Drosophila TRANSGENIC LINES EXPRESSING THE MAMMALIAN N-ACETYLGLUCOSAMINYLTRANSFERASE II AND β 1,4-GALACTOSYLTRANSFERASE

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N-linked glycosylated proteins play a role in different biological processes. The initial steps of protein modifications with glycan in eukaryotic cells are identical. Both mammalian and insect N-glycosylation pathway share the intermediate complex, (For review see: Jarvis 2003; Rendić *et al*., 2008). The N-glycosylation process starts with trimming three glucose residues from the high mannose structure "Glu3Man9GlcNac2-N-Asn", followed by further removing of five mannose residues. One residue of N-acetylglocusamine is added to the structure and the common intermediate structure is achieved by trimming two mannose residues. In insect cells removal of further residues is continue by trimming the terminal N-acetylglucosamine residue forming the major N-glycan product in insect cells, the paucimannose, "Man3GlcNAc2-N-Asn" (Altmann *et al*., 1995). However, in some cases, only extremely low levels of terminal glycosyltransferase activities have been detected in insect cells (Jarvis, 2003). In contrast, the mammalian glycosylation process tends to elongate this intermediate structure and producing more complicated complex of N-glycans with terminal sialic acids (Beyer *et al*., 1979).

The differences in glycosylation pathways between insect and mammalian cells limit the ability of insect-based baculovirus expression systems to produce broad spectrum varieties of N-glycoproteins. In early attempts to overcome such problematic differences, lepidopteran cells were transformed with mammalian genes to shift the insect glycoslation process toward mammalian pathway. The *Spodoptera frugiperda* cells (Sf9) were engineered to express the mammalian β1,4-galactosyltransferase (GalT) and α2,6-sialyltransferase (ST6). The resulted cells, Sf4GalT/ST6, produced a recombinant glycoprotein with mono-antennary structure with only α1,3 arm elongated (Hollister and Jarvis, 2001; Hollister *et al*., 2002). Further transformion with N-acetylglucoseaminyl-transferase II gene created SfSWT-1 cells which produced a recombinant glycoprotein with bi-antennary sialylated N-glycans (Hollister *et al*., 2002).

Our goal is to apply the knowledge gained from engineered insect cells to "humanize" the glycoprotein processing

pathway in intact insects. Here we report the generation of transgenic *Drosophila* lines expressing the human *N*-acetylglucosaminyltransferase II and a bovine β1,4-galactosyltransferase.

MATERIALS AND METHODS

Plasmids

The donor plasmid pXLBacII-TetO1.GalT/GnTII-DsRed.A cl3 (Fig. 1) was provided by Dr. Donald Jarvis, Wyoming State University (Shi *et al*., 2007). The plasmid was designed to encode a human *N*-acetylglucosaminyl-transferase II (GnTII; Tan *et al*., 1995) and a bovine β1,4-galactosyltransferase (GalT; Shaper *et al*., 1986) under the "*PCMVmin-TetO7-PCMVmin*" dual tetra-cycline-inducible transcriptional element flanked with the *piggyBac* terminal repeats using pXLBacII vector (Li *et al*., 2005) as a template construct. The dual transcriptional control element consisted of back-to-back minimal cytomegalovirus immediate early promoters (*PCMVmin*) separated by a tetracycline-inducible operator (*TetO7*; Gossen *et al*., 1995). Both GnTII and GalT were terminated with DNA fragments containing the bovine growth hormone polyadenylation signal, BGHpolyA, (Goodwin and Rottman, 1992). The red fluorescent marker gene (DsRed1), under the transcriptional control of the eye-specific promoter, 3xP3 (Horn *et al*., 2000), was also incorporated into the plasmid. The phspBac plasmid (Handler and Harrell, 1999) was used for microinjection as a source of *piggyBac* transposase.

Drosophila culture and microinjection

The *D. melanogaster* w^{1118} white eye strain was used for microinjection experiments. A total concentration of 0.6 µg/µl of pXLBacII-TetO1.GalT/GnTII-DsRed.A cl3, and 0.4 µg/µl of the phspBac were coinjected. Drosophila rearing and preblastodermal microinjec-tion conditions are described before (Mohammed *et al*., 2010). The progeny adults were screened using Olympus SZX12 fluorescent microscope equipped with Red filter set.

Southern hybridization

Southern analysis was applied on genomic DNA that was extracted from transformat flies using DNAZol (Molecu-lar Research Center, Inc). Both donor DNA plasmid, as positive control, and genomic DNA were digested with *Hind*III-restriction enzyme and trans-ferred overnight onto Nylon membrane then cross-linked to using a UV crosslinker (Stratagene). The membrane was allowed to hybridize overnight with the donor plasmid (as probe) which was previously radio-labeled with ^{32}P dCTP (Amersham) using Prime-a-Gene Label-ing System (Promega).

IPCR

Inverse PCR was performed as described before (Mohammed *et al*., 2010) using different primer sets (for oligonucleotide sequences see Table (1). For first PCR round, 5'SauInverFWD57-1/5' SauInverRVS57-1 was used for the 5' end and 3'TaqInverFWD/3'TaqInverRVS

for the 3'end. Two microliters of the first-round PCR products were used as templates for the second-round using the primer pairs 5'SauInverNestFWD57-1 /5'SauInverNestRVS57-1 for the 5'end and 3'SauInverFWD57-1/3'SauInver RVS57-1 for the 3' end. DNA sequence was determined on ABI Prism 310 DNA sequencer using BigDye terminator v3.1 cycle sequencing kit (Applied Biosys-tems). Amplified products were deter-mined 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

To induce both GntII and GalT4, the transgenic lines should be combined with a source of the tet-response protein, rtTAM2-alt, to drive gene expression in the presence of Doxycycline. The five responder lines (57-1 through 57-5) were crossed with driver line (M2-1; Mohammed *et al*., 2010). A total of 5 crosses, 2 driver males were crossed with 5 responder virgin females. The progeny of each cross was kept separately and allowed to grow on the regular insect diet. The emerged adults were screened for both EGFP and DsRed expression in their eye. The EGFP/DsRED positive flies were selected to achieve homogeneity for each cross. For each cross, 20 adult flies were added to diet supplemented with 100 µg/ml Dox and similar number of adults was added to Dox free-diet. The adults were allowed to lay their eggs and the hatched larvae were allowed to feed on the diet for 5-7days. The larvae were picked up from the diet and used for RNA extraction.

The total RNA was extracted from the homogenized larvae using the Trizol reagent (Invitrogen). First strand cDNA was prepared from the total RNA to be used as a template for RT-PCR analysis as previously described (Mohammed *et al*., 2010). The sequences of primer oligonucleotides used for the PCR reaction are presented in Table (1).

RESULTS AND DISCUSSION

Transformation experiments

Drosophila melanogaster w^{1118} were microinjected with pXLBacII-TetO1.GalT/GnTII-DsRed.A cl3 and phspBac at a concentration of 0.6 and 0.4 µg/µl, respectively. Seven experiments with a total of 3278 embryos were microinjected. The hatchability percent-age ranged from 7.5 to 32 (Table 2). The emerged adults were individually backcrossed with w^{1118} flies in separate families and the progenies (G0) were screened for EGFP expression. Three out of seven injection experiments yielded five positive flies showing green fluoresces (Fig. 2). 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 3.8-14.3%. The positive flies were

backcrossed with w^{1118} flies for few generations. Every generation, the flies were selected for red expression then crossed with their siblings and the flies which did not show the marker expression were immediately isolated and destroyed. Simply, the putative transgenic individuals were marked as 57-1 through 57-5 and so, their progenies were designated as line.

Southern hybridization

The plasmid DNA digested with *Hind*III yield two internal fragments at 1.09 and 1.20 kb, present within all transgenic lines, and one external fragment at 7.05 kb (Fig. 3). The lines show different signal patterns which may reveal the 5 transgenic individuals were originated from different piggyBac transposition. Line 1 may includes 3 different insertions while line 2 may also has 3 insertions but the signals are stronger. Lines 3 and 4 may have two inserts however, they are different and line four show stronger signal. Line 5 shows one strong signal.

RT-PCR analysis

The GnTII and GalT transcripts within the larval bodies were verified using RT-PCR analysis. The responder transformants encoding GnTII and GalT were crossed with driver flies expressing rtTAM2, and the progeny were selected according to dual eye marker. The putative dual transgenic flies were allowed to lay their eggs on Dox-contained diet and on naïve diet as induction control. After 5-7 days, the larvae were collected from both diets and used for RT-PCR analysis (Fig. 4).

The RT-PCR detects GnTII and GalT transcripts in larvae that were fed Dox-diet. Every two lanes in Fig. (4) represent the two transcripts for each cross. For most crosses show expression of GnTII (adjacent to *piggyBac* 3'end) more than GalT. Similar results were detected before in dual *piggyBac*-mediated vectors (Shi *et al*., 2007). However, GalT transcript shows different size bands which may reveal that some transcripts are spliced or endogenous transcripts may present within the larval bodies. Unexpected RT-PCR products are detected for both GnTII and GalT within larvae fed on Dox-free diets. These results indicate that the Tet-off regulatory system is leaking in the current case. In contrast to Stebbins *et al*. (2001) stated that rtTAM2 is more efficient and gene induction can be modulated by the Dox-concentration within the insect food.

Insertion site determination

The *piggyBac*-mediated chromosomal transposition was verified by inverse PCR. The PCR products were cloned and sequenced. The sequencing data were analyzed by DNA alignment with the *piggyBac* terminal sequences and identification of TTAA duplicate target site, the characteristic for all *piggyBac* integrations (Elik *et al*., 1995). Finally, the DNA sequence proximal to the TTAA was Blast analysis (Altschul *et al*., 1990) to identify genomic insertion site se-

quences. The 5' and 3' *piggyBac* junctions for three out of five lines were recovered showing the duplicate TTAA sequences (Table 3). All three sites are located on different chromosome locations at third and second chromosomes. Only the 5' junction was recovered for lines 57-2. The PCR reaction failed to yield any product for line 57-5. Although the results from hybridization analysis showing different sites for some of these lines (57-1, 57-3 and 57-4), the inverse PCR didn't identify these different sites.

Due to deficiency of endogenous genes such as GalT, GlcNacT-II, sialyl-transferase, SAS and CMP-SAS, insect cells are unable to produce mammalian type N-glycans (Tomiya *et al*., 2003). In the effort to modify the insect N-glcyosylation process pathway, six mammalian genes; GnTII, GalT, ST6GalI, ST3GalIII, SAS and SMP-SAS have been selected to transform *Drosophila melanogaster*. Previously, two drosophila transgenic lines expressing ST6GalI, ST3GalIII**,** SAS and SMP-SAS have been developed (Mohammed *et al*., 2009 a&b)**.** The current study is a report of generating transgenic drosophila expressing GnTII and GalT within the fly bodies under the regulation of Tet-regulatory transactivator. In the future, all six mammalian genes will be combined together in a single line by crossing the three transgenic lines to achieve drosophila strain that is capable of producing recombinant glycoprotein with bi-antennary sialylated N-glycans.

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SUMMARY

One way of producing glycoproteins that have therapeutically values is insect-mediated baculovirus expression vectors. However, this system has limitation due to the insect glycosylation pathways. Modification of insect cell lines by introducing mammalian gene showed some success of altering the glycosylation process of the expressed recombinant proteins. The current study is a part of transgenic experiment series to produce *Drosophila* capable of processing the glycoproteins as mammalian glycosylation pathways. The w^{1118} eye mutant strain of *Drosophila melanogaster* was transformed with *piggyBac*-derived construct carrying DNA fragment encoding both mammalian *N*-acetylglucosaminyl-transferase II and a bovine β1,4-galactosyltransferase under the "*PCMVmin-TetO7-PCMVmin*" dual tetracycline-inducible transcriptional element. The transformation experiments yielded five positive flies showing red fluorescence. The putative transgenic flies were further analyzed.

REFERENCES

Altmann, F., H. Schwihla, E. Staudacher, J. Glossl and L. Marz (1995). Insect cells contain an unusual, membrane-bound B-N-acetyl gluciosaminidase probably involved in the processing of protein N-glycans. J. Biol. Chem., 270: 17344-17349.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). Basic local alignments search tool. J. Mol. Biol., 215: 403-410.

Beyer, T. A., J. I. Rearick, J. C. Paulson, J. P. Prieels, J. E. Sadler and R. L. Hill (1979). Biosynthesis of mammalian glycoproteins. Glycosylation pathways in the synthesis of the nonreducing terminal sequences. J. Biol. Chem., 254: 12531-12541.

Elick, T. A., C. A. Bauser, N. Principe and M. J. Fraser (1995). PCR analysis of insertion site specificity, transcription, and structural uniformity of the Lepidopteran transposable element IFP2 in the TN-368 cell genome. Genetica, 97: 127-139.

Goodwin, E. C. and F. M. Rottman (1992). The 3'-flanking sequence of the bovine growth hormone gene contains novel elements required for efficient and accurate polyadenylation. J. Biol. Chem., 267: 16330-16334.

Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen and H. Bujard (1995). Transcriptional activation by tetracyclines in mammalian cells. Science, 268: 1766-1769.

Handler, A. M. and R. A. Harrell (1999). Germline transformation of *Drosophila melanogaster* with the *piggyBac* transposon vector. Insect Mol. Biol., 8: 449-457.

Hollister, J. and D. L. Jarvis (2001). Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1,4-galactosyl-transferase and α2,6-sialyltransferase genes. Glycobiology, 11: 1-9.

Hollister, J., E. Grabenhorst, M. Nimtz, H. Conradt and D. L. Jarvis (2002). Engineering the protein N-glycosylation pathway in insect cells for production of biantennary complex N-glycans. Biochemistry, 41: 15093-15104.

Horn, C., B. Jaunich and E. A. Wimmer (2000). Highly sensitive, fluorescent transformation marker for *Drosophila* transgenesis. Dev. Genes Evol., 210: 623-629.

Jarvis, D. L. (2003). Developing baculovirus-insect cell expression systems for humanized recombinant glycoprotein production. Virology, 310: 1-7.

Li, X., R. A. Harrell, A. M. Handler, T. Beam, K. Hennessy and M. J. Fraser (2005). *piggyBac* internal sequences are necessary for

efficient transformation of target genomes. Insect Mol. Biol., 14: 17-30.

Mohammed, A., L. Sun and M. J. Fraser (2009a). Germline Transformation in *Drosophila melanogaster* expressing the mammalian sialic acid synthase and CMP-sialic acid synthetase. Arab J. Biotech., 12: 303-314.

Mohammed, A. L. Sun and M. J. Fraser (2009b). The *piggyBac*-mediated germline transformation of *Drosophila melanogaster* expressing the mammalian α2,3-sialyltransferase and α2,6-sialyltransferase. Arab J. Biotech., 12: 315-324.

Mohammed, A., L. Sun, T. Fraser and M. J. Fraser (2010). *PiggyBac* tarnsposon-based tetracycline-dependent transactivator expression in Drosophila. Egypt. J. Genet. Cytol., 39: 73-86.

Rendi, D., I. B. H. Wilson and K. Paschinger (2008). The Glycosylation Capacity of Insect Cells. Croatica Chemica Acta. 81: 7-21.

Shaper, N. L., J. H. Shaper, J. L. Meuth, J. L. Fox, H. Chang, I. R. Kirsch and G. F. Hollis (1986). Bovine galactosyltransferase: identification of a clone by direct immunological screening of a cDNA expression library. Proc. Natl. Acad Sci. USA, 83: 1573-1577.

Shi, X., R. L. Harrison, J. R. Hollister, A. Mohammed, M. J. Fraser and D. L. Jarvis (2007). Construction and characterization of new *piggyBac* vectors for constitutive or inducible expression of heterologous gene pairs and the identification of a previously unrecognized activator sequence in *piggyBac*. BMC Biotechnol., Jan., 18: 7-5.

Stebbins, M. J., S. Urlinger, G. Byrne, B. Belo, W. Hillen and J. C. P. Yin (2001). Tetracycline-inducible systems for *Drosophila*. Proc. Natl. Acid Sci., USA, 98: 10775-10780.

Tan, J., A. F. D'Agostaro, B. Bendiak, F. Reck, M. Sarkar, J. A. Squire, P. Leong and H. Schachter (1995). The human UDP-N-acetylglucosamine: alpha-6-D-mannoside-beta-1,2-N-acetylglucosaminyltransferase II gene (MGAT2). Cloning of genomic DNA, localization to chromosome 14q21, expression in insect cells and purification of the recombinant protein. Eur. J. Biochem, 231: 317-328.

Tomiya, N., D. Howe, J. J. Aumiller, M. Pathak, J. Park, K. B. Palter, D. L. Jarvis, M. J. Betenbaugh and Y. C. Lee (2003). Complex-type biantennary N-glycans f recombinant human transferrin from Trichoplusiani insect cells expressing mammalian B-1,4-galactosyltransferase and B-1,2-N-acetylglucosaminyltransferaseII. Glycobiology, 13:23-34.

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

Primer	Sequence
Inverse PCR primers	
TaqInverFWD	5'-CGTCACAATATGATTATCTTTCTAGGG-3'
TaqInverRVS	5'-CGCATGTGTTTTATCGGTCTGTATATC-3'
SauInverFWD57-1	5'-CGCATGATTATCTTTAACGTACGTCAC-3'
SauInverRVS57-1	5'-CAAAACTTTTATCGAATTCCTGCAGCC-3'
SauInverFWD57-1	5'-ATCAAGCTTATCGATACCGTCGACCTCG-3'
SauInverRVS57-1	5'-CACTTACCGCATTGACAAGCACG-3'
SauInverNestFWD57-1	5'-TTCGCCCTATAGTGAGTCGTATTAAGA-3'
SauInverNestRVS57-1	5'-CGACTGAGATGTCCTAAATGCACA-3'
RT-PCR primers	
GntIIFWD	5'-AGGGCACTCTGAGTGGATGGTC-3'
GntIIRVS	5'-AAGGAAGAACGAGGCCCTCG-3'
GntIIFW-2	5'-CCTAGGAATTTGAGGAACCAGCAC-3'
GntIIRVS-2	5'-GCCGGGGTGAATTTCTGTCC-3'
GalTFWD	5'-ATGAAGTTTCGGGAGCCGCT-3'
GalTRVS	5'-CGTCCCGATGTCCACTGTGA-3'
GalTFWD-2	5'-GTCGCACCGCCGCCGCCTTT-3'
GalTRVS-2	5'-CCTCCCCAGCCCCAGTAGTT-3'
DsRedORF-FWD	5'-GCGCTCCTCCAAGAACGTCATCAAG-3'
DsRedORF-RVS	5'-GGCTTCTTGGCCATGTAGATGGACT-3'

Table (2): 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	743	56	7.5	10	0	0.0
2	363	53	9.0	16	0	0.0
3	514	51	10.0	7	1	0.143
4	525	94	18.0	26	1	0.038
5	445	40	9.0	16	0	0.0
6	575	117	20.0	25	3	0.120
7	93	30	32.0	13	0	0.0

Table (3): Insertion sites of transformed *Drosophila* lines.

Line	Chromo-some location	Insertion site sequence 5' junction		Insertion site sequence 3' junction
57-1	3R	AataaggggattaccctTTAA	…*piggyBac*…	TTAAtttcaccttgtgagacg
57-2	2R	agccatgtcaccccacTTAA	…*piggyBac*…	ND*
57-3	2R	cagagtttcctgataatTTAA	…*piggyBac*…	TTAAtaaacacgtaatactg
57-4	2L	aaatgtccctagaagtTTAA	…*piggyBac*…	TTAAagcgttttcgggagtg
57-5	ND*	ND*		ND*

* The junction sequences are not resolved by inverse PCR.

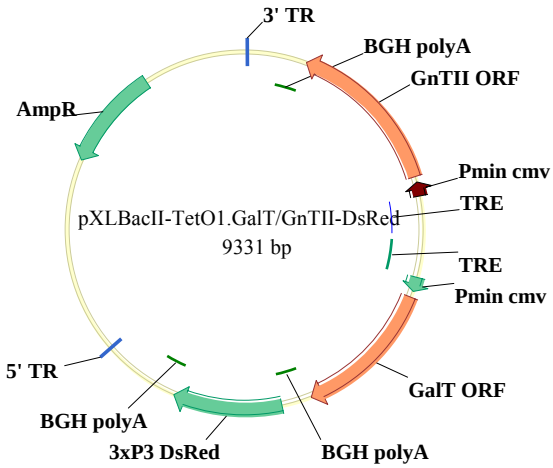

Fig. (1): The map of the donor plasmid pXLBacII-TetO1.GalT/GnTII -DsRed.A cl 3

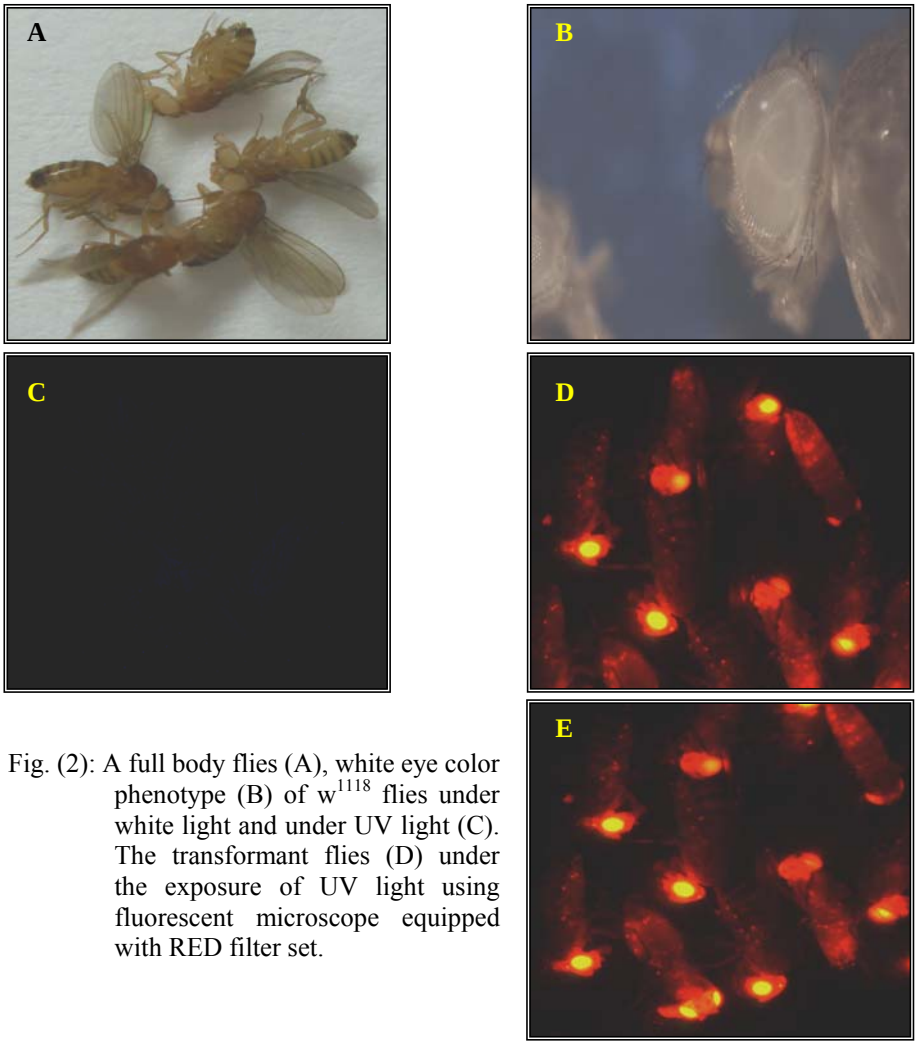

Fig. (2): A full body flies (A), white eye color phenotype (B) of w^{1118} flies under white light and under UV light (C). The transformant flies (D) under the exposure of UV light using fluorescent microscope equipped with RED filter set.

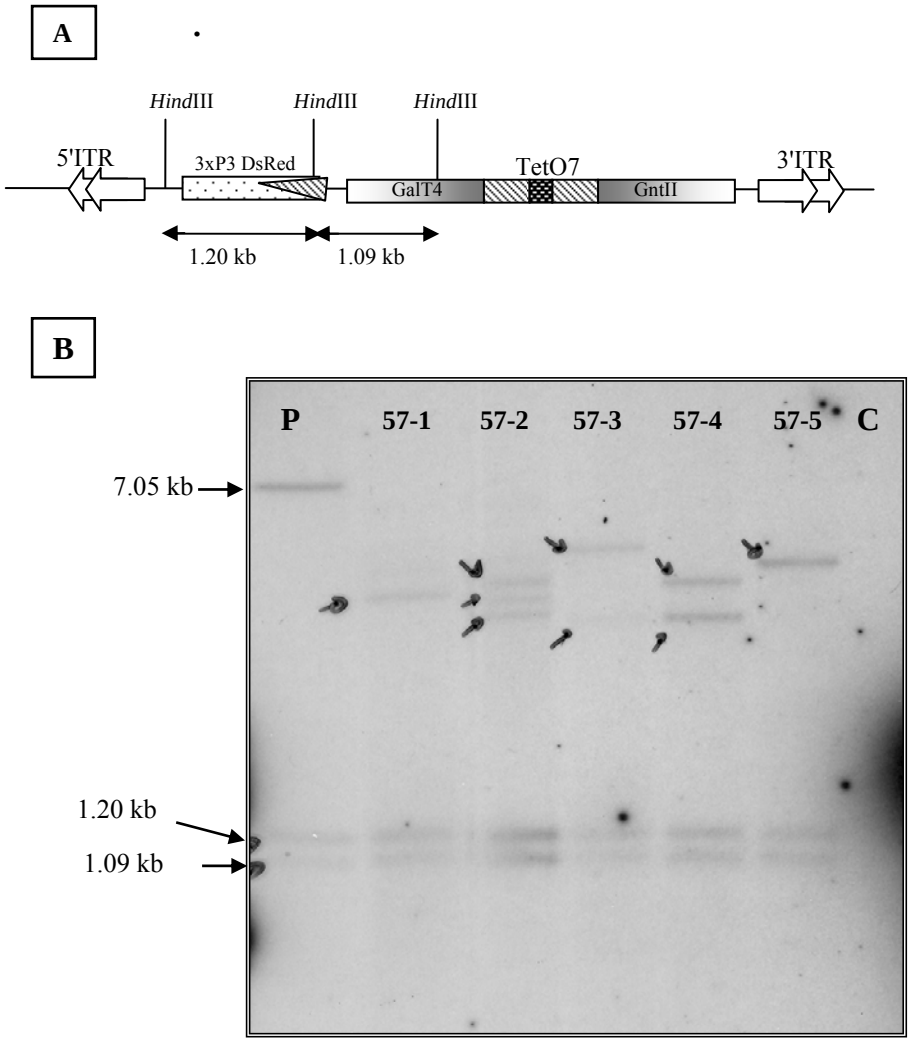

Fig.(3): Southern DNA hybridization analysis of *D. melanogaster* transformants. A) Scheme of the pXLBacII-TetO1.GalT/GnTII-DsRed.A cl 3 vector showing the location of HindIII restriction sites. B) DNA hybridization representing flies from each transgenic line (57-1 through 57-5). All lines have two internal fragments (1.195 and 1.085 kb) and extra-variable size fragment(s). Lines have single insertion such as 57-1 and 57-5. Lines 57-3 and 57-4 show two variable signals while line 57-2 shows three signals**.** The positive control is the plasmid DNA (P) and the negative control is the w^{1118} flies (C).

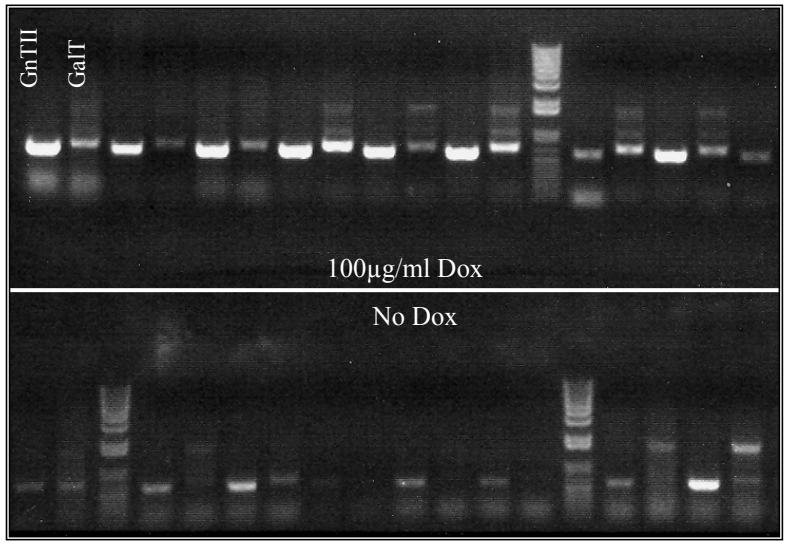

Fig. (4): The transcripts N-acetylglucosaminyltransferase II (GnTII) and β1,4-galactosyltransferase (GalT) were verified for each crossing using RT-PCR.