The *piggyBac*-mediated germline transformation of *Drosophila melanogaster* expressing the mammalian α2,3-sialyltransferase and α2,6-sialyltransferase

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ABSTRACT

The insect N-glycosylation processes produce truncated glycoproteins with much less complexity than that of mammals. Due to the inability to produce biantennary complexes, insect cell-based baculovirus expression systems produce glycoproteins with N-linked oligosaccharide lacking the terminal sialic acid residues. Modifying the insect N-glycosylation process is critical for expanding the utility of insect expression systems. In the current study we attempt to modify the N-glycosylation pathway of drosophila to process glycoprteins to sialylation. The w^{1118} eye mutant strain of melanogaster was transformed with piggyBac-derived constructs carrying both mammalian α2,3-sialyltransferase and α2,6-sialyltransferase under the "ie1-hr5-ie1" dual back to back constitutive promoter. The transformation experiments yielded seven positive flies showing eye-specific-cyan fluorescence with two transgenic lines expressing both mammalian enzymes. No detectable effect on fecundity or survivability.

Key words: *PiggyBac, mediated germline, transformation, D. melanogaster .*

INTRODUCTION

Insect-based baculovirus expression systems exhibit effective protein processing capabilities such as folding, modification, assembling newly synthesized ploypeptides and others. However, the insect protein glysylation pathway is not equivalent to those of higher eukaryotes (Kost *et al.*, 2005). While both insect and mammalian N-glycan processing pathways share a common intermediate, in insect cells, exoglycosidases catalyze trimming reactions of the common intermediate yielding highly trimmed product known as "paucimannose" (Man3-GlcNac2-N-Asn). In contrast, mammalian cell glycosyltransferases catalyze elongation reactions yielding biantennary complex structures. Lepidopteran cell lines transformed with mammalian genes encoding N-glycan processing enzymes produce authentic mammalian glycoproteins during baculovirus infection (see review, Jarvis 2003; Hollister and Jarvis 2001, Hollister *et al.*, 2002). *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (BTI Tn 5B1 4) cell lines were doubly transformed with mammalian 4Gal-T and 2,6 sialyltransferase (ST6GalI) genes (Hollister *et al.* 1998; and Breitbach and Jarvis 2001). Analyses of the final N-glycan product showed that these transformed cells (ST6) cells could produce terminally sialylated glycoproteins.

These results lead to the conclusion that engineering lepidopteran insect cells with mammalian genes encoding functions that are missing or limited in insect cells, extends N-glycan processing pathways similar to those in mammalian cells.

Our goal is to apply the knowledge gained from engineered insect cells to "humanize" the glycoprotein processing pathway in intact insects and assess the effect of this attention of the glycosylation pathway on the insects viability. Here we report the generation of transgenic *melanogaster* lines expressing the rat α2,6-sialyltransferase and the mouse α2,3-sialyltransferase. The transgenic lines were analyzed for the expression of both genes at the molecular level.

MATERIALS AND METHODS

Plasmids

The donor plasmid, pXLBacII-ST6.1/ST3.3M-CFP#21-1, was provided by Dr. Donald Jarvis, Wyoming State University (Shi *et al.* 2007).

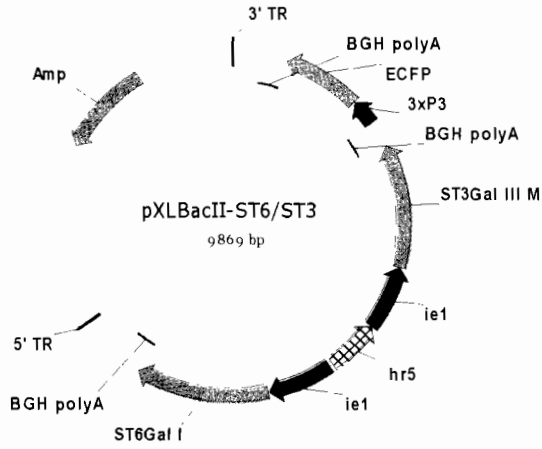

Fig. (1): The map of donor plasmid, pXLBacII- ST6.1/ST3.3M-CFP#21-1.

The pXLBacII (Li *et al.*, 2005) was utilized as backbone construct. The plasmid was designed to encode a rat α2,6-sialyltransferase (ST6GalI) (Weinstein *et al.*, 1987) and a mouse α2,3-sialyltransferase (ST3GalIII) (Weikert *et al.*, 1999; and Kono *et al.*, 1997) under the "*ie1-hr5-ie1*" dual constitutive transcriptional control element and terminated with the bovine growth hormone polyadenylation signal, BGHpolyA. The cyan fluorescent marker gene (*ECFP*), under the eye-specific promoter, 3xP3, was also incorporated into the plasmid. The helper plasmid, phspBac (Handler and Harrell, 1999), was used for microinjection as a source of *piggyBac* transposase.

Drosophila culture

Drosophila strains were reared under standard laboratory conditions (Roberts, 1998). The preblastodermal embryos were microinjected as previously described (Rubin and Spradling 1982). The *D. melanogaster* w^{1118} white eye strain was used for microinjection with a concentration of 0.6μg/μl of pXLBacII- ST6.1/ST3.3M-CFP M#89F50 #21-1, and 0.4μg/μl of the phspBac. All injected embryos were subjected to one

hour heat shock at 37°C for 24 hours past injection to induce expression of the *piggyBac* transposase. The injected embryos were allowed to grow to adulthood at 28°C. Emerging adults were individually mated with w^{1118} flies, and their progeny were screened as adults for fluorescent eye color using an Olympus SZX12 fluorescent microscope equipped with CFP 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

Genomic DNA was prepared from adult flies using DNAzol (MBL) and 25ug was digested overnight with *Xho*I restriction enzyme then transferred overnight onto Nylon membrane and cross-linked using a UV crosslinker (Stratgene). The donor plasmid DNA was used as a positive control as well as a probe. The probe was radio-labeled with ^{32}P dCTP (Amersham) using Prime-a-Gene Labeling System (Promega).

IPCR and Sequence analysis

Inverse PCR was performed by digesting 5µg of the genomic DNA from the transformed strain with *Sau*3AI followed by heat inactivation of the restriction enzyme and DNA precipitation, and finally self ligation in a 100µl volume overnight. The inverse PCR reaction was performed in two steps. The reaction conditions and primers sets were essentially as described in some where else in the current volume (Mohammed *et al*., 2009).

RT-PCR

The total RNA was extracted from the homogenized flies using the Trizol reagent (Invitrogen). The first stand cDNA synthesis and amplification reaction conditions were as described in the current volume (Mohammed *et al*., 2009). The α2,6-sialyltransferase (ST6GalI) was amplified using the primer set: ST6GalIFWD: 5'-ATGATTCATACCAACTTGAAG-3' and ST6GalIRVS:5'-CAACAACGAATGTTCCGGAA-3'. While the primer set: ST3GalIIIFWD: 5'-TCAGATACCGCTGCTTAAGT-3' and ST3GalIIIRVS: 5'-ATGGGACTCTTGGTATTTGT-3' was used to amplify the α2,3-sialyltransferase. The enhanced cyan fluorescent protein encoding gene was amplified using the primer set used by Mohammed *et al*., (2009).

RESULTS AND DISCUSSION

Transformation experiments

From eight separate transformation experiments, a total of 4205 embryos were injected and 1833 larvae were hatched (Table 1).

Table (1): Transformation experiments.

Injection Experiment	Number of injected embryos	Number of hatched larvae	Percentage of hatching	Number of backcrosses	Number of cyan-expressing flies	Transformation frequency %
1	135	62	40	12	1	8.3
2	440	124	28.18	24	2	8.3
3	450	85	18.88	18	3	1.66
4	178	44	24.72	4	1	2.5
5	490	72	14.69	18	0	0
6	1117	861	77.1	123	0	0
7	650	344	52.92	88	0	0
8	725	241	33.24	ND	0	0

The emerged adults were separately backcrossed with the w^{1118} flies. Only seven positive flies were expressing eye-specific cyan fluorescence protein (Fig. 2). The transformation frequencies ranged from 8.2-25%. The transformation frequency is calculated by dividing the number of the G0 transgenic individuals by the number of crossed families. The flies were marked as 21-1 through 21-7. One of the seven flies, 21-1, died and so was eliminated from the experiment. Six homozygous transgenic lines were developed by backcrossing the positive flies, (21-2 through 21-7), with w^{1118} flies for few generations, then were analyzed for legitimate *piggyBac* transposition.

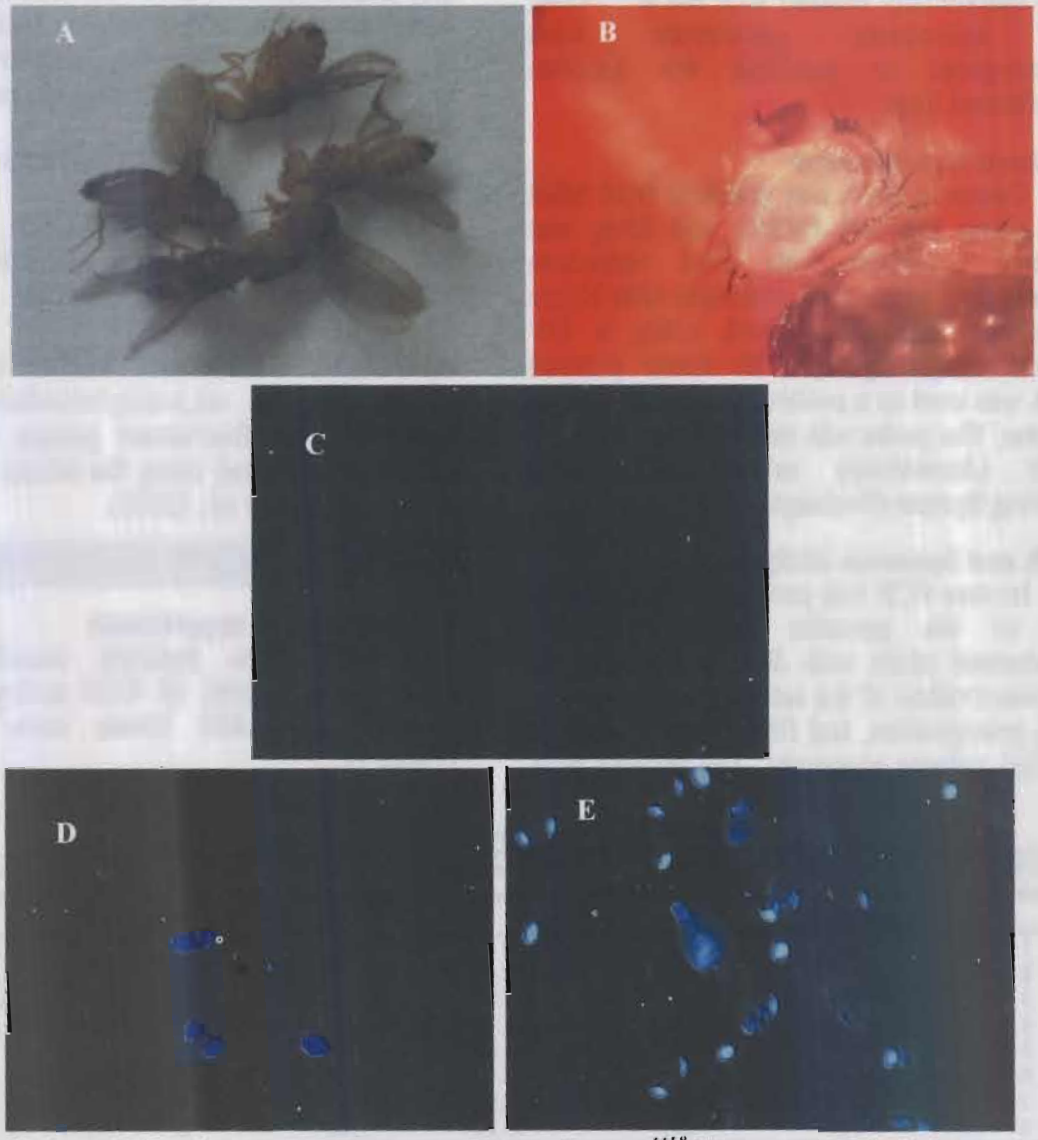

Fig. (2): Full body flies (A), white eye color phenotype (B) of w^{1118} flies under white light and under UV light (C). The transform ants (D and E) flies under the exposure of UV light using fluoreseent microscope equipped with CFP filter set.

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) and successfully used as genetic tool to study the gene function. Bonin and Mann (2004) designed a *piggyBac* gene trap system to study the gene expression and function in drosophila. The trap vector contains the *piggyBac* transposon flanked with splicing acceptor fused to the enhanced green fluorescent protein with transcriptional terminator and integrated into drosophila genome. The driver line expresses the *piggyBac* transposase under the inducer heat shock promoter. Mobilization of the trap sequence (PBss) within the drosophila genome was accomplished by crossing the two transgenic drosophila lines. Insertion of PBss into active gene led to expression of the fused EGFP and protein that is encoded by the trapped gene. In different studies, *piggyBac* with *Hermes* elements were used as insertional mutagenesis system in Drosophila (Horn *et al.*, 2003 and Häcker *et al.*, 2003). Previous experiences with *piggyBac*-based system in insect transformation represent an excellent choice for remodeling the insect N-glycosylation pathway in *Drosophila*.

Insertion analysis

Integration of the transgene DNA into the insect genome was confirmed by Southern blot analysis. The *Xho*I-digested plasmid yields two bands; one finger-print band at 4.44kb which represents the two heterologus genes (ST3 and ST6) and second band at 5.429kb representing the rest of the plasmid (Fig. 3.A). The *piggyBac*-transformed flies should exhibit an internal fragment at 4.4Kb and extra variable fragment(s) that differ according to the insertion site and number of inserts within the insect genome. Fig. (3) shows that the inserted *piggyBac* DNA was transmitted to the progeny generations. The w^{1118} flies had no insert whereas all ECFP-positive flies had at least one insert. Transgenic lines (21-2, 21-4, 21-5, 21-6 and 21-7) showed fragments at the predicted size. Longer exposure times allowed detection of extra faint fragments across the lines (data not shown). The differences in fragment intensity across the lines could be due to degradation of genomic DNA d uring handling and/or the number of insertions or their locations within the fly genome.

One extra fragment of variable size is evident for lines 21-4 and 21-6 which represent one of the terminal repeats flanked with the genomic sequences. The different size of the fragments reveals that the insertion sites in these two lines are at different locations of the fly genome and they may originate from different transposition events in the embryonic germ cells. The signals, representing the *piggyBac* terminal repeats are flanked by genomic DNA. The southern blot for line 21-5 was repeated as shown in the Figure (3.B). The line 21-3 hybridization shows two signals at unexpected sizes. This result suggests that the initial transformation of the ECFP-positive fly was due to illegitimate recombination

RT-PCR

The α2,6-sialyltransferase and α2,3-sialyltransferase expression, in five transgenic lines (21-2, 21-4, 21-5, 21-6 and 21-7), was confirmed using reverse transcriptase PCR (Fig.3). In the four lines, there was a significant difference in abundance of the PCR products demonstrating that the α2,3-sialyltransferase transcript is expressed more than the α2,6-sialyltransferase. The unexpected position effect is due to putative transcriptional activator closer to the 3' domain. In the dual piggyBac-derived vector system, the heterologous genes adjacent to the 3'- terminal region are consistently expressed

at higher levels than those adjacent to the 5'-terminal region of the *piggyBac* element (Shi *et al.*, 2007).

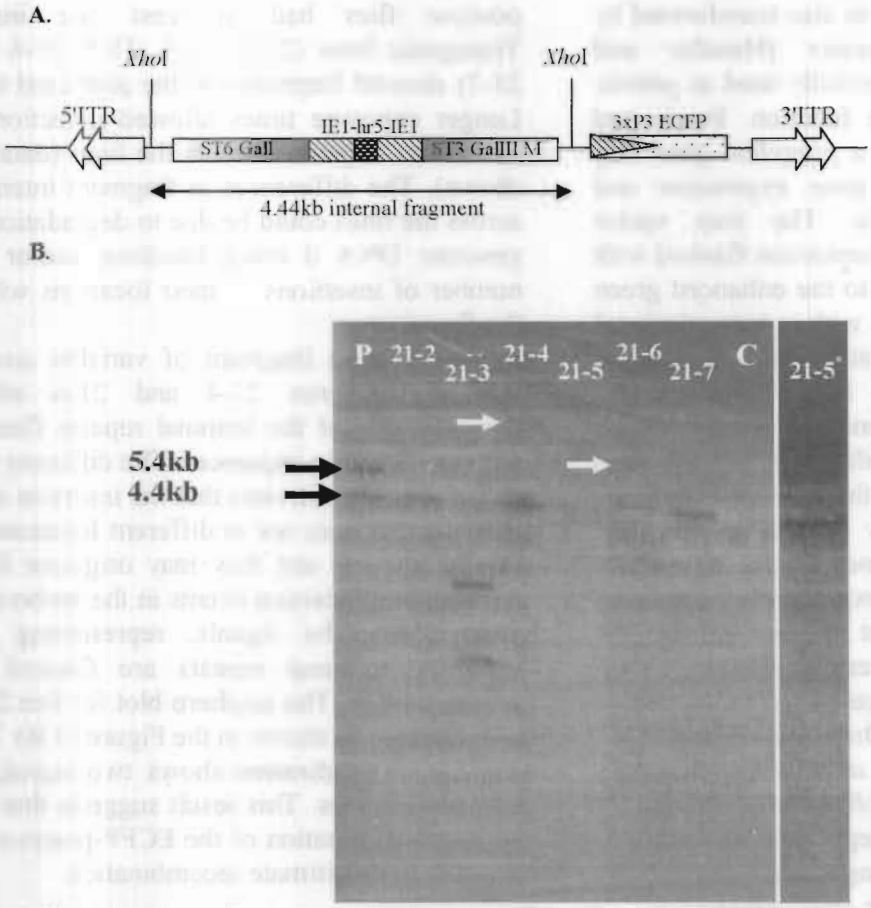

Fig. (3): Southern DNA hybridization analysis of D. melanogaster transformants lines. A) Scheme of the pXLBacII- ST6.1/ST3.3M-CFP #21-1 vector showing the location of XhoI restriction sites. B) Southern DNA hybridization of transgenic lines (21-2 through 21-7). All lines have a fragment of 4.44kb, expect 21-3. Additional S. blot for 21-5 line is shown at the right side. The positive control is the plasmid DNA (P) and the negative control is w^{1118} flies (C).

Insertion site determination

Integration of the sialyltransfersae genes and determination of the insertion sites on the fly chromosomes were verified using inverse PCR. The 5' and 3' junctions of the *piggyBac* terminal repeats were sequenced and Blast analyzed (Altschul *et al.*, 1990) to identify genomic insertion site. All sequences and matching results are shown in Table (2). One out of the six flies displayed illegitimate recombination. The sequence of both 5' and 3' termini of line 21-3 match the internal sequence of the donor plasmid, confirming the southern results shown in Figure (3.B). The sequences of the 5' junctions for all five lines match the fly genome. The 3' junction was

only recovered for lines 21-4 and 21-6. Both junction sequences show duplicates of TTAA. the hallmark of all *piggyBac* integrations (Elik *et al.*, 1995). Other 3' junction sequences were not achieved due to failure of inverse PCR. However, the matching results of 5' junction for lines 21-2 and 21-6 show that *piggyBac*-mediatd transpositions at the same site on the third chromosome. The same is also shown for lines 21-4 and 21-5, on the fourth chromosome. These results suggest that these flies might originate from the same initial transposition at the same germ cell. The line 21-7 insertion site is unique and located at the X chromosome.

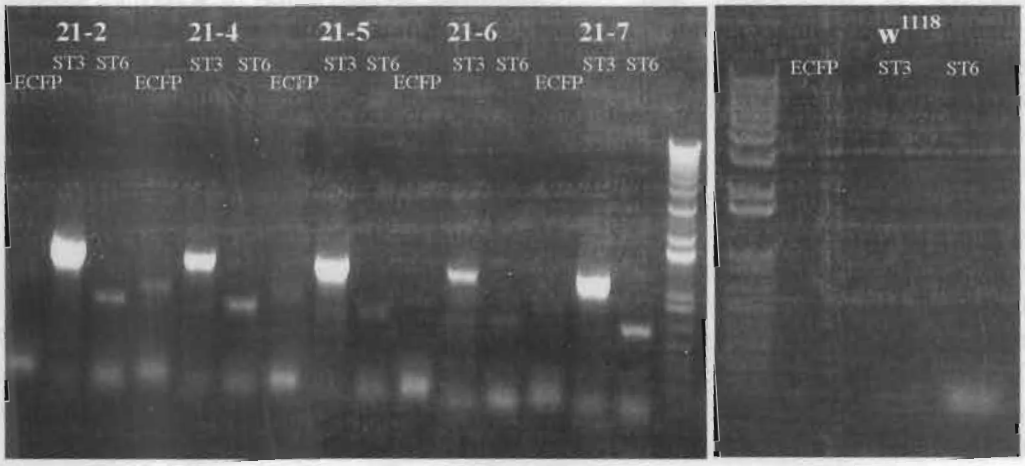

Fig. (4): The transcripts of enhanced cyan fluorescence protein (ECFP), α2,3-Sialyltransferase (ST3) and α2,6-Sialyltransferase (ST6) were verified using RT-PCR for lines 21-2, 21-4, 21-5, 21-6 and 21-7 as well as the negative control of w^{1118} flies.

Table (2): Insertion sites of transformed Drosophila lines.

Line	Chromosome location	Insertion site sequence 5' junction		Insertion site sequence 3' junction
pXLBacII-clone#	------	ctgatgcagctagaTTAA	..*piggyBac*..	TTAAtctagctgcgtgttctg
21-1				
21-2	4	aaaaaatgacttcaTTAA	..*piggyBac*..	ND*
21-3**	ND	tcgtattaagatctaaTTAA	..*piggyBac*..	ATATacagaccgataaa
21-4	3L	ttaagcgtgaatagtTTAA	..*piggyBac*..	TTAAtcactggcgtcctcgt
21-5	3L	ttaagcgtgaatagtTTAA	..*piggyBac*..	ND
21-6	4	aaaaaatgacttcaTTAA	..*piggyBac*..	TTAAaaataattattaaat
21-7	X	caaaggcaaataacTTAA	..*piggyBac*..	ND

* The junction sequence is not resolved by inverse PCR.
** The 5' and 3' junction sequences of 21-3 line match with internal sequence of the pXLBacII-21-1.

Sialyltransferases are a family of glycosyltransferases which catalyzes the transfer of sialic acid to terminal positions on the carbohydrate groups of glycoproteins and glycolipids (Beyer *et al.* 1981; and Kornfeld and Kornfeld 1985). Like many other glycosyltransferases, they are found primarily in the Golgi apparatus of cells, where they participate in post-translational glycosylation pathways (Schachter *et al.*, 1970; and Fleischer, 1981). At least 10-12 different sialyltransferases are required to synthesize all the sialyloligosaccharide sequences known. The rat liver β-galactoside α2,6-sialyltransferase (Weinstin *et al.*, 1987), Golgi apparatus enzyme, is involved in the terminal sialylation of N-linked carbohydrate groups of glycoproteins. It has been used to transform lepidopteran cells, Sf9 and BTI-Tn-5B1-4 (Hollister *et al.* 1998; and Breitbach and Jarvis 2001). The transgenic cells produced terminally sialylated glycoproteins. Both α2,6-sialyltransferase and α2,3-sialyltransferase are expressed in the *Drosophila* transgenic lines and their transcripts have been detected. Further biochemical reactions are required to determine the activities of the expressed enzymes.

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REFERENCES

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

Beyer, T. A.; Sadler, J. E.; Rearick, J. I.; Paulson, J. C. and Hill, R. L. (1981). Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. Adv. Enzymol. Relat Areas Mol Biol., 52:23-175.

Breitbach, K. and Jarvis, D. L. (2001). Improved glycosylation of a foreign protein by Tn-5B1-1 cells engineered to express mammalian glycosyltransferases. Biotch. Bioengr., 74:230-239.

Bonin, C. P. and Mann, R. S. (2004). A *piggyBac* transposon gene trap for the analysis of gene expression and function in Drosophila. Genetics 167: 1801–1811.

Elick, T. A.; Bauser, C. A.; Principe, N. and Fraser, M. J. (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.

Fleischer, B. (1981). Orientation of glycoprotein galactosyltransferase and sialyltransferase enzymes in vesicles derived from rat liver Golgi apparatus. J. Cell Biol., 89: 246-255.

Häcker, U.; Nystedt, S.; Barmchi, M. P.; Horn C. and Wimmer E. A. (2003). *piggyBac*-based insertional mutagenesis in the presence of stably integrated *P* elements in *Drosophila*. PNAS USA 100:7720-7725.

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

Horn, C.; Offen, N.; Nystedt, S.; Häcker, U. and Wimmer, E. A. (2003). *piggyBac*-Based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. Genetics 163: 647–661.

Hollister, J.; Shaper, J. H. and Jarvis, D. L. (1998). Stable expression of mammalian β1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. Glycobiology 8: 483-4780.

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

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

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

Kono, M.; Ohyama, Y.; Lee, Y. C.; Hamamoto, T.; Kojima, N. and Tsuji, S. (1997). Mouse beta-galactoside alpha 2,3-sialyltransferases: comparison of in vitro substrate specificities and tissue specific expression. Glycobiology. 7(4):469-479.

Kornfeld, R. and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Annu. Reu. Biochem. 54:631-664

Kost, T. A.; Condreay, J. P. and Jarvis, D. L. (2005). Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. 23:567-575.

Li, X.; Harrell, R. A.; Handler, A. M.; Beam, T.; Hennessy, K. and Fraser, M. J. (2005). piggyBac internal sequences are necessary for efficient transformation of target genomes. Insect Mol. Biol. 14: 17–30.

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

Roberts, D.B. (1998). Drosophila: A practical approach. IRL Press. Oxford.

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

Schachter, H.; Jabbal, I.; Hudgin, R. L.; Pinteric, L.; McGuire, E. J. and Roseman, S. (1970). Intracellular localization of liver sugar nucleotide glycoprotein glycosyltransferases in a Golgi-rich fraction. J. Biol. Chem. 245,1090-1100.

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

Weinstein, J.; Lee, E. U.; McEntee, K.; Lai, P. H. and Paulson, J. C. (1987). Primary structure of β-galactoside alpha 2,6-sialyltransferase. Conversion of membrane-bound enzyme to soluble forms by cleavage of the NH2-terminal signal anchor. J Biol Chem. 262:17735-17743.

Weikert, S.; Papac, D.; Briggs, J.; Cowfer, D.; Tom, S.; Gawlitzek, M.; Lofgren, J.; Mehta, S.; Chisholm, V.; Modi, N.; Eppler, S.; Carrol, I. K.; Chamow, S.; Peers, D.; Berman, P. and Krummen, L. (1999). Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat Biotechnol. 17:1116-1121.

الملخص العربي

التحول الوراثى عن طريق بيجى باك للخلايا الجرثومية فى درسوفيلا ملانوجاستر لانتاج الأنزيمين الثدييين الفا ٣,٢ سياليل ترانسفريز و الفا ٦,٢ سياليل ترانسفريز

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**معهد بحوث الهندسة الوراثية الزراعية – مركز البحوث الزراعية – جيزة - مصر

طرق معلاجة البروتينات بالمواد الكربوهيدراتية فى أجسام الحشرات تنتج جليكوبروتينات مبتوره وأقل تعقيدا عما يحدث داخل أجسام الثدييات. ونتيجة لعدم القدرة على تخليق مركبات ثنائية القرن فأن أنظمة التعبير الجينى للباكولوفيرس التى تعتمد على خلايا الحشرات تنتج جليكوبروتينات ن-مرتبطة بعديد السكريات ولكنها تفتقر الى أحماض السليك الطرفية . تعديل طرق معلاجة البروتينات بالمواد الكربوهيدراتية داخل أجسام الحشرات هى خطوة ضرورية لتوسيع نطاق الفائدة من أنظمة تخليق البروتينات المعتمدة على الحشرات. في الدراسة الحالية تم محاولة تعديل مسار معلاجة الجليكوبروتينات داخل حشرة الدرسوفيلا. تم أجراء عدة تجارب تحول وراثى على سلالة طفرة العين البيضاء [و١١١٨] من ذبابة الفاكهة درسوفيلا ملانوجاستر بأستخدام العنصر القافز بيجى باك المحتوى على شظايا الحمض النووى الذى يرمز الى كل من الأنزيميين الثدييين الفا ٣،٢ سياليل ترانسفريز و الفا ٦،٢ سياليل ترانسفريز تحت تأثير العنصر الحافز المستمر المزدوج [إي١-هر٥-ي١]. أنتجت تجارب التحول الوراثى سبع ذبابات إيجابيّة تظهرن الوميض الأزرق السماوى فى أعينهن عند تعرض أجسامهن للأشعة فوق البنفسجية. تم أخضاع الأجيال المستنبطة من هؤلاء الأفراد السبعة الى أجراء تجارب تحليلية تأكيدية.