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Methods for transferring gene into silkworm and expression of hGM-CSF in silk glands of transgenic silkworm

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ABSTRACT

To explore efficient of different gene transfer methods and to express human granulocyte-macrophage colony-stimulating factor (hGM-CSF) in silk glands of transgenic silkworm, a novel transgenic vector containing a hGM-CSF controlled by silkworm fibroin heavy chain gene promoter (Pfib-H) with a neomycin resistance gene (neo) driven by BmNPV (B. mori nucleopolyhedrovirus) immediate-early gene ie-1 promoter (Pie-1) was constructed. The transgenic vector was transferred into the B. mori by using sperm-mediated gene transfer, particle delivery system gene transfer, eggs puncture gene transfer, and gonadal injection gene transfer, respectively, and the effectiveness of different transfer methods was investigated. The results showed that the ratio of fluorescence silkworm in G0 generation for four transform methods was 3.44%, 2.07%, 1.25% and 0.02%, respectively, indicating exogenous DNA can be introduced into silkworms eggs by the four methods mentioned above. The obtained transgenic silkworms by using sperm-mediated gene transfer were screened with neo and gfp genes as selecting markers, and verified with PCR and Southern blot. The hGM-CSF were expressed to about 13.67 ± 0.59 and 14.55 ± 0.65 ng per gram freeze-dried powder of silk glands from G5 and G8 generation transgenic silkworm, respectively. A specific 22 kD-band of hGM-CSF was detected in western blot. Furthermore, bioactivity assay indicated that the hGM-CSF expressed in posterior silk glands of transgenic silkworm could stimulate the proliferation of K562 cells. These results provide interesting information for further research of exogenous genes in the silk glands of transgenic silkworm by using diverse methods.

Key words: *piggyBac*; transgenic silkworm; gene transfer; human granulocyte-macrophage colony-stimulating factor.

INTRODUTION

The silkworm (*Bombyx mori*, Lepidoptera: Bombycidae) is a kind of silking-producing insect that was domesticated from the wild silkworm (*B. mandarina*) in ancient times. Comparing with other animals and plants, the technique of transgenic silkworm takes some advantages in research of fine structure, function and expression regulation of organism's genome. The silkworm is not only a very useful animal model for research, but also an economically important insect. Moreover, with the development of biotechnology, *B. mori* has become an important bioreactor for recombinant protein production [1,2]. In recent years, researchers have devoted themselves to the investigation of exogenous gene expression in the silkworms[3-5] . The silkworm baculovirus expression vector system [6] and the techniques of transgenic silkworms [1,7-9] have been established. As a whole, the investigations on the transgenic

silkworms and bioreactors of silk glands are still in starting stage. The silk gland of silkworm is a highly specialized organ that has tremendous ability to synthesize and secrete fibroin. It has extensive prospect of application as a good bioreactor.

The mobility and transposition functions of *piggyBac* were established and exploited to develop an important system for insect germline transformation[9-11] . Secretory expression of exogenous genes in the silk gland of transgenic silkworm has been successfully achieved under the control of a silk gland-specific promoter, using a *piggyBac*-based vector. Expression of human type III procollagen in the cocoon fiber has been carried out by use of a *fib*-L promoter [2] The same approach was also applied to the secretory expression of prolyl hydroxylase α-subunit and human collagen in the posterior silk gland [12]. Hino et al. succeeded in expressing a fusion protein of fibroin and human basic fibroblast growth factor in the posterior silk gland of transgenic silkworm that secreted to the fibroin layer of cocoon[13] . But the biological activity of the expression product was rather low because of denaturing and refolding in protein. In addition, Ogawa et al. successfully expressed human serum albumin (rHSA) in the sericin using sericin promoter *ser*-1[14]. They achieved as high as 3.0 μg rHSA in one mg of cocoon and obtained an active rHSA with 99% purity by a two-step process that consisted of phosphate buffered solution extraction and ammonium sulfate precipitation. Furthermore, Kurihara et al. designed a feline interferon (FeIFN) protein fused with N- and C-terminal sequences of the fibroin H-chain respectively that was expressed in the posterior silk gland, and separated from interferon by protease. Although the expressed fusion proteins from transgenic silkworms had almost no activity, they recovered a very high biological activity after eliminating the Fib-H derived N- and C-terminal sequences by protease cleavage [15]. We had used the fibroin heavy chain gene (*fib*-H) promoter [16] with its downstream signal peptide sequence to secrete the expressed hIGF-I in silkglands into the lumen of silk gland and ultimately reached the fibroin layer of cocoon. Moreover, we had reported that blood glucose level in TIDM mice could be reduced by orally administering the silk glands of transgenic hIGF-I silkworms [16]. Collectively, these approaches for exogenous gene expression applied a strategy of protein fusion with silk protein. In the design of most *piggyBac* transposon-based vectors, it is usual to introduce one exogenous gene (besides the fluorescence-expression gene) into the genome. At present, there are reporter genes has been used at areas of research transgenic silkworm such as fluorescence-expression gene, *neo*, *CAT* gene, but all these reporter genes were used alone, the transgenic silkworm are screened with double reporter genes may be more effective.

Currently, microinjecting transgenic vector into the early stage of fertilized eggs is now commonly used in silkworm transgenic studies; however, the hard shell of silkworm egg is a physical hindrance to microinjection. Usually, the exogenous DNA can be microinjected after an egg was penetrated first by a fine tungsten needle. However, the survival rate of microinjected eggs is very low because outflow of the egg contents at the moment of breaking the shell. Moreover, silkworm is in diapause in the egg stage, this diapause can be broken by immediate artificial hatching with hydrochloric acid at the early stage of fertilized eggs. Clearly, the microinjected injected eggs are not fit for treatment with hydrochloric acid. Therefore, gene transfer via microinjection into the fertilized silkworm egg is successful in only a few cases, greatly hampering silkworm transgenic research. It is thus necessary to develop new approaches for the introduction of exogenous genes into silkworm eggs.

Human granucyto-macrophage colony-stimulating factor (hGM-CSF) is a kind of glycoprotein that regulates hematopoiesis and immunoregulation. The oral administered hGM-CSF from silkworm expression by recombinant baculovirus could be absorbed into the blood[17]. Therefore it will be a possible way to make oral administered medicine directly by expressing hGM-CSF in silk glands of transgenic silkworms. We had reported that expression of the hGM-CSF in the silk glands of germline of gene-targeted silkworm [5,3] and had confirmed that the silk glands containing expressed hGM-CSF performed the function of significantly increasing leukocyte count of CY-treated mice in a time-and dose- dependent manner[3] . In this study, to develop new approaches for the introduction of exogenous genes into silkworm eggs and to study the non-secretory expression of hGM-CSF in the silk gland of transgenic silkworms, we constructed a transgenic vector that contains a neomycin resistance gene (*neo*), the green fluorescent protein gene (*gfp*), and a expression cassette of h*GM-CSF* gene. Our work investigated the effectiveness of different gene transfer methods and the expression of an exogenous gene in the silk glands of transgenic silkworms, which provides useful information for further developing new approaches for the introduction of exogenous genes into silkworm eggs and research of exogenous genes in this system.

EXPERIMENTAL SECTION

Vector and Host-bacteria

The transgenic vector pigA3GFP, the transposase-expression helper plasmid pigA3 with the *piggyBac* transposase sequence under the control of the *B. mori A3* promoter, the h*GM-CSF*-containing vector pBacPAK-hGM-CSF [18], the vector pcDNA3.1 containing the resistance gene *neo* (Invitrogen Corporation, Carlsbad), the vector pIE-Neo[19] containing the *neo* gene regulated by the immediate early stage gene (*ie*-1) promoter ($P_{ie\text{-}1}$) from the silkworm baculovirus, the vector pSK-FH-DsRed-PolyA[20] containing *DsRed* gene regulated by the silkworm fibroin heavy chain gene (*fib*-H) promoter ($P_{fib\text{-}H}$, GenBank accession No. EF540776), the vector pigA3GFP-fib-L-intron1 with the partial intron 1 (fib-L-intron 1) sequence of the fibroin light chain gene (*fib*-L)[21], the vector pBluescript II SK (+) (Stratagene, La Jolla), host bacteria *E. coli* strainTG1 were provided or previously constructed by the Laboratory of Molecular Biology of the College of Basic Medicine and Biological Sciences, Soochow University.

Enzyme and Reagents

Restriction enzymes, PCR reagents, the gel extraction kit and the T4 DNA ligase were purchased from the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Sangon, Shanghai). G418, lipofectin was the product from Gibco BRL (Rockville).

Primers and PCR

The primers for the *fib*-H promoter, fib-H-1 (gcg aat tcg aga atg tct gga cag, the underline indicates an *Eco*RI site) and fib-H-2 (gcg ata tct tga gag ttg gaa ccg, the underline indicates an *Eco*RV site) were designed based on the sequence published in GenBank (AF226688). The primers, hGM-CSF-1 (tgg ata tca tgt ggc tgc aga gcc tgc, the underline indicates an *Eco*RV site) and hGM-CSF-2 (atc tcg aga agc tta tca ctc ctg gac tgg ctc cc, the underline indicates an *Xho*I site and a *Hin*dIII site) were designed for h*GM-CSF* based on the sequence published on the GenBank (M11220). The primers for tailing signal sequence (*fib*L-PA) of the silk fibroin light chain gene (*fib*-L) were TPFib-L-3 (ggc tcg agc aaa ttg tgt ttg cgt tag g, the underline indicates a *Xho*I site) and TPFib-L-4 (gcg gta ccc act gtc caa tcc acc gtc, the underline indicates a *Kpn*I site). The primer for neo-2 (agc tcg agc tag agg tcg acg g, the underline indicates an *Xho*I site) was designed based on the sequence of plasmid pcDNA3.1 with neomycin gene. The primer for IE-1 (ttc gaa ttc gat ttg cag ttc ggg ac, the underline indicates an *Eco*RI site) was designed based on *ie*-1 promoter region of the whole genomic sequence of the BmNPV-T3 strain published on the GenBank (L33180). The primer for *gfp* gene was designed based on its sequence, termed DEGFP-1 (tgg aat tca tgg tga gca agg gcg agg, the underline indicates an *Eco*RI site) and DEGFP-2 (ttg gat cct tac ttg tac agc tcg tcc atg, the underline indicates a *Bam*HI site).The *gfp*, *fib*-L-intron1, h*GM-CSF*, *neo*, $P_{fib\text{-}H}$ and *fib*L-PA were amplified using a standard PCR protocol.

Construction of vector pigA3GFP-hGM-CSF-neo

To construct the plasmid pSK-fibH-hGM-CSF-fibL-PA, the fragment of the h*GM-CSF* gene, which encodes the peptide of hGM-CSF, was amplified from the plasmid pBacPAK-hGM-CSF with primers hGM-CSF-1 and hGM-CSF-2, digested with *Xho*I and *Eco*RV, and then ligated into pSK-FH-DsRed-polyA. Fluorescent protein gene (*DsRed*) was excised in this construction.

The hGM-CSF expression cassette from the plasmid pSK-fibH-hGM-CSF-fibL-PA was put into plasmid pigA3GFP-fib-L-intron1 digested with *Kpn*I and *Eco*RI to generate plasmid pigA3GFP-hGM-CSF-fib-L-intron1. The *ie*-1-promoter-driven *neo* gene was isolated from the plasmid pIE-Neo by *Eco*RI, and cloned into *Eco*RI site of the plasmid pigA3GFP-hGM-CSF-fib-L-intron1 to construct pigA3GFP-hGM-CSF-neo (Fig. 1).

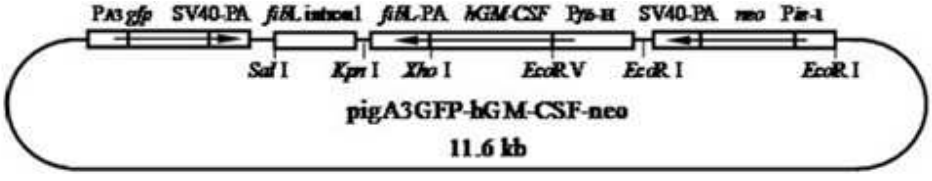

Fig. 1 Construction of transgenic vector pigA3GFP-hGM-CSF-neo

P_{A3}: A3 promoter of silkworm actin; *gfp*: green fluorescent protein gene; SV40-PA: polyA signal sequence of SV40; *fib*-L intron 1: the promoter sequence of intron 1 of fibroin light chain gene; *fib*L-PA: polyA signal sequence of

fibroin light chain gene; *hGM-CSF*: human granulocyte-macrophage colony-stimulating factor gene; P_{fib-H}: promoter of fibroin heavy chain gene (*fib*-H); *neo*: neomycin resistance gene; P_{ie-1}: The promoter of immediate-early gene (*ie*-1) of silkworm baculovirus.

Introduction of transgenic vectors into silkworms

Sperm-mediated gene transfer was performed according to the methods described by Zhou et al., [22] and Cao et al.,[18]. Moths were free to copulate. The transgenic vector at a concentration of 2 μg / μL was mixed with helper plasmid pigA3 at a ratio of 1:1 and injected into the copulatory pouch of female moths (Strain Gaobai). Then female moths oviposited.

Particle bombardment system gene transfer was performed according to the methods of the reference [23]. Gold microcarriers (50 μL, 3 μg) was put to a 1.5 mL centrifuge tube, then 2.5 μL of helper pigA3 vector DNA (1 μg/μL), 2.5 μL of transgenic vector pigA3GFP-hGM-CSF-neo DNA (1 μg/μL), 50 μL of 2.5 mol/L $CaCl_2$ and 20 μL of 100 mmol/L spermidine were added and vortexed for 2-3 min. According to PDS-1000/He apparatus (BIO-RAD Corporation) manufacturer's application, the silkworm eggs spawned within 2 hours (Strain Gaobai) were bombarded.

Eggs puncture gene transfer was performed as follows: the silkworm eggs spawned within first 2 hours were disinfected with 2% of formaldehyde for 5 min; the eggs were fixed on the table. The mixture of the vector pigA3 DNA and the transgenic vector pigA3GFP-hGM-CSF-neo DNA (1:1) were coated on the surface of eggs, the eggs were punctured gently by using disinfected acupuncture needle, and sealed with 1% of low melt point agarose gel.

Gonadal injection gene transfer: the transgenic vector pigA3GFP-hGM-CSF-neo and the helper plasmid pigA3 were mixed with cationic lipofectin reagent (Roche, Mannheim), and injected into the gonads of silkworm (Strain Gaobai) at the second day of the 4^{th} instars. The silkworms were fed normally, the moths were copulated each other and oviposit. The eggs incubation and silkworms rearing were performed by standard protocols.

Extraction of moth genomic DNA

Moths were washed with ultrapure water and then grinded in 1 mL of TE buffer (100 mmol / L Tris-HCl and 10 mmol/L EDTA, pH8.0). One mL Tris-equilibrating phenol (Shanghai Sangon) was added to the homogenate and mixed well. After incubation of 30 min, the mixture was centrifuged (13,000 g, 4°C) for 10 min. The supernatant was transferred to a fresh tube and processed again with Tris-equilibrating phenol as described above. An equal volume of phenol/chloroform (1:1) was then added into the supernatant, stirred gently for 30 min and centrifuged (13,000 g, 4°C) for 10 min. The DNA was spooled out with a glass rod after adding one-tenth volume of sodium acetate solution (3 mol/L) and two volumes of absolute ethanol into the final supernatant. The DNA precipitate was washed with 70% ethanol, dried and dissolved in 100 μL of TE buffer, and then stored in -20°C.

Examination of transgenic silkworms

The G0 generation silkworms were fed by standard protocols and screened under the fluorescent microscope (Olympus SZX12). Moths of G0 generation were copulate between male and female of positive fluorescence individuals. The larvae newly molted second instars of G1 generation were fed 1 day with mulberry leaves coated with 10 μg/mL G418, the surviving silkworms were then fed with normal mulberry leaves and were screened at different stages by fluorescent microscope, and the obvious fluorescence individuals were copulate each other to make G2 generation. The silkworms of G2 to G8 generation were treated with same methods mentioned above.

The fluorescent transgenic silkworms' genomic DNA of G0, G1 and G2 generation were identified by PCR with primer pairs DEGFP-1/ DEGFP-2 and hGM-CSF-1/ hGM-CSF-2, respectively. PCR products were recovered with the BioFlux Kit (Bioer Technology, Hangzhou) and subjected to direct sequencing. The fluorescent transgenic silkworms' genomes of G8 generation were identified by PCR with primer pairs DEGFP-1/ DEGFP-2, hGM-CSF-1/ hGM-CSF-2 and IE-1/ neo-2, respectively.

The genomic DNA of the G1 generation moth was digested by *Kpn*I for 4-5 h in total volume of 50 μL, about 13 μg of DNA sample for each lane was separated with 1% agarose gel. DNA hybridization with a DIG-labeled *gfp* probe, membrane wash and signal detection were carried out according to the manufacturer's instructions of the DIG DNA labeling and Detection Kit (Roche, Mannheim). Positive and negative controls were also included.

SDS-PAGE and western blotting

The posterior silk glands were from the fourth day of fifth instar of G3 generation transgenic silkworm larvae produced by the sperm-mediated gene transfer method, and were made to freeze-dried powder. 0.01 gram of the powder was dissolved in 150 μL of tissue lysis buffer (10% glycerol, 2.5% SDS, 5% mercaptoethanol, 625 mmol/L Tris-Cl, pH6.8). After centrifugation, the supernatants were mixed with loading buffer, and boiled for 5 min, then loaded on 15% SDS-PAGE. Western blot was performed with a primary antibody of rabbit anti-hGM-CSF (Beijing Biosynthesis Biotechnology) in 1:1000 and a secondary antibody of HRP-conjugated goat anti-rabbit (Beijing Biosynthesis Biotechnology) in 1:1000.

Detection of hGM-CSF expression

The 0.01 g of freeze-dried powder of the posterior silk glands from the transgenic silkworm larvae of G5 and G8 generation was dissolved in 150 μL of tissue lysis buffer (10% glycerol, 2.5% SDS, 5% mercaptoethanol, 625 mmol/L Tri·Cl, pH6.8), deposited for 12 h. After centrifugation (13,000 g, 4°C) for 5 min, the supernatant was subjected to ELISA detection. ELISA was performed according to the manufacturer's instructions (Boster Biotechnology, Wuhan).

Detection of bioactivity for expressed hGM-CSF with MTT

The freeze-dried powder of the posterior silk glands of the silkworm larvae of transgenic silkworm of G8 generation or normal silkworm at the fourth day of fifth instar were ground with RPMI-1640 medium (1:10 W/V, Gibco-BRL, Germany) on ice, after sonication, the homogenate was incubated for 12 h at 4°C and centrifuged (16,000 g, 4°C) for 30 min, then the supernatant was transferred to a fresh tube. According to the previous results of ELISA detection, the supernatant then was diluted to 0.5 ng/mL of hGM-CSF with fresh RPMI-1640 medium. Standard sample of hGM-CSF (SinoBio Biotech, Shanghai) was diluted to 100 ng/mL with RPMI-1640 medium. The chronic myelogenous leukemia cells K562 (provided by Dr. Lan Xu of College of Basic Medicine and Biological Sciences, Soochow University) in logarithmic growth phase were harvested and centrifuged (11,000 g, 4°C) for 5 min, 1×10^6/mL of cells were prepared by adding fresh RPMI-1640 medium with 10% fetal calf serum (FCS), of which 100 μL was added into each well on the 96-well plate. Then the 96-well plate was divided into five groups. ① Test group: 100 μL of the supernatant made of posterior silk glands from transgenic silkworm (SPSG-T) was added to each well; ② Negative control group 1: 100 μL of the supernatant made of silk glands from normal silkworm posterior (SPSG-N) was added to each well; ③ Positive control group 1: 99.5 μL of the SPSG-T and 0.5 μL of the contrast of standard hGM-CSF was added to each well; ④Positive control group 2: 99.5 μL of the fresh RPMI-1640 medium and 0.5 μL of the hGM-CSF standard was added to each well; ⑤ negative control group 2: 100 μL of the fresh RPMI-1640 medium was added to each well. The cells were cultured under 5% CO_2 at 37°C, and 20 μL of MTT (5 mg/ mL) was added to each well after the cells were cultured for 12, 24 and 36 h, respectively. Being incubated for another 4 h, after centrifugation (20,000 g, 4°C) for 5 min, the precipitation of the cells for each well was dissolved with 150 μL of DMSO, 50μL of which was poured to a new 96-well plate, then 4-fold dilution with DMSO was taken and the absorbance for each well at 570 nm wavelength was determined with ELISA detector (2010 type, Anthos Labtec Instrucments Company, Australia).

RESULTS AND DISCUSSION

Identification of the transgenic vector pigA3GFP-hGM-CSF-neo

In the design of most *piggyBac* transposon-based vectors, one exogenous gene (besides the fluorescent gene) is usually introduced into the genome[24]. However, some transgenic approaches require the introduction of multiple heterologous genes into a single target organism. To obtain stably transgenic silkworms continuously expressing exogenous genes through the mediation of *piggyBac* transposon by screen with the neomycin resistance gene, we constructed the transgenic vector pigA3GFP-hGM-CSF-neo that contains the *neo*, *gfp*-tag and h*GM-CSF* expression cassette. In this transgenic vector, the h*GM-CSF* gene was regulated by the *fib*-H promoter, while the *neo* gene was regulated by the *ie*-1 promoter from the silkworm baculovirus. Moreover, the enhancer, a PCR amplified partial sequence of the first intron of the fibroin light chain gene (GenBank accession No. DQ679478) was also cloned into the transgenic vector to elevate the expression level of the exogenous gene. PCR verified the constructed vector pigA3GFP-hGM-CSF-neo using the primer pairs fib-H-1/fib-H-2, hGM-CSF-1/hGM-CSF-2, DEGFP-1/DEGFP-2, TPFib-L-3/TPFib-L-4, IE-1/neo-2 and Fib-L-5 (cgg ata tct atg ggc tcc agt aac c) / Fib-L-6 (gcg tcg acg gtc agg tta gat taa cgg g). The PCR products of 490, 450, 700, 290, 300 and 1 700 bp corresponding with anticipation in size were detected, respectively (Fig. 2). These results showed that the vector pigA3GFP-hGM-CSF-neo was

successfully constructed.

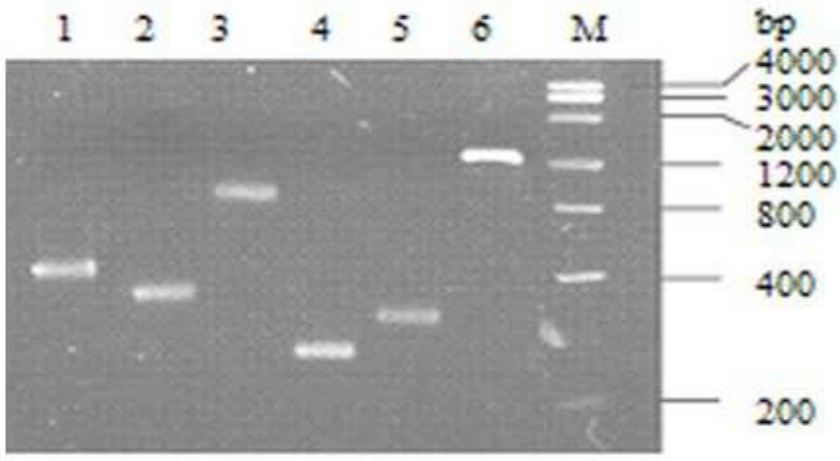

Fig. 2 Verifying the plasmid pigA3GFP-hGM-CSF-neo by PCR

PCR amplification showed the presence of the gene fragments in the plasmid. Lane 1: *fib*-H promoter (P_{fib-H}); Lane 2: *hGM-CSF* gene; Lane 3: *gfp* gene; Lane 4: polyA signal sequence of the fibroin light chain gene (*fib*-L-PA); Lane 5: intron 1 of the fibroin light chain gene (*fib*-L intron 1); Lane 6: *ie*-1promoter-driven neomycin-resistance gene expression cassette; Lane M: 200 bp ladder DNA marker.

Methods comparison of introduction of transgenic vectors into silkworms

The transgenic vectors were introduced into silkworms by using sperm-mediated gene transfer, particle bombardment system gene transfer, gonadal injection gene transfer and eggs puncture gene transfer, respectively. The gonadal injection gene transfer, a new transgenosis method was attempted considering factors such as silkworm gonad development stage and suitable period for gonad injection. In the eight silkworms whose gonad was injected, three male and two female silkworms developed to moths finally, and hybridization each other (in which a male moths were discarded). The hatching rate of eggs and the ratio of fluorescence silkworm in G0 generation for different transgenosis methods were shown in Table 1. The ratio of fluorescence silkworm for gonad injection gene transfer was the highest, reached to 3.44%. The ratio of fluorescence silkworm for eggs puncture gene transfer was up to 2.07%, but the hatching rate of eggs was 11%, this result showed that the eggs were harmed due to puncture. The ratio of fluorescence silkworm for particle bombardment gene transfer was the lowest, that might be relative to the less vectors DNA in eggs because of the thicker eggshell and difficult to puncture with the particle.

Table 1 The hatching rate of eggs and the ratio of fluorescence silkworm in G0 generation

Methods of gene transfer	Total number of silkworm eggs	Number of newly hatched larvae	Number of fluorescence individuals	Rate of fluorescence silkworm (%)
Sperm-mediated	3523	3513	44	1.25
Particle bombardment	1326	1303	3	0.02
Gonadal injection	786	780	27	3.44
Eggs puncture	773	85	16	2.07

Screening and identification of transgenic silkworms

The fluorescent larvae (Fig. 3A) of G0 generation obtained by sperm-mediated gene transfer were fed with normal mulberry leaves, green fluorescence also were observed obviously in pupa stage under a fluorescent microscope (Fig.

3B, 3C), after copulation, oviposit, The months' genomic DNA were extracted and were used for PCR amplification with the primer pairs DEGFP-1/DEGFP-2 and hGM-CSF-1/hGM-CSF-2 to detect *gfp* gene and h*GM-CSF* gene, respectively. The specific PCR product for *gfp* gene (about 720 bp) and h*GM-CSF* gene (about 450 bp) were successfully amplified from the genomes of the fluorescent moths (results not shown). Results showed that the *gfp* gene and h*GM-CSF* gene were integrated into the genome of silkworm.

The newly hatched worms of the G1 generation were screened with antibiotic G418; the surviving silkworms were then fed with normal artificial food. To verify the stable integration of *gfp* and h*GM-CSF* genes into the genome, we then randomly selected ten moths of the G1 generation and extracted genomic DNA for PCR amplification of the *gfp* and h*GM-CSF* genes. The results showed that the 720 bp (Fig. 3D) and 450 bp (Fig. 3E) of specific products were amplified from the genomic DNA of nine moths with the primer pair DEGFP-1/ DEGFP-2 and hGM-CSF-1/ hGM-CSF-2. DNA sequencing result represented the existence of the *gfp* and h*GM-CSF* genes in the G1 generation of silkworm and strongly suggested that these moths were transgenic silkworms.

The silkworms' genomes of G8 generation were also identified by PCR amplification. The results showed that with the primer pairs DEGFP-1/ DEGFP-2, hGM-CSF-1/ hGM-CSF-2 and IE-1/ neo-2 the PCR amplicons at the size of 720 bp (Fig. 3G), 450 bp and 1.7 kb(Fig. 3H) were specifically achieved, respectively, and strongly suggested that the *gfp*, h*GM-CSF* and *neo* gene had stably integrated into the genome.

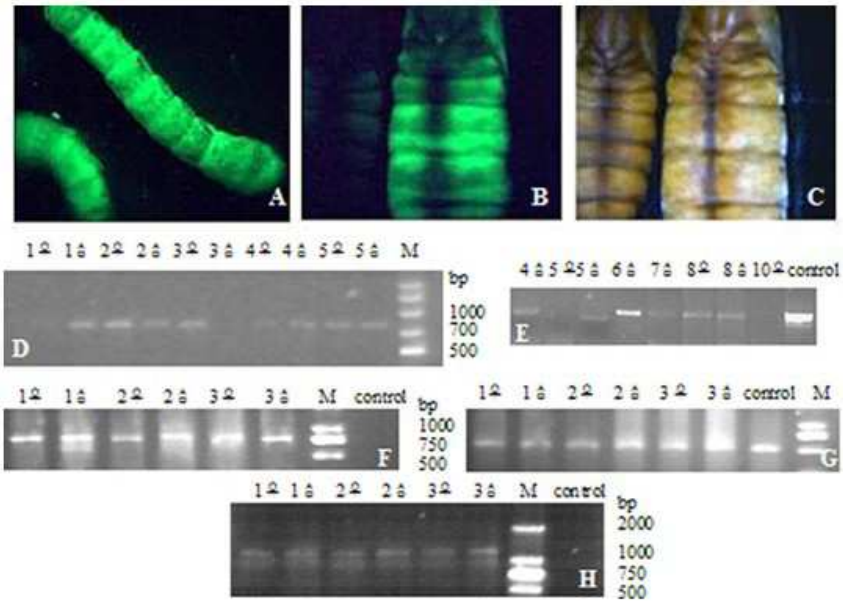

Fig. 3 The fluorescence of transgenic silkworm identification of PCR

(A): The fluorescent silkworm larva of the G0 generation. (B): A fluorescent silkworm pupa of the G0 generation, under a fluorescent microscope. (C): The silkworm pupa of the G0 generation, under a normal vision. (D): PCR identification of G1 generation silkworms that survived the G418 screening using the primer pair DEGFP-1/DEGFP-2. Ten silkworms of generation G1 survived the G418 screening. (E): PCR identification of G1 generation silkworms that survived the G418 screening using the primer pair hGM-CSF-1/hGM-CSF-2, control: pigA3GFP-hGM-CSF-neo vector, M: DNA marker. (F): identification of G8 generation silkworms by PCR with the primer pair DEGFP-1/DEGFP-2, control: normal silkworm, M: DNA marker. (G): identification of G8 generation silkworms with PCR using the primer pair hGM-CSF-1/hGM-CSF-2, control: normal silkworm, M: DNA marker. (H): identification of G8 generation silkworms with PCR with the primer pair IE-1/ neo-2, control: pigA3GFP-hGM-CSF-neo vector, M: DNA marker.

G418 screening of transgenic silkworms

The larvae of G1 generation obtained by the sperm-mediated gene transfer method at newly molted second instar were fed with mulberry leaves coated with 10 μg/mL of G418 for 1 day. The developmental differences were

obvious among the silkworms in response to G418 (Fig. 4), and successive deaths of worms were observed. The fluorescent individuals could be detected in the some of normal growth silkworms. In order to understand the transgenic silkworm's resistance to G418, the silkworms of the G2 generation at newly molted second instars were randomly divided into four groups and fed with mulberry leaves coated with 10 μg/mL of G418 for different days. The results showed that the rate of eclosion for normal silkworm (Strain Gaobai) fed with G418 for 1 day was only 1.2% (Table 2); it indicated that G418 were higher toxicity to non-transgenic silkworm. The larvae of G2 generation were fed with G418 for1 day, 14% of which were kept to the cocooning and eclosion, but the rate of eclosion for silkworms fed with G418 was reduced obviously in the groups treated more days, The results demonstrated that an expression cassette with the *neo* resistance gene in the transgenic vector was convenient and efficient for screening transgenic silkworms.

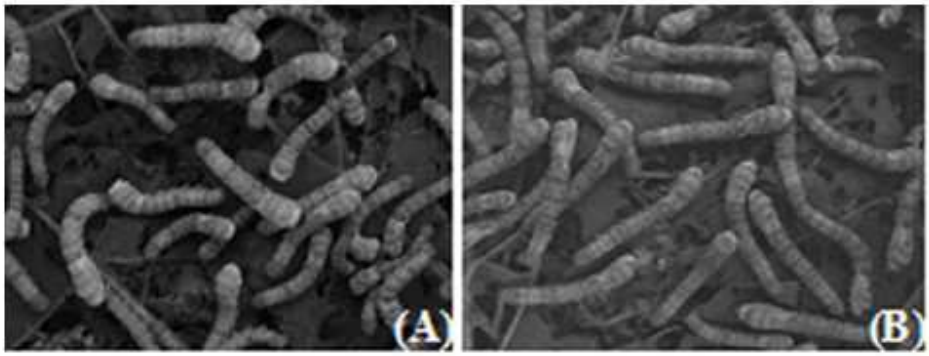

Fig. 4 The larvae of G1 generation fed with G418

(A): G1 generation silkworms were fed with mulberry leaves coated with 10 μg/mL G418; (B): normal (negative control) silkworm rearing were performed by standard protocols.

Table 2 The result of G2 generation were fed with G418

	Transgenic silkworm of G2 generation				strain Gaobai
The time of fed with G418 (d)	1	2	3	0	1
The number of newly hatched larvae	357	363	361	379	394
The number of pupas	63	12	0	351	13
The number of moths	50	8	0	347	5
The rate of eclosion (%)	14	2.2	0	92	1.2

Southern blotting of genomic DNA of transgenic silkworms

The Southern blotting of genomic DNA of transgenic silkworms of G1 generation obtained by the sperm-mediated gene transfer method showed that the *gfp* gene probe could hybridize the genomes of the fluorescent moths (Fig. 5), and indicated *gfp* gene had been present in the genome of transgenic silkworm.

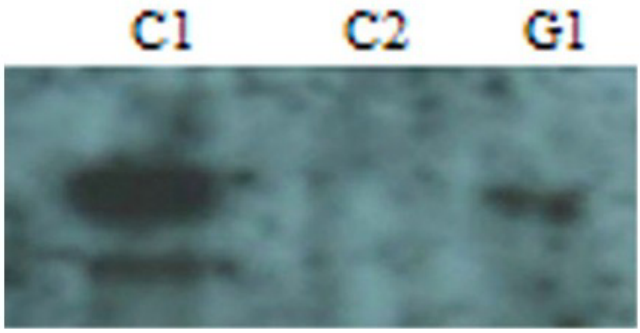

Fig. 5 Southern blot analysis for genomic DNA of transgenic silkworm

C1: Transgenic vectors pigA3GFP-hGM-CSF-neo; C2: Genomic DNA from normal silkworm was digested with *Kpn*I; G1: Genomic DNA from silkworm of G1 generation was digested with *Kpn*I.

The western blotting analysis of hGM-CSF expressed in silk gland of transgenic silkworm

The three transgenic silkworms of G3 generation obtained by the sperm-mediated gene transfer method displaying green fluorescence and resistance to G418 showed that the *gfp* and neomycin-resistance genes had been expressed correctly. After the silk glands of G3 generation were treated with organization lysis buffer, SDS-PAGE analysis and western blotting analysis were performed. No strong expressed protein bands appear on SDS–PAGE; however, a 22 kD protein band was detected by specific antibody on western blotting (Fig. 6). It was corresponding to the recombinant hGM-CSF expressed with baculovirus expression system in *B. Mori*,The results showed that hGM-CSF had been expressed in silk glands transgenic silkworms, and the expressed hGM-CSF had been modified during the processing of post translation.

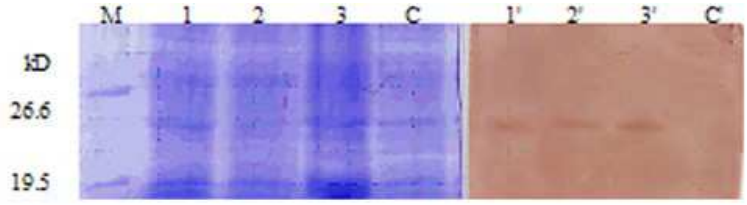

Fig. 6 SDS-PAGE and western blot analysis of hGM-CSF expressed in transgenic silkworm silk glands

M: Protein Marker; 1-3: The SDS-PAGE for samples of posterior silk glands from the different larvae of G3 generation; C: The SDS-PAGE for posterior silk gland from normal silkworm; 1'-C': The western blotting was corresponding with 1-C.

Determination of hGM-CSF expression in transgenic silkworm silk glands

The transgenic silkworms of G5 and G8 generation obtained by the sperm-mediated gene transfer method were used to estimate the expression levels of hGM-CSF with an ELISA kit. The expression of hGM-CSF from transgenic silkworms of G5 and G8 generation were 13.67 ± 0.59 and 14.55 ± 0.65 ng per gram freeze-dried powder of posterior silk glands, respectively.

Bioactivity assay for expressed hGM-CSF

The chronic myelogenous leukemia cell line K562 is a multipotent cell line, capable of differentiating along the megakaryocytic, erythroid, and monocytic pathway[25]. hGM-CSF plays an important role in promoting differentiation, survival, and proliferation of colony-forming unit-granulocyte-macrophage progenitor cells[26] and stimulating burst promoting activity for burst-forming units, erythroid[27].It was reported that hGM-CSF activates a set of genes such as c-*fos*, c-*jun*, and c-*myc* genes in both hematopoietic and nonhematopoietic cells[28]. In this study, the effect of hGM-CSF expressed in posterior silk glands of transgenic silkworms on proliferation of K562 cells was investigated. The results showed the SPSG-T promoted the proliferation of K562 cells compared to that of the SPSG-N (Fig. 7), and bio-function of SPSG-T was similar to that of hGM-CSF standard, therefore, the hGM-CSF expressed by transgenic silkworm was supposed to own the biologic activity.

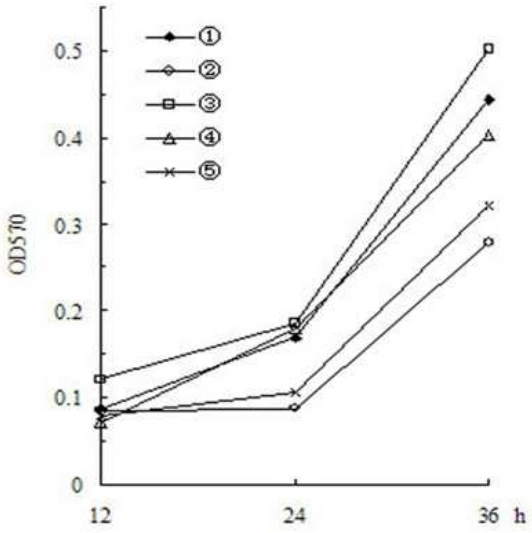

Fig. 7 Effect of hGM-CSF expressed in posterior silk glands on the proliferation of K562 cells

① Test group: supernatant of posterior silk glands from transgenic silkworm; ② Negative control group 1: supernatant of silk glands from normal silkworm posterior ③ Positive control group 1: supernatant of normal silkworm posterior silk gland with hGM-CSF standard; ④ Positive control group 2: hGM-CSF standard; ⑤ Negative control group 2: RPMI-1640 medium.

DISCUSSION

The investigations of the bioreactors of transgenic silkworms and silk glands are still in the starting stage and confronting many challenges. First, only a few transgenic vectors are available. So, it is necessary to construct new vectors to meet different needs. Second, although there are several reporter genes, such as *gfp* and *DsRed*, which can be used in screening, the workload associated with screening is still very large and more efficient methods need to be developed. Third, exogenous genes may be lost in the transgenic silkworms. A previous work reported that the expression of exogenous genes in silkworms beyond generation G3 was notably decreased, indicating a possible loss of the exogenous genes [29]. Thus it is also important to retain the stability of exogenous genes in transgenic silkworms. Finally, microinjection is commonly applied for the introduction of exogenous genes into eggs. However, this method requires a high level of experience and is not feasible for processing a large number of silkworm eggs in a short period of time.

Many genes have been successfully expressed in the Lepidoptera insect and its cells through the baculovirus expression vector system. However, the recombinant proteins expressed in the baculovirus-infected insect and its cells often fail to be completely processed [30]. To overcome this problem, a vector-based stable expression system has been developed [31] and the post-translation modification of recombinant protein could be improved [32]. Recently, the *piggyBac* transposon system was applied in the transformation studies in several types of animals [33-34]. The efficiency of transgenic silkworms with *piggyBac* transposon vector was quite low. Although the reporter gene of fluorescence was helpful for screening the transgenic silkworms, the workload of screening is still very large. Notably, our strategy to introduce an expression cassette with the *neo* resistance gene into the transgenic vector successfully overcomes this problem. When the offspring of the moths processed by gene transfer were fed on G418-containing mulberry leaves, the worms without the *neo* resistance gene successively died. Therefore, it may be concluded that *neo* expression cassette cloned into the transgenic vector, not only facilitate the screening of transgenic silkworm, but also prevent loss of exogenous genes in transgenic silkworm under the pressure of G418 selection.

CONCLUSION

The most widely used strategy currently to express exogenous genes in the silk gland of silkworms is the fusion of

exogenous genes with the fibroin gene[2,12-15]. However, this strategy often results in low activities of gene expression products[13,15]. In our study, non-secretory expression of the h*GM-CSF* gene was achieved under the regulation of the *fib*-H promoter, although the expression level was rather low. The expression level of hGM-CSF of transgenic silkworms in the posterior silk glands of G5 and G8 generation were about 13.67 ng and 14.55 ng per gram freeze-dried powder, respectively and were similar to that in the posterior silk glands of gene-targeted silkworm [3,5]. The theoretical molecular weight of hGM-CSF is 16.29 kD, while a 22 kD of the specific protein band was detected in the posterior silk glands of transformation silkworms by Western blotting. However, the molecular weight of recombinant protein is corresponding with that of recombinant hGM-CSF expressed with baculovirus expression system in *B. mori* [35]. The result might due to post translational modification of hGM-CSF expressed in the posterior silk glands of transformation silkworms It has been reported that the hGM-CSF expressed in silkworm with recombinant baculovirus had bioactivity, the oral administered hGM-CSF could be absorbed into the blood [17]. In this study, we found that the hGM-CSF expressed in posterior silk glands of transgenic silkworm could stimulate the proliferation of K562 cells; therefore, the silk glands of transgenic silkworms with hGM-CSF would possibly be directly used as peroral administered medicine [3]. The expression level may be influenced by the non-secretory expression, the integration site of the exogenous gene in the silkworm genome, or the intrinsic characteristics of the h*GM-CSF* gene. The low expression level of hGM-CSF was also possible closely related to the target gene copy number comparing with that of baculovirus expression vector system, or might related to the short half-life of mRNA of target gene in the silk glands cells. Therefore it is the challenge to improve expression of hGM-CSF in transgenic silkworms.

There have been many approaches for the induction of exogenous genes into silkworm eggs. Among these approaches, microinjection is the most popular method. However, this method has several shortcomings, such as high requirements of equipment and techniques, limited manipulation time and low survival rate of eggs following injection. Moreover, since the injected eggs are not fit for immediate artificial hatching with hydrochloric acid, this method is not appropriate for the transgenic studies in the silkworms of currently practical varieties. It is thus necessary to develop new approaches for the introduction of exogenous genes into silkworm eggs. Recently, Ando and Fujiwara established a rapid genetic functional analysis system for non-model insects using a low-cost electroporator designed for somatic transformation with the *piggyBac* transposon [36]. The method of sperm-mediated gene transfer has several advantages, no specific equipments required and no limitation of voltine of varieties. In addition, this method makes it possible to process a large quantity of virgin moths in a short period of time. The efficiency of transgenic silkworm depend on gene transfer methods and process phase, if the transgenic vector were injected into the copulatory pouch of virgin moths before copulation, as the mating process of silkworm requires1-2 h, the most of the exogenous DNA previously injected into copulatory pouch may has been degraded, that resulted in lower transgenic efficiency. Here, the transgenic vector was injected into the copulatory pouch of female moths after copulation, improving the transgenic efficiency and increasing the proportion of transgenic individuals. The transgenic efficiency for particle bombardment system gene transfer was not recommended for silkworm's transgenosis because of the lowest efficiency. In addition, as previous studied, though some technique like electroporation and biolistics could be used in *B. mori* [36-37],they had some drawbacks for restricting on our purpose. However, gonadal injection gene transfer and eggs puncture gene transfer methods were very efficient for silkworm's transgenosis. So it was considered that transfer exogenous DNA into spermatocytes and oocytes during the development of germ cells is an effective transgenosis method. The survival rate of eggs treated with eggs puncture gene transfer method was quite low (about 10%) because of handwrought manipulation, but the proportion of transgenic silkworms in survival eggs was more than 2%. Therefore, the eggs puncture gene transfer method could be used if there was no microinjector equipment in the lab.

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REFERENCES

[1] Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M. Kômoto N, Thomas J L, Mauchamp B, Chavancy G, Shirk P, Fraser M. *Nat Biotechnol*., **2000** , 18,81-84.
[2] Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura *T*, Yoshizato

K. *Nat Biotechnol*., **2002**, 21,52–56.
[3] Xue RY, Chen HM, Cui LL, Cao GL, Zhou WL, Zheng XJ, Gong CL. *Transgenic Res*., **2012** ,21(1),101-111
[4] Li Y, Cao G, Wang Y, Xue R, Zhou W, Gong C. *Biotechnol Lett*., **2011**, 33 (3),489-494.
[5] Li Y, Cao G, Chen H, Jia H, Xue R, Gong C. *Biochem Biophys Res Commun*., **2010** ,391(3),1427-1431.
[6] O'Reilly D, Miller L K, Luckow V A. *New York : WH. Freeman and Company*., **1992,** NY. 24–50.
[7] Imamura M, Nakai J, Inoue S; Quan GX, Kanda T,Tamura T. *Genetics*., **2003** ,165(3),1329−1340
[8] Xue R, Wang Y, Cao G, Pan Z, Zheng X, Zhou W, Gong C. *J Agric Food Chem*., 2012**,** ,60(26),6559-64.
[9] Zhang H, Cao G, Zhang X, Wang X, Xue R, Gong C. *Agric Food Chem*., **2012** ,28:60(12),3173-9
[10] Handler A M, McCombs S D, Fraser M J, Saul S H. *Proc Natl Acad Sci U S A.*,**1988** , 95 (13),7520–7525.
[11]Tan A, Fu G, Jin L, Guo Q, Li Z, Niu B, Meng Z, Morrison NI, Alphey L, Huang Y.*Proc Natl Acad Sci U S A.*, **2013**, 110(17),6766-70.
[12] Adachi T, Tomita M, Shimizu K, Ogawa S, Yoshizato K. *J Biotechnol*., **2006**, 126(2),205–219.
[13] Hino R, Tomita M, Yoshizato K.. The generation of germline transgenic silkworms for the production of biologically active recombinant fusion proteins of fibroin and human basic fibroblast growth factor. *Biomaterial*s., **2006**, 27(33),5715–5724.
[14] Ogawa S, Tomita M, Shimizu K, Yoshizato K. *J Biotechnol*., **2007**, 128(3),531–544.
[15] Kurihara H, Sezutsu H, Tamura T, Yamada K. *Biochem Biophys Res Commun*., **2007**,355:976–980.
[16] Cong L, Cao G, Xue R, Pan Z,Zheng X, Zhou W, Gong C. *Biochem Biophys Res Commun*., **2011**,410(4),721-725.
[17] Zhang W, Lv Z, Nie Z, Chen G, Chen J, Sheng Q, Yu W, Jin Y, Wu X, Zhang Y. *PLoS ONE*., **2009**, 4(5),5353.
[18] Cao G, Xue R, He Z, Zheng X, Sheng W, Gong C. *Acta Sericologica Sinica*., **2006**, 32,324–327.
[19] Zhou W, Wang C, Liu B, Cao G, Xue R, Shen W, Gong C*Acta Sericologica Sinica*., **2007**, 33,30–35.
[20] Zhou W, Cao J, Ye A, Cao G, Xue R, Gong C. *Acta Sericologica Sinica*., **2008**, 34,72–77.
[21] Zhao Y, Li X, Cao G, Xue R, Gong C. *Science in China Series C Life Sciences*., **2009**,52(12),1131–1139.
[22] Zhou WL, Gong CL, Xu RY, Cao GL, Cao JR, Ye AH, Weng HB, Wang YQ *Biol Reprod*., **2012**, 87(6),144,1–5.
[23] Zhao Y, Zhang F, Chen X, Xu A, Feng X, Huang J, Lu C. *High Technology Letters*., **1999** , 6,16–19
[24] Shi X, Harrison R L, Hollister J R, Mohammed A, Fraser Jr M J, Jarvis D L. *BMC Biotechnol*., **2007**, 7,5.
[25] Sutherland J A, Turner A R, Mannoni P, McGann L E, Turc J M. *J Biol Response Mod*., **1986**, 5(3),250–262.
[26] Gasson J C. *Blood*., **1991**,77(6),1131–1145.
[27] Sieff C A, Emerson S G, Donahue R E, Nathan D G, Wang E A, Wong G G, Clark S C. *Science*., **1985**, 230(4730),1171–1173.
[28] Watanabe S, Kubota H, Sakamoto K M, Arai K. *Blood*., **1997**, 89(4),1197–1206.
[29] Wu X F, Cao C P. *J Zhejiang Univ Sci*., **2004**, 5,644–650
[30] Harrison R L, Jarvis D L. *Methods Mol Biol*., **2007**, 388,341–356.
[31] Jarvis D L, Finn E E. *Nat Biotechnol*., **1996**, 14,1288–l292.
[32] Okada T, Ihara H, Ito R, Nakano M, Matsumoto K, Yamaguchi Y, Taniguchi N, Ikeda Y,*Glycobiology.*, **2010**, 20(9),1147-1159.
[33] Balu B, Shoue D A, Fraser Jr M J, Adams J H. *Proc Natl Acad Sci U S A.*, **2005**, 102(45),16391–16396.
[34] Ding S, Wu X, Li G, Han M, Zhuang Y, Xu T. *Cell*., **2005**, 122(3),473–483.
[35] Gong C, Xue R, Cao G, Shi X, Shen W. *Prog Biochem Biophys*., **2005**, 32,947–952.
[36] Toshiya Ando and Haruhiko Fujiwara.*Development*., **2013**, 140,454-458.
[37] Thomas JL. *Journal of Insect Science*., **2003**, 3,17.