOff and TetOn, have been derived from the bacterial Tet system. In the Tet-Off system, the bacterial TetR is fused with the C-terminal of the herpes simplex virus (VP16) activation domain (Triezenberg *et al*., 1988). The resulted hybrid protein that is known as the tetracycline-controlled transactivator (tTA), efficiently activates gene expression in the absence of Tc or doycycline (Dox). In contrast, the Tet-On system is based on a "reverse" Tet repressor (rTetR) which was created by changing four amino acid (aa) in TetR (Hillen and Berens, 1994; Gossen *et al*., 1995). Three of the four aa are located in the protein core (Hinrichs *et al*., 1994; Orth *et al*., 1998) at the Tc or Dox binding site. Therefore, the rTetR binds to tetO only in the presence of Tc or Dox. Fusion of rTetR to the transcriptional activation domain of herpes simplex virus protein VP16 produces a eukaryotic reverse tTA (rtTA) which is widely used in transformation of different eukaryotic organisms. However, rtTA regulatory systems showed some limitations, therefore, the rtTA^s-M2 has been generated with increased induction capabilities and tighter regulation (Urlinger *et al*., 2000). Stebbins *et al*. (2001) modified both rtTA and rtTA^s-M2 by deleting a putative cryptic splice site of TetR gene, adjusting codon usage of the TetR moiety and flanking the cassette with the *Drosophila* boundary sequences SCS and SCS', producing the altered rtTA (rtTA-alt) and rtTA-M2-alt. The transgenic *Drosophila* expressing the rtTA-M2-alt under the constitutive promoter actin5C demonstrated its utility in adults, embryos, and larvae. With this activator, a 70-fold of transgene induction level has been reported compared with the original rtTA transactivator (Stebbins *et al*., 2001).

The current work is a part of transformation experimental series to generate transgenic line of *Drosophila melanogaster* capable to process the N-glycan as mammalian pathways. Previously, we developed transgenic *Drosophila* ubiquitously expressing sialic acid

synthase, CMP-sialic acid synthetase, α2,6-sialyltransferase, and α2,3-sialyltransferase (Mohammed *et al*., 2009a&b). The constitutive expression of N-acetylglucosaminylteransferase II (GntII) and, β 1,4-galactosyltransferase (GalT), have an adverse effect on the insect. Therefore, strong tight induction system to express both GntII and GalT in *Drosophila* tissues is the recommended choice (see review for gene expression systems in *Drosophila*, McGuire *et al*., 2004). This study aimed to develop a transgenic *Drosophila* line expressing the rtTAM2-alt under the constitutive hr5-ie1 enhancer-promoter element. This transgenic line will be used to induce and tightly regulate the expression of such mammalian enzymes within the progeny flies by crossing with other transgenic lines bearing mammalian genes.

MATERIALS AND METHODS

Plasmids

Tetracycline transactivator constructs

Four plasmids (Fig. 1) were designed to drive the expression of rtTA and rtTAM2-alt genes under the regulatory sequences of the *Bombyx mori* 3C cytoplasmic actin gene promoter (Ac3C) and the bacoluviral immediate early enhancer-promoter (hr5-ie1). Each cloning step included amplification of the DNA fragments using high fidelity platinum pfx polymerase (Invitrogen) followed by subcloning step into pCR2.1 plasmid (Invitrogen). The fragment was released using the restriction enzyme(s) that previously designed on the primer sets, gel extracted and finally cloned into the construct. A 237 bp DNA fragment, carrying the simian virus 40 (SV 40) polyadenylation site, was amplified using pBSII-3xp3ECFP ITR (Li *et al*., 2001) as DNA template and two specific primers SV40-SpeFWD and SV40-NotREV (see Table 1; for primer sequences). The *SpeI* and *NotI* digested SV 40 fragment was released from pCR2.1 and inserted into the pBluescript II SK (Stratagene) forming pBS.SV40. One kb DNA fragments encoding rtTA and rtTAM2-alt open reading frames (ORFs) were amplified using pTet-On (Clonetech) and pYJ2006 (Stebbins *et al*., 2001) as templates and TetON-orf FWD and TetON-orfREV, as specific primer set. One unite of high fidelity platinum pfx polymerase (Invitrogen) was used to amplify each ORF. The amplification conditions were as follows; preheating period for 5 min at 95°C, followed by 30 amplification cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min finished with incubation at 72°C for 5 min and the reaction was held at 4°C. The PCR products were separately cloned into pCR2.1. The DNA fragments were then released using *Eco*RI/*Bam*HI restriction enzymes and finally cloned into the pBS.SV40 to create pBS.rtTA and pBS.rtTAM2. The hr5-ie1 enhancer-promoter sequence was amplified using pIE1-3pigORF (Mohammed and Coates, 2004) and hr5IE1KpnBglFWD and hr5IE1SmaRVS as specific primers. The *Kpn*I/*Sma*I fragment was ligated to corresponding sites of pBS.rtTA and pBS.rtTAM2 forming pBS.IE1.rtTA and

pBS.IE1.rtTAM2, respectively. The pBS.Act3C.rtTA and pBS.Act3C.rtTAM2 were constructed by amplifying the 672 bp DNA fragment which encodes the silkworm actin3C using pPIGA3GFP (Tamura *et al*., 2000) and BmA3_for and BmA3_rev as specific primers. A *Kpn*I/*Sma*I-fragment was cloned into pBS.rtTA and pBS.rtTAM2.

Donor construct

According to luciferase assay results, the pBS.IE1.rtTAM2 plasmid was selected to create the donor plasmid, pBS-rtTAM2.EGFP (Fig. 2), which is a necessary element for transformation experiments. A 1.21 kb cartridge fragment carrying the *piggyBac* terminal repeats sequences was amplified using pBSII-3xp3ECFPITR as template (Li *et al*., 2001) and two specific primers, MCS-ITRKpnF and MCS-ITRKpnR. The *Kpn*I-digested cartridge fragment was released from pCR2.1, and then cloned into pBS.IE1.rtTAM2.SV40. Finally, the insect marker gene 3xp3.EGFP.SV40 was cloned to construct the donor plasmid, pBS-rtTAM2.EGFP. A 1.286 kb DNA fragment encoding the enhanced green fluorescent protein under the regulation of the universal insect eye specific promoter (3xp3) was amplified using two specific primers, UnivMarkBglFWD and UnivMarkBglRVS, and pBSII.3xp3-EGFP.SV40 as DNA template (Berghammer *et al*., 1999).

Cell cultures and transfections

The Cell cultures and transfection conditions were performed as described by Carter *et al*. (2008). The *Spodoptera furgiperda* (Sf9) cells were grown in suspension of ExCell 401 (JRH Biosciences) supplemented with 10%fetal bovine serum (Atlanta Biologicals) and an antibiotic/antimycotic solution consisting of 10,000U/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B (Invirtrogen). For each transfection, Sf9 cells were transfected in triplicate in wells of a six-well plate with two μg plasmid DNA with equal amounts of pTRE2hyg-Luc and one the four constructs (pBS.IE1.rtTA, pBS.IE1.rtTAM2, pBS.Act3C.rtTA or pBS.Act3C.rtTAM2) using transfectin reagent (Bio-Rad). The cells were incubated for 24 hours at 28°C in ExCell 401 medium supplemented with 35 μg/m1 hygromycin, as well as 1 μg/m1 Dox (Clontech) to induce Tet activation system, meanwhile, Dox was eliminated from the medium for non-induction system.

Luciferase assay

The induction activity of Tet-On system and the transcriptional activity of the promoters were determined using the dual-luciferase reporter assay system (Promega). After 24 hours of DNA transfection, cells were lysed and each well was analyzed for *Renilla luicferase* activity on the LMaxII348 luminometer (Molecular Devices).

Drosophila culture and microinjection

Drosophila strains were reared under standard laboratory conditions (Roberts, 1998). The preblastodermal embryos

were microinjected as described in (Rubin and Spradling 1982). The *D. melanogaster* w^{1118} white eye strain was coinjected with pBS.rtTAM2-EGFP and phspBac (Handler and Harrell, 1999) at equal concentrations of 0.4µg/µl. Injection and screening procedures were performed as described in Mohammed *et al*., (2009a). The progenies were screened as adults for fluorescent eye color using an Olympus SZX12 fluorescent microscope equipped with GFP filter set. Positive adults were individually crossed with the w^{1118} flies, and subsequent generations were homozygosed to establish the separate transformed lines.

Southern hybridization

Southern hybridization analysis was performed according to Ausubel *et al*. (1994) with minor modifications. The genomic DNA was extracted from transformed flies using DNAZol (Molecular Research Center, Inc) according to the manufacture procedures. A *Puv*II-digested gDNA was transferred onto Nylon membrane and cross-linked using a UV crosslinker (Stratgene). The probe was prepared by labeling the pBS-rtTAM2.EGFP plasmid using random labeling kit Prime-a-Gene Labeling System (Promega) and ^{32}P dCTP (Amersham). Finally, the membrane was exposed to X-ray films (Kodak).

Inverse PCR

Inverse PCR (IPCR) was performed by completely digesting 5µg of the genomic DNA from the transformed strain with *Hin*P1I for the 3'end or *Taq*1 for the 5'end. The digested DNAs were circularized by overnight self-ligation then precipitated and resuspended in 20µl ddH_2O. A 5µl of the self ligated DNA was used as a template for the first amplification round using specific primer sets for both 5' and 3'ends (see Li *et al*., 2005 for oligonucleotide primer sets sequences). The primer pairs (IFP2_R1 + MF14) were used for the 5' end and (JF3 + IFP_Lb) for the 3'end. The first PCR round started with 5 min at 95°C followed by 10 cycles of 95°C for 1 min, 40°C for 2 min and 72°C for 1.4 min followed by 30 cycles of 95°C for 45 sec, 48°C for 1 min and 72°C for 1.4 min and finally 72°C for 5 min. A 2µl of the first-round PCR products was used as templates for the second-round PCR (94°C 1 min, 50°C 1 min, 72°C 2 min, thirty-five cycles) using the primer pairs (iPCR_R1 + iPCR_6) for the 5'end and (iPCR_L1 + IFP2_L-R) for the 3' end. All PCR products were cloned into the pCR2.1 (Invirtogen). DNA sequence was determined on ABI Prism 310 DNA sequencer using BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). Amplified products were determined on an ABI Prism 373 DNA Sequencer using BigDye termination DNA sequencing kit containing AmpliTaq FS polymerase (Perkin Elmer Cetus, Foster City, CA). Sequences were subjected to the Blast search of the NCBI database to identify the location of the insertions.

RT-PCR

The total RNA was extracted from the homogenized bodies of flies collected

from the two transgenic lines using the Trizol reagent (Inovitrogen). First strand cDNA was prepared from the total RNA as standard procedures (Kotewicz *et al*., 1988) and used for RT-PCR with modification as described in Mohammed *et al*., (2009a). The TetON-orf FWD /TetON-orfREV primer set to amplify the rtTAM2alt transcripts. The green fluorescent protein transcripts were amplified using the primer set; EGFPORF-FWD and EGFPORF-RVS. The amplification conditions were 94°C for 5 minutes followed by 35 cycles of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 1 min followed by 5 min at 72°C.

RESULTS AND DISCUSSION

Promoter and tetracycline induction

The induction activities of four plasmids; pBS.IE1.rtTA, pBS.Act3C.rtTA pBS.IE1.rtTAM2 and pBS.Act3C. rtTAM2 were determined within Sf9 cell line. The Dox-induction is calculated according to the relative ratio of Renilla luciferase values of Dox- to free medium. The ratios in Table (2) indicate that both rtTA plasmids (pBS.IE1.rtTA and pBS.Act3C.rtTA) are non-functional and the luciferase activity may due to background expression, whereas, both promoters show no significant differences in luciferase expression. On the other hand, the luciferase expression was highly activated by rtTAM2-alt (Fig. 3). The induction activity of rtTAM2-alt driven by the actin3C promoter is 126 fold higher than rtTA. While hr5ie1 enhancer-promoter is showing a relatively higher expression range reaches 168 fold. Therefore, hr5-ie1 enhancer promoter cassette expressing rtTAM2 was selected for deriving Tet-on system within transgenic *Drosophila*.

Here, the significant differences of the induction level are clearly demonstrated between rtTA and its mutant rtTAM2-alt. About 126 and 168 fold of the luciferase level are induced by rtTAM2-alt under actin3C and hr5ie1 promoters, respectively. These results are in consistent with previous reports for the higher activities of the mutated Tet protein. In *Saccharomyces cerevisiae*, the mutant transactivator, rtTA2^{S}-M2, functions at a10-fold lower Dox concentration than rtTA, with no background expression in the absence of Dox (Urlinger *et al*., 2000). Further mutation of the TetR gene produced rtTAM2-alt which confers a sixty eight fold better performance compared to the rtTA protein in *Drosophila* flies (Stebbins *et al*., 2001).

Transformation of D. melanogaster w^{1118} *with genes encoding a mutated reverse Tet-responsive transactivator*

Four transformation experiments were performed to generate tetracycline-inducible system in *D. melanogaster* w^{1118}. A total of 2833 embryos were coinjected with pBS.rtTAM2-EGFP and phspBac. Excluding the third experiment, the hatchability percentage ranged from 39 to 51 (Table 3). The emerged adults were individually backcrossed with w^{1118}

flies in separate families and the progenies (G0) were screened for EGFP expression. Two out of four injection experiments yielded four positive flies showing green fluoresces (Fig. 4). The transformation frequency is calculated by dividing the number of the G0 transgenic individuals by the number of crossed families. The transformation frequencies ranged from 2.9 to 3.6%. However, only two transgenic lines 1 and 2 (Fig. 4) were established from the four individuals.

Drosophila, the first insect model to be transformed using *P*-element (Rubin and Spradling, 1982), and was also transformed by *piggyBac*-mediated vectors (Handler and Harrell, 1999). The transformation frequencies (2.9-3.6%), reported in the current transformation experiments, are variable among the experiments due to different factors. In previous studies, the transformation frequencies using *piggyBac*-derived vectors in *Drosophila* ranged 5.1-7.5% (Mohammed *et al*., 2009a), 8.2-25% (Mohammed *et al*., 2009b) and 1-26% (Handler and Harrell, 1999).

Southern hybridization

Both plasmid DNA (pBSrtTAM2.EGFP) and gDNA were extracted from the two transgenic lines were digested overnight at 37°C with *Pvu*II restriction enzyme. The *Pvu*II digestion resulted in five fragments; four internal fragments, represented within the transgenic lines (2.513, 1.896, 0.867 and 0.843 kb) and one external 1.665 kb which includes the 1.1K *piggyBac* cartridge (Fig. 5). Both 0.867 and 0.843 kb bands appear as one single band so the four internal fragments are represented by three signals within the transgenic lines as in the plasmid. The external band disappears from the transgenic lines while, instead, faint signal at different size appears on both lines. Line 1 has signal less than 1.5 Kb and line 2 has signal more than 2 kb while may reveal that they are two different inserts. And these two lines are originated from two different *piggyBac* insertions.

RT-PCR

The transcripts of the rtTAM2 within M2-1 and M2-2 transgenic lines were confirmed using RT-PCR (Fig. 6). One microgram of DNA-free total RNA was used as a template for the synthesis of the first strand which was used for further amplification. EGFP-specific primers were used for positive control reaction. The primer set, used in cloning, is ORF-specific for rtTA and rtTAM2. Hence, it was also used to amplify rtTAM2 transcript within transgenic flies.

Insertion site determination

The *piggyBac*-mediated transposition is verified by localization of *piggyBac*-insertion site(s) on the insect chromosome. The duplicate TTAA target sequence, the hallmark of legitimate *piggyBac*-based insertion, is identified within inverse PCR products by DNA alignment with *piggyBac* terminal repeats. The DNA sequence proximal to the TTAA was

Blast analysis (Altschul *et al*. 1990) to identify genomic insertion site sequences. The 5' junctions of four insertion sites were identified for lines M2-1 and M2-2 (Table 4). However, only one 3' junction for M2-1 line was recovered. The DNA alignment yielded that three out of the four sites are at different locations on the third chromosome. Both 5' and 3' junctions are identified for the insert that located on the fourth chromosome.

The generated inducible *Drosophila* transgenic lines using *piggyBac* vector encode rtTM2-alt driven by strong enhancer-promoter hr5-ie1. The induction efficiency of this transactivator lines is not demonstrated in the current study. However, the tight regulation of GnTII and GalT expression will be evaluated by crossing M2-1 line to other transgenic *Drosophila* lines encoding GnTII and GalT under the tetracycline-inducible operator TetO7 sequences.

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SUMMARY

The reverse tetracycline-controlled transactivator (rtTA) and its derivatives have been used as tight regulator of target gene expression. The functional activities of rtTA and the altered rtTA^{s}-M2 (rtTAM2-alt) in lepidopteran cells, Sf9, under either the silkworm actin3C or hr5-ie1 promoters were determined. The *luciferase* gene expression under the control of the tetracycline-response element (TRE) was used as a reporter for the induction activity. The expression of *luciferase* was highly regulated by rtTAM2 compared to rtTA. Furthermore, we generated transgenic *Drosophila* line to drive the expression of rtTAM2-alt under the constitutive enhancer-promoter hr5-ie1 to be used for future work.

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Table (1): A listing of primers that were used in cloning, and RT-PCR experiments.

Primer	Sequence
Cloning primers	
SV40-SpeFWD	5'-GCTAACTAGTACTCTCGAGTCATAATC-3'
SV40-NotREV	5'-GCTAGCGGCCGCTAGTAGCAAGCTTTTTGGACAAACCACAAC-3'
TetON-orf FWD*	5'-ATGCGAATTCCATGTCTAGATTAGATAAAAGTAAAGTGATTAACAGCGC-3'
TetON-orfREV*	5'-ATGCGGATCCCTACCCACCGTACTCGTCAATTCCAAGG-3'
BmA3_for	5'-ACTGAAGCTTGCGCGTTACCATATATGGTGAC-3'
BmA3_rev	5'-ACTGAATTCCTTGAATTAGTCTGCAAGAAAAG-3'
hr5IE1KpnBglFWD	5'-GCTAGGTACCAGATCTGCGTAAAACACAATCAAGTATGAG-3'
hr5IE1SmaRVS	5'-GCTACCCGGGCACTTGGTTGTTCACGATCT-3'
MCS-ITRKpnF	5'-CGATGGTACCGGGCCCCCCCTCGAGGTCGAC-3'
MCS-ITRKpnR	5'-CGATGGTACCTAGTGGATCCCCCGGGCTGC-3'
UnivMarkBglFWD	5'-GCTAAGATCTGTGTTCCCACAATGGTTAATTCG-3'
UnivMarkBglRVS	5'-GCTAAGATCTCTAGGCGCGCCGTACGCGTA-3'
RT-PCR primers	
EGFPORF-FWD	5'-GCGACGTAAACGGCCACAAGTTCAG-3'
EGFPORF-RVS	5'-CAGCTCGTCCATGCCGAGAGTGATC-3'

* The primer set was used for both cloning and RT-PCR**.**

Table (2): Luciferase induction of four plasmids with and without Doxycycline.

Rep.	Act3C-rtTA			hr5IE1-rtTA			Act3C-rtTAM2			hr5IE1-rtTAM		
	ND	Dox	Ratio	ND	Dox	Ratio	ND	Dox	Ratio	ND	Dox	Ratio
1	482	637	1.32	473	1016	2.14	366	98776	269.8	936	291426	311.3
2	391	657	1.68	463	610	1.31	513	111279	216.9	1099	320369	291.5
3	353	570	1.61	454	589	1.29	1156	320369	92.8	1459	286761	196.5

ND: no doxycycline Dox: induction by doxycycline Ratio: The luciferase expression ratio between Dox/ND.

Table (3): Transformation experiments.

Injected Experiment	Number of injected embryos	Number of hatched larvae	Percentage of hatching	Number of backcrosses	Number of G0 cyan-expressing flies	Transformation frequency
1	768	300	39	55	2	0.036
2	390	197	51	67	2	0.029
3	525	ND*	ND*	39	0	0.0
4	1150	527	46	ND*	0	0.0

* Data is not recoded.

Table (4): Insertion sites of transformed *Drosophila* lines**.**

Line	Chromosome location	Insertion site sequence 5' junction	Insertion site sequence 3' junction
M2-1	4	tcaaaaaatgacttca TTAA.....*piggyBack*...TTAAaaataattattaaattcc	
M2-1	3L	ctagagtgcgccact TTAA.....*piggyBack*.....	ND*
M2-2	3L	cttaagcgtgaatagtTTAA.....*piggyBack*.....	ND*
M2-2	3L	aacggatctcagtatgtTTAA....*piggyBack*.....	ND*

* The junction sequence is not resolved by inverse PCR.

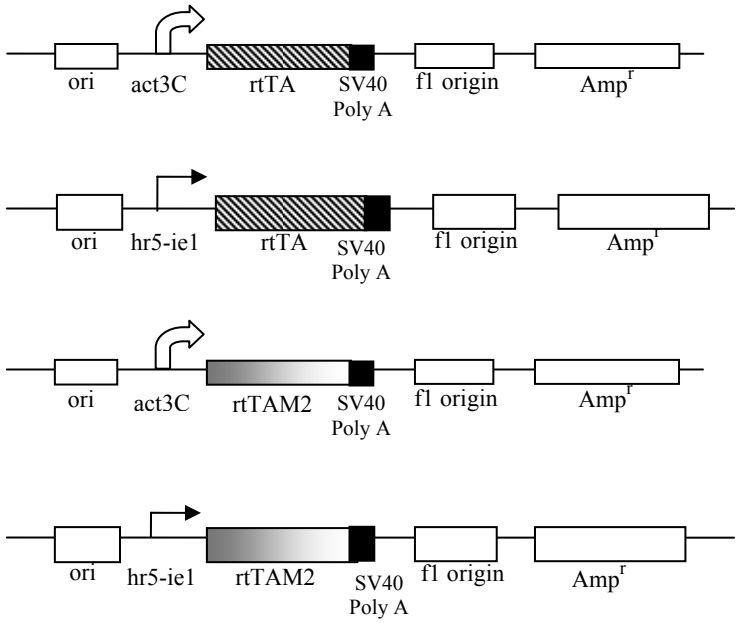


Fig. (1): Schematic illustration map of the four constructed plasmids encoding both the reverse tetracycline transactivator gene (rtTA) and its mutant (rtTAM2) under the regulation of the silkworm actin3C promoter and the baculovirus enhancer promoter fragment hr5-ie1 and terminated by the simian virus40 polyadenylation site**.**

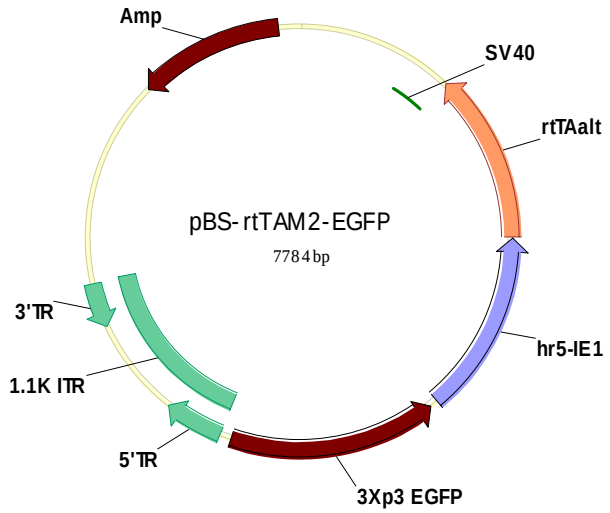


Fig. (2): The map of donor plasmid, pBS-rtTAM2-EGFP.

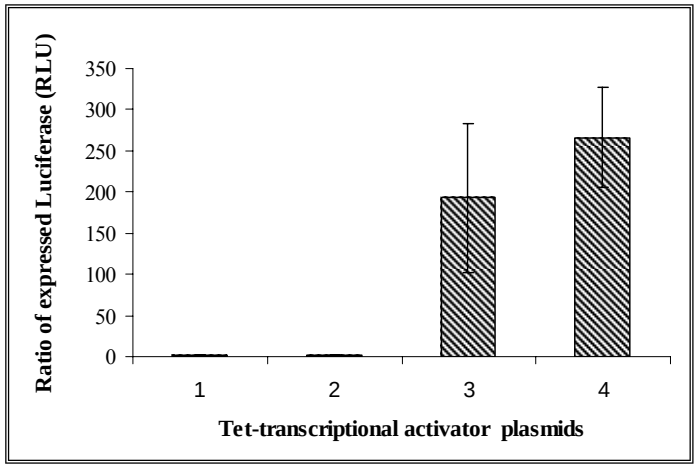


Fig. (3): Fold induction level of luciferase expression. The X axis represents the four plasmids; (1) pBS.IE1.rtTA, (2) pBS.Act3C.rtTA, (3) pBS.IE1.rtTAM2, and (4) pBS.Act3C.rtTAM2 and the Y axis represents the ratio of luciferase expression between dox-induced and dox-free media in relative light unit (RLU).

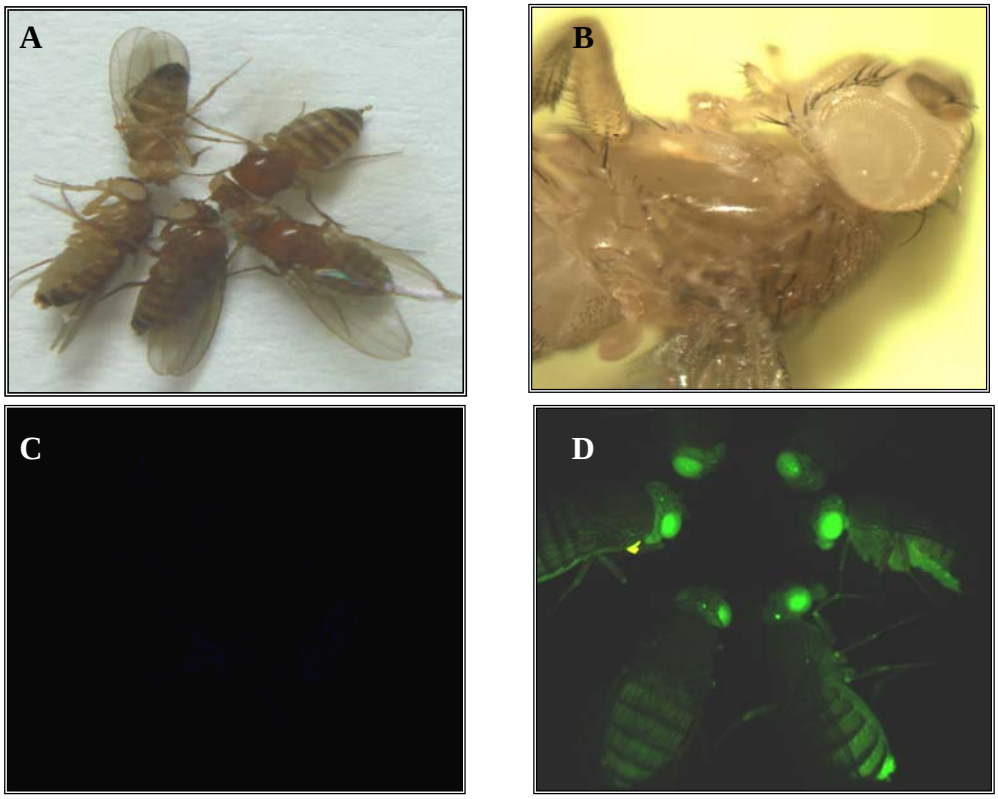
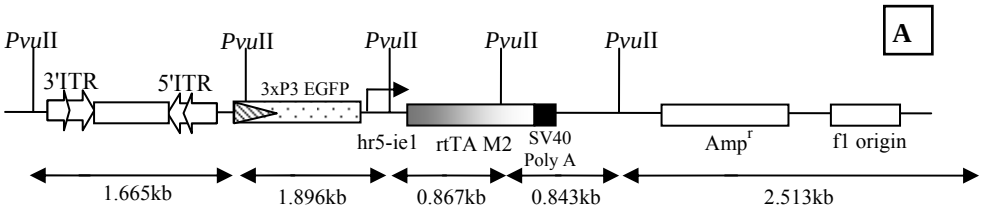


Fig. (4): A full body flies (A), white eye color phenotype (B) of w^{1118} flies under white light and under UV light (C). The transformant flies (D) under the exposure of UV light using fluorescent microscope equipped with GFP filter set.



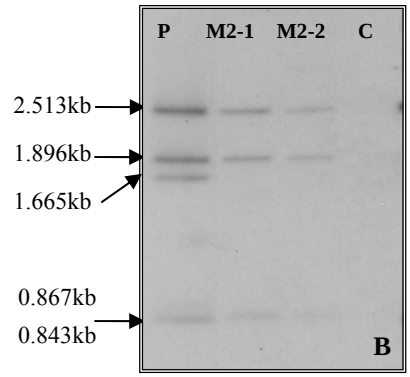


Fig. (5): Southern DNA hybridization analysis of *D. melanogaster* transformants. A) Scheme of the pBS-rtTAM2.EGFP vector showing the location of PuvII restriction sites. B) Southern DNA hybridization of transgenic lines (M2-1 and M2-2). Both lines have four fragments 2.513, 1.896, 0.867 and 0.843 kb (the last two appears as one) and one extra with variable size. The positive control is the plasmid DNA (P) and the negative control is w^{1118} flies (C).

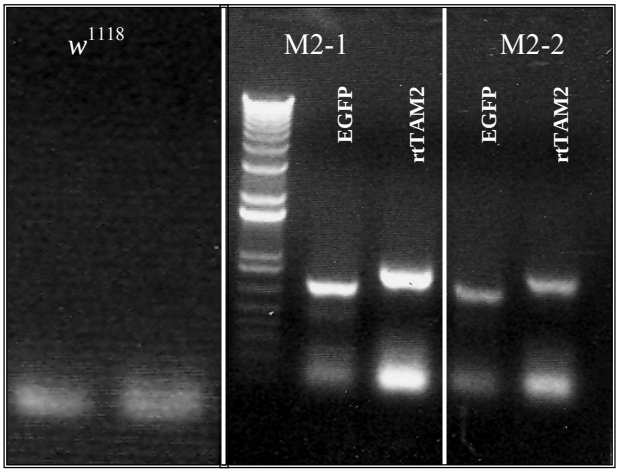


Fig. (6): RT-PCR; the transcripts of enhanced green fluorescence protein (EGFP) and the altered reversed tetracycline transactivator mutatnt-2 (rtTAM2-alt) for the two transgenic lines as well as the negative control of w^{1118} flies.