Journal of Animal and Veterinary Advances 11 (20): 3785-3791, 2012
ISSN: 1680-5593
© Medwell Journals, 2012

Expression of the B Subunit of *Escherichia coli* Heat-Labile Enterotoxin in Transformed *Bombyx mori* BmN Cells

[1]Wen-Lin Zhou, [1]Jin-Ru Cao, [1]Ai-Hong Ye, [2, 3]Cheng-Liang Gong, [1]Hong-Biao Weng, [1]Li-Hua He, [2, 3]Ren-Yu Xue, [2, 3]Guang-Li Cao and [1]Yong-Qiang Wang
[1]Zhejiang Academy of Agricultural Sciences, Sericulture Research Institute, 310021 Hangzhou, P.R. China
[2]College of Pre-Clinical Medical and Biological Science, Soochow University, 215123 Suzhou, P.R. China
[3]National Engineering Laboratory for Modern Silk, 215123 Suzhou, China

Abstract: The non-toxic B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) is a potent mucosal immunogen and immunoadjuvant for coadministered antigens. To obtain transformed silkworm cell line stably expressing LTB, researchers fused the LTB coding sequence, neomycin-resistance gene (Neo^R) and *gfp* gene into piggyBac-based transponson vector and transduced into silkworm BmN cells. After screening against antibiotic G418, the positive rate of cells emitting green fluorescence was 70.79%. PCR detection indicates the existence of exogenous LTB coding sequence, Neo^R and gfp in the transformed cell genome. Western blot analysis also confirms the predicated ~60 kDa band of LTB protein. These results demonstrated that the strategy was practicable.

Key words: *Escherichia coli* heat-labile enterotoxin, piggyBac transposon, *Bombyx mori*, BmN cell, genetic transformation

INTRODUCTION

Escherichia coli (*E. coli*) heat-labile enterotoxin (LT) is an enterotoxin that is easily inactivated by heating at 65°C for 30 min and causes severe diarrhea in young animal and human (Gyles, 1992). LT and Cholera Toxin (CT) belong to the same toxin family, share 80% homology in their primary structure and possess similar tertiary structures. The antiserum to either one cross-react with the other (Nataro and Kaper, 1998). The crystal structure of LT revealed that it is a ring-like hexamer composed of one A subunit (LTA, 27 kDa) and five non-covalently associated B subunits (LTB, 11.6 kDa each) (Loc *et al*., 2010). LTA, as the major virulence factor has an ADP-ribosylation activity that causes constitutive activation of adenylate cyclase leading to the increase in intracellular cAMP and the subsequent severe diarrhea (Spander, 1999). LTB is able to bind to GM1-gangliosdie, a glycosphingolipid found ubiquitously on the membrane of mammalian cells and to interact with other related receptors such as GD1b-gangliosdie, asialo-GM1, lactosylceramide and certain galactoproteins (Williams *et al*., 1999). Extensive investigations have shown that LTB is non-toxic but is a potent mucosal immunogen and immunoadjuvant for co-administered antigens. LTB has been expressed in several bacterial, yeast and plant systems such as *Saccharomyces cerevisiae* (Rezaee *et al*., 2005; Lim *et al*., 2009), Siberian ginseng (Kang *et al*., 2006a), potato (Arntzen, 1997; Ravin *et al*., 2008), maize (Chikwamba *et al*., 2002), tobacco (Kang *et al*., 2004a, b, 2006b; Wagner *et al*., 2004), lettuce (Kim *et al*., 2007), carrot (Rosales-Mendoza *et al*., 2007) and rice (Zhang *et al*., 2009). However, the expression of LTB using animal bioreactor is rarely reported.

The domesticated silkworm *Bombyx mori* (*B. mori*) belongs to Bombycidae of Lepidoptera. It is not only a very useful animal model for research but also an economically important insect. As a completely domesticated insect, *B. mori* can be cultivated in large-scale under sterile condition at a low cost. Therefore, researchers hope that LTB can be produced in future using transgenic silkworm bioreactor. In recent years, transgenic silkworm technology using piggyBac transposon-derived vector was developed and it was demonstrated that the target genes were stably maintained in the transgenic descendant (Tamura *et al*., 2000). The piggyBac is a class II transposable element and

Corresponding Author: Jin-Ru Cao, Zhejiang Academy of Agricultural Sciences, Sericulture Research Institute, No. 198 Shiqiao Road, 310021 Hangzhou, Zhejiang Province, P.R. China

a DNA type transposon originated from Lepidoptera which was initially identified in Baculovirus-infected Trichoplusia ni cell line TN-368 (Fraser *et al*., 1985). It is an autonomous element and can be accurately cut out from the host chromosomes and transpose. The full length of piggyBac is 2.5 kb, containing an approximately 2.1 kb Open Reading Frame (ORF) that encodes a 68 kDa transposase which is indispensable for the high-frequency cut and transposition of the element. Moreover, the piggyBac also contains a 13 bp of Inverted Terminal Repeat (ITR). Since, its insertion into the chromosomes is always at the sequence of TTAA, piggyBac has thus been classified into TTAA-specific transposable element family (Handler, 2002). Construction of transposon-based gene transfer vector was usually done by insertion of foreign gene into the transposase sequence which abolished the transposase function and the ability of transposon to cut out from the host chromosomes. In addition, another plasmid which contains a broken arm and loses the ability to insert but expresses functional transposase was also constructed as the helper vector. When both vectors are introduced into host cells, the helper plasmid provides transposase to help the exogenous fragment-carrying gene transfer vector to transpose and integrate into the host genome; transgenic individuals with stably integrated exogenous genes were thus obtained (Tamura *et al*., 2000).

Insect cell lines have been widely used in gene function analysis, apoptosis study and recombinant protein production via baculoviral expression system (Wickham *et al*., 1992). Transgenic study using cultured cells has the advantage of easy operation and short study period. Although, several insect cell lines have been used in stable transformation, only a few reports regarding *B. mori* cells (Xue *et al*., 2009). The present study has first time constructed transgenic expression vector for LTB based on piggyBac transposon, transformed *B. mori* cell line and successfully expressed LTB in cultured *B. mori* cells, setting up the basis for future expression of LTB using transgenic silkworm bioreactor.

MATERIALS AND METHODS

The construction of plasmid pPIGA3GFP-LTB-Neo: A 631 bp long promoter region of immediate early gene (*ie-1*) of *B. mori* nucleopolyhedrovirus (BmNPV) was cloned from BmNPV genomic DNA template by PCR using primer pair IE-F and IE-R (Table 1). The obtained sequence was deposited in GenBank (ID: AY616665). A 375 bp coding region of LTB was synthesized by GenScript USA Inc. (Piscataway, NJ, USA) based on GenBank (ID: FJ156281).

The neomycin-resistance gene (*Neo*^{R}) and its downstream 325 bp SV40 3' untranslated sequence, totally 1120 bp in length were amplified by PCR with primers Neo-F and Neo-R (Table 1) using pcDNA3.1 (Invitrogen, USA) plasmid as template. These LTB and Neo^R expression cassettes were then ligated tandemly (by GenScrip USA Inc. using homologous recombination method) into the Bgl II site of piggyBac-derived transposon vector pPIGA3GFP which contains *B. mori* A3 promoter-driven *gfp* gene. This constructed transgenic vector was designated as pPIGA3GFP-LTB-Neo (Fig. 1).

Table 1: The Forward (F) and Reverse (R) primers and the estimated size of PCR products

Primer name	Primer sequence (5′-3′)	Target gene	Products size (kb)
IE-F	TTCGAATTCGATTTGCAGTTCGGGAC	*ie-1* promoter region	0.6
IE-R	GCGGAATCAGTCGTTTGGTTGTTCA		
GFP-F	CGCGTTACCATATATGGTGAC	*gfp* expression cassette	1.8
GFP-R	GCGATCCAGACATGATAAGAT		
Neo-F	ATGATTGAACAAGATGGATTGC	*Neo*^{R}	1.1
Neo-R	GCTAGAGGTCGACGGTATAC		
LTB-F	CGGCGTTACTATCCTCTC	*LTB* coding sequence	0.3
LTB-R	GGTCTCGGTCAGATATGTG		

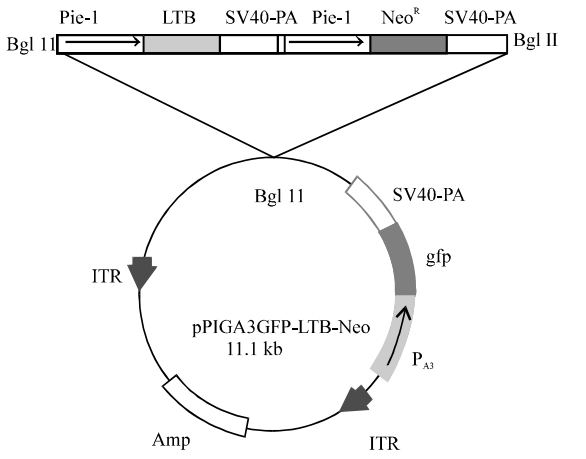

Fig. 1: Construction of the transgenic vector. Recombinant plasmid vector pPIGA3GFP-LTB-Neo contains the immediate early gene promoter-driven *LTB* coding sequence and neomycin resistance gene expression cassette and the *B. mori* A3 cytoplasmic actin gene promoter-controlled gfp expression cassette. All the expression cassettes are ended with repsective SV40 3' untranslated sequence. ITR, Inverted Terminal Repeats of piggyBac transposon; P_{A3}, *B. mori* A3 cytoplasmic actin gene promoter; *gfp*, green fluorescent protein gene; SV40-PA, SV40 3' untranslated sequence; P_{ie-1}, immediate early-stage gene promoter of *B. mori* nucleopolyhedrovirus; LTB, the B subunit of *Escherichia coli* heat-labile enterotoxin coding sequence and *Neo*^{R}, neomycin resistance gene

The cell culture and transfection: The cells were transfected using Liposome-mediated Method (Xue *et al*., 2009). Briefly, pPIGA3GFP-LTB-Neo and helper plasmid (transposase donor) were isolated using QIAGEN Plasmid Mini kit (Germany), mixed at a 1:1 molar ratio to a final concentration of 2.0 μg μL^{-1} of DNA which were then mixed with transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 1:3 mass ratio, incubated at room temperature for 30 min and transfected BmN cells. About 4 days after transfection, G418 was added into the culture at a final concentration of 800 μg mL^{-1} and maintained for 4 weeks. After that the culture was maintained in 400 μg mL^{-1} of G418.

Identification of stably transformed BmN cells: After transfection, the appearance of green fluorescence was monitored under inverted fluorescence microscope to identify the expression of exogenous *gfp* reporter gene which is the indicator of stable transformation after G418 screening. Genomic DNA was then isolated using Gentra Puregene Cell kit (QIAGEN, Germany) from the transformed cells and used as template to determine the existence of gfp expression cassette (A3-gfp-SV40) by PCR using specific primers: GFP-F and GFP-R (Table 1). Sequences of LTB and Neo^R were also detected using primer pairs LTB-F/LTB-R and Neo-F/Neo-R (Table 1), respectively.

Immunoblot detection of expressed LTB protein: According to the literature (Kang *et al*., 2004b; 2006a; Xue *et al*., 2009), transformed BmN cells were harvested, washed twice in PBS (0.1 mol L^{-1}, pH 7.4), resuspended with 5 volumes of PBS, sonicated and centrifuged at 13,000 g for 10 min. The supernatant was collected, mixed with loading buffer and direct loaded onto the gel, without boiling. The cell proteins were separated electrophoretically on 15% polacrylamide gradient gels and the gels were stained with Coomassie Brilliant Blue 250 or used for immunoblotting analysis. The proteins on the gel were transferred to a PVDF membrane which was then reacted with rabbit anti-cholera antiserum (Sigma, USA) and goat anti-rabbit secondary antibody (Bioss, USA) subsequently and visualized by addition of 3, 3-diamonobenzidine tetrahydrchloride (DAB) substrate.

RESULTS AND DISCUSSION

Construction of transgenic vector: As shown in Fig. 1, LTB and Neo^R expression cassettes were successfully recombined into pPIGA3GFP to form the new construct pPIGA3GFP-LTB-Neo which is suitable to transfer genes into BmN cells. The results of restriction enzyme digestion of the new vector was shown in Fig. 2. Bgl II digestion released a ~3.1 kb band indicating that LTB and Neo^R expression cassettes were successfully cloned into pPIGA3GFP. The full length sequencing of pPIGA3GFP-LTB-Neo (GenScript USA Inc.) further confirmed the successful construction of this transgenic vector.

Screening of the transformed cells: About 48 h after the transfection of BmN cells, very few cells are observed to express green fluorescence under the microscopy. About 4 days after transfection, the cells were selected against antibiotics G418 at a final concentration of 800 μg mL^{-1}. About 4 weeks after selection, the proportion of green fluorescent cells increased significantly but continuous selection did not further significantly increase this ratio. Then, the cells were maintained in culture medium with 400 μg mL^{-1} of G418 (Fig. 3). After selected against antibiotics G418, the number of total cells and fluorescent cells were calculated from randomly-selected six visual fields of fluorescent inverted microscope (Nikkon, TE2000U). Statistical result indicates that the positive rate was 70.79% (Table 2).

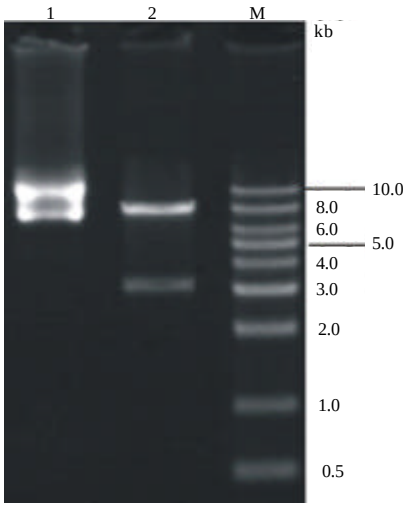

Fig. 2: Restriction enzyme digestion of pPIGA3GFP-LTB-Neo. The Lane 1: pPIGA3GFP-LTB-Neo plasmid; Lane 2: Bgl II digestion of pPIGA3GFP-LTB-Neo and M: DNA marker

Table 2: The survey of transformed *B. mori* BmN cells

Visual fields	Number of cells	Number of fluorescent cells	Positive rate (%)
1	218	138	63.30
2	227	152	66.96
3	188	145	77.13
4	194	132	68.04
5	233	171	73.39
6	241	183	75.93
Total	1301	921	70.79

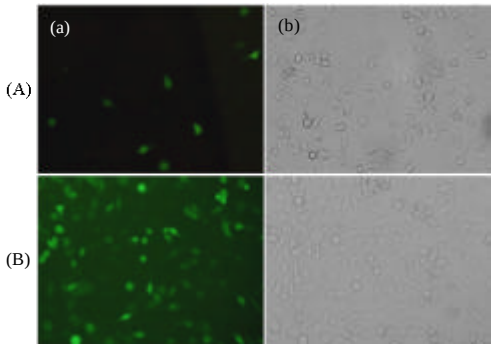

Fig. 3: The screening of stably transformed *B. mori* BmN cells. A) 48 h after transfection, a few cells with green fluorescence can be observed under microscope; B) 4 weeks after screening against 800 μg mL^{-1} G418, the number of cells emitting green fluorescence was constant at 70.79%; a) dark field; b) bright field

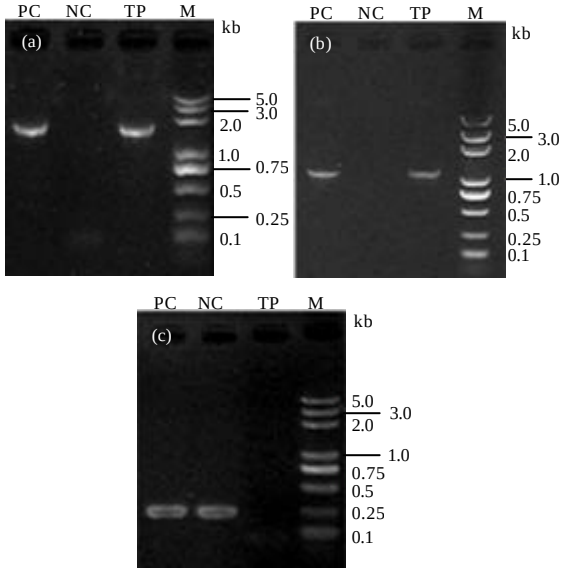

Fig. 4: PCR amplification of the transgenes from genomic DNA of the tranformed cells. a) PCR of *gfp* gene using GFP-F/GFP-R primers; b) PCR of Neo^R using Neo-F/Neo-R primers and c) PCR of LTB coding region using LT-F/LT-R primers. NC: Negative Control using genomic DNA from control cells as template; PC: Positive Control using plasmid pPIGA3GFP-LTB-Neo as template; TP using genomic DNA form G418-selected green fluorescent cells as template; M: DNA marker

Identification of the transgenic cells: Genomic DNA was extracted from the cells 4 weeks after G418 selection and used as template for PCR using gfp specific primers GFP-F and GFP-R. An approximately 1.8 kb specific band is clearly observed (Fig. 4a). PCR amplification of Neo^R sequence using Neo-F/Neo-R primers produced specific band at the predicated size of ~1.1 kb (Fig. 4b), PCR amplification of LTB coding region with primers LT-F/LT-R generated ~300 bp specific band which is consistent with the predicated size (Fig. 4c). All PCR products were finally confirmed by sequencing (Sangon Biotech (Shanghai) Co., Ltd.).

Determination of LTB expression in transformed BmN cells: The proteins isolated from the collected stably transformed cells were analyzed by SDS-PAGE and Western blot. The results were shown in Fig. 5. While SDS-PAGE failed to show significantly specific band, a ~60 kDa band was clearly observed in Western blot analysis which is consistent with the expected molecular size of LTB (five non-covalently associated LTB of 11.6 kDa forming a ring-like pentamer).

Stable expression of exogenous genes in transformed cells can be obtained through antibiotics selection. Generally, there are two ways to select transformed mammalian cells with G418 screening (Zhang *et al*., 2005): one is to use high concentration (800 μg mL^{-1}) of G418 to select for 5 days, i.e., to kill the non-transformed cells and to use a lower concentration (200 μg mL^{-1}) for maintenance; another one is to use minimum lethal dose to do one-step screening, e.g., used the minimum lethal dose (450 μg mL^{-1}) of G418 to screen for 2 weeks and achieved a complete killing of the negative control mouse fibroblast cells (NIH/3T3) (Li *et al*., 2000). The present study has adopted the former approach using 800 μg mL^{-1} of G418 to screen the transgenic BmN cells for 4 weeks. However, there are still nearly 30% of the cells failed to show green fluorescence (Fig. 3, Table 2), suggesting that in those BmN cells, the *gfp* gene did not express or the complete *gfp* gene expression cassette was not integrated into the genome. In other words, the possibility that exogenous DNA was randomly integrated into the genomes in BmN cells cannot be excluded. Jarvis *et al*. (1999) have transfected sf-9 cells with plasmid carrying Neo^R marker and IE-1 promoter-driven β-galactosidase expression cassette and obtained the stably transformed sf-9 cell line after selection against G418; the sequence of integrated plasmid was detectable after 50 passages (~6 months) and the expression of β-galactosidase remained at the same level after 100 passages (Jarvis *et al*., 1990). Recently, Invitrogen has developed a vector (pIZT/V5-His) which can stably express exogenous genes in insect cells; it uses the *IE*-2 promoter of *Orgyia pseudotsugata* NPV to drive the

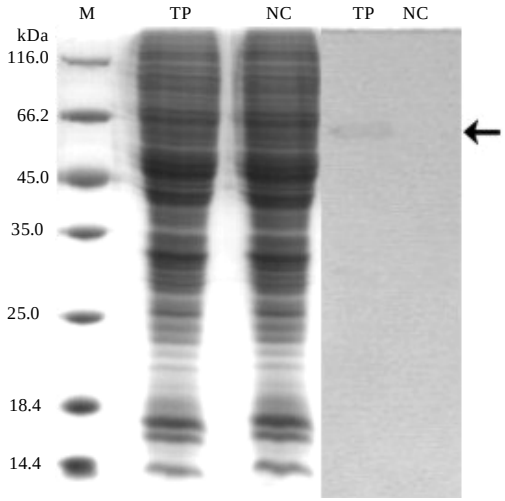

Fig. 5: Immunoblot analysis of control and stably transformed cells. Protein marker (Lane M); protein from the transformed cells (Lane TP) and protein from normal BmN cells (Lane NC). Western blot with corresponding lanes was shown side by side. The concentrations of the stacking gel and the separating gel in the SDS-PAGE were 5 and 15%, respectively. In the Western blot, the primary antibody was rabbit anti-cholera antiserum and the secondary antibody was HRP-conjugated goat anti-rabbit IgG

expression of exogenous gene and uses Zeocin resistance as marker to screen for stably transformed cell lines (Yu *et al*., 2002).

Furthermore, researchers also noticed that the intensity of stimulated green fluorescence varied significantly among transformed cells, suggesting the possible differences in copy numbers of the integrated gfp expression cassettes or the differences in integration sites in the genome.

Expression of exogenous genes using transformed cell lines has its intrinsic advantages. Since, the exogenous genes in Baculoviral Expression Vector System (BEVS) are commonly expressed in the late stage of viral infection, the abnormal cell state at the time often leads to insufficient processing of the expressed products which eventually affects their natural activity (Kitts and Rjostad, 1993). In addition, BEVS is a transient non-passageable expression system which needs repeated inoculation and is thus with heavy workload and low efficiency. Furthermore, after passages, the ability of virus to infect insect cells declines and the exogenous genes are lost easily resulting in the alleged defective viruses which also affects the expression efficiency (Kobayashi *et al*., 1992). During the whole process of *exogenous* gene expression using stably transformed cell line, there are no viral protein expression and viral genome contamination resulting in a relatively improved biosafety. In addition, the integration of exogenous gene into the cellular genome avoids the adverse effect of viral infection on cell functions which results in a more efficient processing of the expressed product. If the serum-free technology of insect cell culture is combined with the application of secreting expression technology, the purification of secreted product becomes much easier.

However, the expression level of exogenous gene by transformed insect cells is relatively low compared with that by BEVS (~1%) (Jarvis *et al*., 1990). This study has successfully expressed exogenous LTB in transformed BmN cells; however, SDS-PAGE analysis fails to show significantly specific bands which can only be detected by Western blot analysis indicating that the expression level of exogenous gene level is really low. Therefore, raising the expression level of exogenous gene in transformed insect cells is an immediate concern that needs to be addressed. *B. mori* is a insect with strong ability to secret silk.

The silk protein, composed of fibroin and sericin is mainly synthesized in the middle and late stages of fifth instar larvae. In just 4-5 days, each cell in the posterior silk gland of *B. mori* synthesizes about 300 μg of fibroin (6×10^8 fibroin molecules per second in average) which is more than 60 times faster than the synthesis of serum albumin by liver cells (Lu, 1990). Such a high intensity of protein synthesis is rarely observed in other animal cells. Fibroin promoter is one of the strongest in nature and exhibits high spatial and temporal specificity (Zhu *et al*., 2002).

CONCLUSION

The present study has confirmed that LTB can be expressed by piggyBac transposon-mediated gene transformation in silkworm cell line. Therefore, further manipulation of transgenic vector using fibroin promoter to drive LTB expression, to generate individual transgenic *B. mori* and produce LTB using such transgenic silkworm bioreactor will significantly improve the efficiency of LTB production.

ACKNOWLEDGEMENTS

This research was supported by grants from Zhejiang Province Natural Science Foundation of China (No. Y3080005), Zhejiang Province Public Welfare Technologies Research Projects (No. 2010C32028) and

Zhejiang Provincial Major Sci-Tech Projects (No. 2011C312008). The researchers are also grateful to the helps from Zhejiang Provincial Sci-Tech Innovation Team of Sericulture and Zhejiang Provincial 12·5 Mulberry and Silkworm Breeding Research Collaboration Team. Moreover, we are also grateful to Dr. Xiaolong Hu, Dr. Pengjie Zhang and Mr. Bo Liu.

REFERENCES

Arntzen, C.J., 1997. High-tech herbal medicine: Plant-based vaccines. Nat. Biotechnol., 15: 221-222.

Chikwamba, R.K., J. Cunnick, D. Hathaway, J. Mc Murray, H. Mason and K. Wang, 2002. A functional antigen in a practical crop: LT-B producing maize protects mice against *Escherichia coli* heat Labile Enterotoxin (LT) and Cholera Toxin (CT). Transgenic Res., 11: 479-493.

Fraser, M.J., J.S. Brusca, G.E. Smith and M.D. Summers, 1985. Transposon-mediated mutagenesis of a baculovirus. Virology, 145: 356-361.

Gyles, C.L., 1992. *Escherichia coli* cytotoxins and enterotoxins. Can. J. Microbiol., 38: 734-746.

Handler, A.M., 2002. Use of the piggyBac transposon for germ-line transformation of insects. Insect. Biochem. Mol. Biol., 32: 1211-1220.

Jarvis D.L., C. Oker-Blom and M.D. Summers, 1990. Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. J. Cell. Biochem., 42: 181-191.

Kang, T.J., S.C. Han, M.O. Jang, K.H. Kang, Y.S. Jang and M.S. Yang, 2004b. Enhanced expression of B-subunit of *Escherichia coli* heat-labile enterotoxin in tobacco by optimization of coding sequence. Applied Biochem. Biotechnol., 117: 175-188.

Kang, T.J., S.C. Han, M.S. Yang and Y.S. Jang, 2006a. Expression of synthetic neutralizing epitope of porcine epidemic diarrhea virus fused with synthetic B subunit of *Escherichia coli* heat-labile enterotoxin in tobacco plants. Protein Expres. Purif., 46: 16-22.

Kang, T.J., S.C. Han, M.Y. Kim, Y.S. Kim and M.S. Yang, 2004a. Expression of non-toxic mutant of *Escherichia coli* heat-labile enterotoxin in tobacco chloroplasts. Protein Expres. Purif., 38: 123-128.

Kang, T.J., W.S. Lee, E.G. Choi, J.W. Kim, B.G. Kim and M.S. Yang, 2006b. Mass production of somatic embryos expressing *Escherichia coli* heat-labile enterotoxin B subunit in Siberian ginseng. J. Biotechnol., 121: 124-133.

Kim, T.G., M.Y. Kim, B.G. Kim, T.J. Kang and Y.S. Kim *et al*., 2007. Synthesis and assembly of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic lettuce (*Lactuca sativa*). Protein Expres. Purif., 51: 22-27.

Kitts, P.A. and L.B. Rjostad, 1993. Method for producing recombinant baculovirus expression vectors at high frequency. Biotechniques, 14: 810-817.

Kobayashi, J., S. Imanishi, H. Inoue, K. Ohsuye, K. Yamaichi, N. Tsuruoka and S. Tanaka, 1992. High level expression of a frog alpha-amidating enzyme, AE-II, in cultured cells and silkworm larvae using a *Bombyx mori* nuclear polyhedrosis virus expression vector. Cytotechnology, 8: 103-108.

Li, Y., B.L. Han, H.X. Wang, J.Y. Long and K. Li, 2000. Expression and identification of human TGF-&beat;-1 gene in NIH/3T3 cells. Acta. Acad. Med. Mil. Tert., 22: 68-71.

Lim, J.G., J.A. Kim, H.J. Chung, T.G. Kim and K.R. Lee *et al*., 2009. Expression of functional pentameric heat-labile enterotoxin B subunit of *Escherichia coli* in *Saccharomyces cerevisiae*. J. Microbiol. Biotechnol., 19: 502-510.

Loc, N.H., N.H. Bach, T.G. Kim and M.S. Yang, 2010. Tissue culture and expression of *Escherichia coli* heat-labile enterotoxin B subunit in transgenic *Peperomia pellucida*. Protein Expression Purif., 72: 82-86.

Lu, H.S., 1990. The Sericultural Science in China. Shanghai Scientific and Technical Publishers, Shanghai, China.

Nataro, J.P. and J.B. Kaper, 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142-201.

Ravin, N.V., V.V. Kuprianov, L.A. Zamchuk, A.V. Kochetov, Y.L. Dorokhov, J.G. Atabekov and K.G. Skryabin, 2008. Highly efficient expression of *Escherichia coli* heatlabile enterotoxin B subunit in plants using potato virus X-based vector. Biochemistry, 73: 1108-1113.

Rezaee, M.A., A. Rezaee, S.M. Moazzeni, A.H. Salmanian and Y. Yasuda *et al*., 2005. Expression of *Escherichia coli* heat-labile enterotoxin B subunit (LTB) in *Saccharomyces cerevisiae*. J. Microbiol., 43: 354-360.

Rosales-Mendoza, S., R.E. Soria-Guerra, M.T. de Jesus Olivers-Flores, R. Lopez-Revilla and G.R. Arguello-Astorga *et al*., 2007. Expression of *Escherichia coli* heat-labile enterotoxin b subunit (LTB) in carrot (*Daucus carota* L.). Plant Cell Rep., 26: 969-976.

Spander, B.D., 1999. Structure and fuction of chooleratoxin and the related *Escherichia coli* heat-lableentero toxin. Microbiology, 56: 622-643.

Tamura, T., C. Thibert, C. Royer, T. Kanda and E. Abraham *et al*., 2000. Germline transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. Nat. Biotechnol., 18: 81-84.

Wagner, B., K. Hufnagl, C. Radauer, S. Wagner and K. Baier *et al*., 2004. Expression of the B subunit of the heat-labile enterotoxin of *Escherichia coli* in tobacco mosaic virus-infected *Nicitiana benthamiana* plants and its characterization as mocusal immunogen and adjuant. J. Immunol. Methods, 287: 203-215.

Wickham, T., T. Davis, R. Granados, M. Shuler and H. Wood, 1992. Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression systems. Biotechnol. Bioeng., 8: 391-396.

Williams, N.A., T.R. Hirst and T.O. Nashar, 1999. Immune modulation by the cholera-like enterotoxins: From adjuvant to therapeutic. Trends Immunol., 20: 95-101.

Xue, R., X. Li, Y. Zhao, X. Pan, X. Zhu, G. Cao and C. Gong, 2009. Elementary research into the transformation BmN cells mediated by the piggyBac transposon vector. J. Biotechnol., 144: 272-278.

Yu, A., B.M. Kneller, A.E. Rettie and R.L. Haining, 2002. Expression, purification, biochemical characterization and comparative function of human cytochrome P450 2D6.1, 2D6.2, 2D6.10 and 2D6.17 allelic isoforms. J. Pharmacol. Exp. Ther., 303: 1291-1300.

Zhang, M.Y., H. Li, W.W. Dong, W. Yang and Y. Wang *et al*., 2005. Development of a G418-resistant feeder layer of embryonic stem cell of mice. J. Chin. Med. Univ., 34: 1-3.

Zhang, X., Z. Yuan, Q. Duan, H. Zhu, H. Yu and Q. Wang, 2009. Mucosal immunity in mice induced by orally administered transgenic rice. Vaccine, 27: 1596-1600.

Zhu, C.G., Y.F. Jin, F. Shi, R.Z. Jin, Y.C. Wu and Y.Z. Zhang, 2002. Construction of transgenic silkworm gene targeting vectors based on fibroin light chain. J. Agr. Biotechnol., 10: 171-175.