IJIE Int. J. Indust. Entomol. 34(1) 11-15 (2017)
ISSN 1598-3579, http://dx.doi.org/10.7852/ijie.2017.34.1.11

Expression of the cyan fluorescent protein in fibroin H-chain of transgenic silkworm

Tae-Won Goo[1], Kwang-Ho Choi[2], Seong-Ryul Kim[2], Seung Won Park[3], and Seong-Wan Kim[2]*

[1]*Department of Biotechnology, Catholic University of Daegu, Daegu 38430, Republic of Korea*
[2]*Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Jeonju 55365, Republic of Korea*
[3]*Department of Biochemistry, School of Medicine, Dongguk University, Gyeongju, 38066, Republic of Korea*

Abstract

We constructed the fibroin H-chain expression system to produce enhanced cyan fluorescent proteins (ECFP) in transgenic silkworm cocoon. Fluorescent cocoon could be made by fusing ECFP cDNA to the heavy chain gene and injecting it into a silkworm. The ECFP fusion protein, each with N- and C-terminal sequences of the fibroin H-chain, was designed to be secreted into the lumen of the posterior silk glands. The expression of the ECFP/H-chain fusion gene was regulated by the fibroin H-chain promoter. The use of the 3xP3-driven EGFP cDNA as a marker allowed us to rapidly distinguish transgenic silkworms. The EGFP fluorescence became visible in the ocelli and in the central and peripheral nervous system on the seventh day of embryonic development. A mixture of the donor and helper vector was micro-injected into 1,020 Kumokjam, bivoltin silkworm eggs. We obtained 6 broods. The cocoon was displayed strong blue fluorescence, proving that the fusion protein was present in the cocoon. Accordingly, we suggest that the ECFP fluorescence silk will enable the production of novel biomaterial based on the transgenic silk.

Int. J. Indust. Entomol. 34(1), 11-15 (2017)

Received : 30 Mar 2017
Revised : 30 Mar 2017
Accepted : 31 Mar 2017

Keywords:
Fluorescent,
Transgenic silkworm,
ECFP,
Bombyx mori,
Silk

Introduction

Almost 5,000 years ago, Chinese legend has it that a silkworm cocoon from a mulberry tree fell into a cup of tea of the princess Xi Ling Shi and its silk thread started unraveling. Silkworm, Bombyx mori, produces a vast quantity of silk proteins and spins them into threads to build cocoons. Thus, the silkworm has a prominent capability to efficiently synthesize silk proteins. The principal constituent of silk proteins is fibroin, making up 70% of cocoon proteins in weight. Fibroin is composed of a 350 kDa fibroin heavy chain (FibH), a 25 kDa fibroin light chain (FibL), and fibrohexamerin (Fhx) at a molar ratio of 6:6:1 (Inoue *et al*., 2000). These proteins are accumulated in a large quantity for massive synthesis of fibroin in the posterior silk glands (PSGs) during the fifth instar before spinning, indicating that expressions of their genes (fibH, fibL, and fhx) are under the strict developmental and organ-specific control (Tanaka *et al*., 1999). Specialized cells at the posterior end of the silk glands devote 85% of their protein synthesis activity to silk production (Zhou *et al*., 2000; Zhou *et al*., 2003). The genome of these cells is amplified several thousand fold, resulting in a single nucleus that contains the equivalent of 400,000 haploid genomes. The

***Corresponding author.**
Seong-Wan Kim
Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Jeonju 55365, Republic of Korea.
Tel: +82-63-238-2864 / FAX: +82-63-238-3832
E-mail: tarupa@korea.kr

specific protein synthesis activity in the posterior gland cells is remarkable: a total of approximately 1,000 cells produce up to 300 mg of protein per gland in 4 days. This corresponds to a specific productivity of about 80 ug protein/cell/day. By comparison, the best recombinant high-yield mammalian cell culture systems only produce about 50 pg protein/cell/day (Altman *et al*., 2003; Yamada *et al*., 2001).

Since the piggyBac transposon-based expression system was developed in silkworm, silkworm transgenesis is a powerful tool for producing recombinant proteins (Tamura *et al*. 2000; Tatemastu *et al*., 2012). Especially, the silk gland of silkworm is a useful tool to produce some valuable recombinant proteins, which can be widely used in the biological, biotechnical and pharmaceutical application fields (Kurihara *et al*., 2007; Thomas *et al*., 2002). Recently, Silk-based materials with improved antibacterial properties, mechanical properties, biodegradation and biocompatibility, have been used for applications in bioengineering of bone, skin, cartilage, ligaments, tendons, vascular, neural, ocular, cardiac, and bladder tissues etc (Tatemastu *et al*., 2012; Zhao *et al*., 2009). Kim *et al*. (2015) reported on the expression of BmcecB1 antimicrobial peptide in silkworm.

Also, using this transgenic silkworm system, the stable production of the recombinant protein into the cocoon was investigated. The cocoon is made mainly by silk fibroin, which is synthesized by the posterior silk gland (Hino *et al*., 2006; Ogawa *et al*., 2007). Silk fibroins consist of two subunits, fibroin H- and L-chains, which are linked by a disulfide-bond. Tomita *et al*. (2003) reported on the expression of human type-III-collagen into the cocoon as a fibroin L-chain fusion protein using the piggyBac transposon vector. To secrete the collagen into the cocoon, the gene encoding collagen was connected with the cDNA C-terminal of fibroin L-chain with the L-chain promoter (Inoue *et al*., 2005). In this study, we demonstrated the fibroin H-chain expression system using the piggyBac transposon-derived vector to produce the enhanced cyan fluorescent protein (ECFP) in the cocoon.

Materials and Methods

Silkworm strains

The *B. mori* bivoltine strain, Kumokjam (Jam140 × Jam125) was obtained from the National Academy of Agricultural Science (Jeonju, Korea). The silkworms were reared at 25°C and fed with mulberry leaves and an artificial diet. DNA-injected eggs were maintained at 25°C in moist petri dishes. The hatched larvae were fed on an artificial diet and reared in groups under standard conditions.

Plasmid DNA Construction

The transition vector pG-3xP3-EGFP-pFibH was constructed as follows. The EGFP cDNA was used as a marker and was amplified by PCR using specific primers with the NheI/AflII sites from pEGFP (Clontech, USA), (NheI-DsRed-F: 5'-GCTAGCATGGCCTCCTCCGAGAAC-3' and DsRed-AflII-R: 5'-CTTAAGCTACAGGAACAGGTGGTGGCG-3').. From the genomic DNA of *B. mori*, the DNA fragment (GenBank Accession No.AF226688, nt. 61312–63870) including promoter domain (1124 bp), N-terminal domain 1 (NTD-1, 142 bp), first intron (871 bp), and N-terminal domain 2 (417 bp, NTD-2) was amplified by PCR using specific primers with the AscI/NotI/BamHI sites, (pFibHN-F: 5′-GGCGCGCCGTGCGTGATCAGGAAAAAT-3′ and pFibHN-R: 5′-GGATCCAGCGGCCGCTGCACCGACTGCAGCACTAGTGCTGAA-3′). The resultant DNA fragment was cloned into the pGEM-T Easy Vector System (Promega, USA) and named pGEMT-pFibH-NTR. The DNA fragment (GenBank Accession No. AF226688, nt. 79021–79500) including the C-terminal domain (179 bp, CTD) and poly(A) signal domain (301 bp) of the H-chain was amplified by PCR using specific primers with the SalI/SbfI/FseI sites, (pFibHC-F: 5′-CCTGCAGGAAGTCGACAGCGTCAGTTACGGAGCTGGCAGGGGA-3′ and pFibHC-R: 5′-GGCCGGCCTATAGTATTCTTAGTTGAGAAGGCATA-3′). The resultant DNA fragment was cloned into the pGEM-T Easy Vector System (Promega, USA) and named pGEMT-pFibH-CTR. The pFibH-NTR fragment was excised from pGEMT-pFibH-NTR with ApaI/SalI and subcloned into a pBluescriptII SK(-) vector (Stratagene, CA) that had been disgested with ApaI/SalI to generated pFibH-NTR-null. Next, CTR fragment was excised from pGEMT-CTR with SalI/SacI and subcloned into a pFibH-NTR-null that had been disgested with SalI/SacI to generated pFibHNC-null. The fluorescent gene (ECFP) was synthesized from the BIONEER (Korea) and N- and C-termial had the NotI and SbfI restriction sites. The fluorescent gene has been disgested with NotI/SbfI and

subcloned into a pFibHNC-null that had been disgested with NotI/SbfI to generated pFibHNC-ECFP. The vectors was disgested with AscI/FseI and subcloned into a pG-3xP3- EGFP. The resulting vectors were generated pG-3xP3-EGFP-pFibH-ECFP and the helper vector pHA3PIG (Tamura *et al*. 2000) were provided by Dr M. Jindra (Academy of Sciences of the Czech Republic, Prague,Czech Republic). Each of the vectors was purified with an EndoFree Plasmid Maxi Kit (QIAGEN Gmbh, Hilden, Germany) and used to generate transgenic silkworms.

Transgenesis and screening of silkworms

For egg preparation, male and female moths were allowed to mate for at least 4 h at 25°C. The mating moths were stored overnight at 4°C. The female moths were placed on a plastic sheet and left in dark boxes for 1 h. The laid eggs were immersed in HCl (specific gravity 1.0955, 25°C) for 30 min at 25°C, rinsed with distilled water, and finally dried. The transition vector pG-3xP3-EGFP-pFibH-ECFP and the helper vector pHA3PIG were dissolved in 5 mM KCl and 0.5 mM phosphate buffer (pH 7.0) at a concentration of 0.2 μg/μL and mixed at a ratio of 1:1. Approximately 5–10 nL of this mixture were injected using an IM300 microinjector (Narishige Scientific Instrument Lab., Japan) into pre-blastoderm embryos at 2–8 h after oviposition (Tamura *et al*. 2000). Injected embryos were allowed to develop at 25°C in moist chambers. G1 embryo and larvae were screened under a fluorescence stereomicroscope equipped with a GFP filter (Leica, Switzerland).

Inverse PCR

Genomic DNA was purified using a QIAamp DNA Mini Kit (QIAGEN Gmbh, Germany) from G1 moths. Purified genomic DNA was digested with *Sau*3AI (NEB, UK) and circularized by overnight ligation at 16°C with T4 DNA ligase (Promega, USA). PCR was performed on the ligated DNA using primer sequences between the restriction site and the end sequence of the transition vector. For the 5 ’ junction, the primer pair was 5 ’-ATCAGTGACACTTACCGCATTGACA-3 ’ and 5 ’-TGACGAGCTTGTTGGTGAGGATTCT-3 ’. For the 3 ’ junction, the primer pair was 5 ’-TACGCATGATTATCTTTAACGTA-3 ’ and 5 ’-GGGGTCCGTCAAAACAAAACATC-3 ’ (Tamura *et al*. 2000). PCR products were cloned into the pGEM-T-easy vector (Promega, USA) and sequenced. Sequencing data were

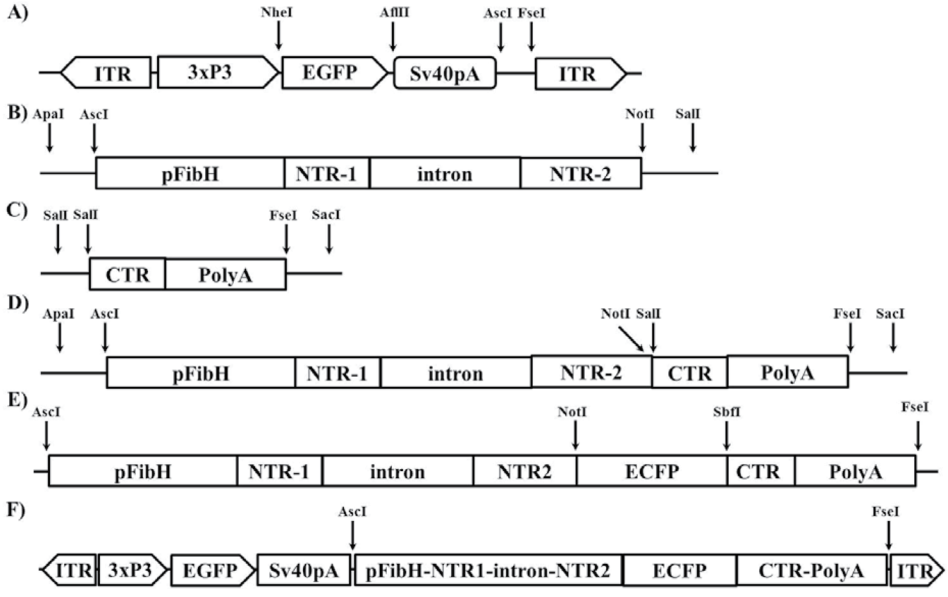

Fig. 1. The structures of transformation vectors for generating transgenic silkworms. The vector was constructed for germline transgenesis. A) pBac-3xP3-EGFP. B) pGEMT-pFibH-NTR. C) pGEMT-CTR. D) pFibHNC-null. E) pFibHNC-EYFP. F) The transformation vectors pBac-3xP3-EGFP-pFibH-EYFP. Transformation vector was piggyBac-derived vector. The restriction enzyme sites for the construction of recombinant vectors were indicated. The nucleotide sequences of pFibH-NTR and CTR were derived from Genebank Accession No. AF226688. pFibH, promoter domain (1124 bp); NTR1, N-terminal region 1 (142 bp); intron, first intron (871 bp); NTR2, N-terminal region 2 (417 bp); CTR, C-terminal region (179 bp); PolyA, poly(A) signal region (301 bp); ECFP, enhanced Cyan fluorescence protein gene; ITR, inverted repeat sequences of piggyBac arms; 3xP3, 3xP3 promoter; SV40, SV40 polyadenylation signal sequence.

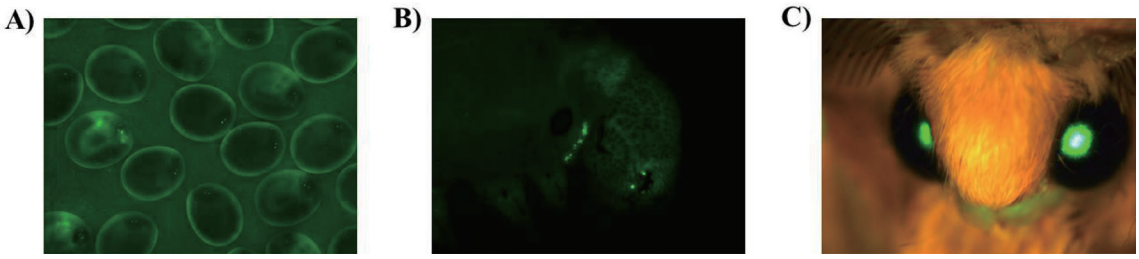

Fig. 2. Fluorescence expression of EGFP in transgenic silkworms. A) Eggs were expressed in the eyes and the abdominal nervous system of seven days old F1 embryo. Arrows point to eyes and nervous system in panel. B) Larva was expressed in the eyes of a F1 1th instar larva. C) fluorescent images of moth.

analyzed using the DNASTAR LaserGene program (DNASTAR Inc., USA).

Results and Discussion

We constructed an pG-3xP3-EGFP-pFibH-ECFP vector (Fig 1.). This vector was mixed with the helper plasmid pHA3PIG and micro-injected into 1,020 eggs of bivoltin silkworms, Kumokjam. The 60 larvae (G0) were hatched and allowed to develop to moths. Moths were mated within the same family or backcrossed with the wild-type moths. The resulting G1 generation was obtained 20 broods and selected 6 broods containing at least one EGFP-positive embryo (Table 1.). The rate of successful transgenesis for G1 broods was 30%. The EGFP-positive G1 moths were 32 and backcrossed with their wild-type moths.

The G1 progeny eggs were screened under an EGFP fluorescent stereomicroscope. The EGFP fluorescence became visible in parts of the embryo segments on the seventh day of G1 embryonic development (Fig. 2a). Strong fluorescence was also observed in the wings of a seven-day old G1 pupae and G2 moths (Fig. 2b, Fig. 2c). The expression of EGFP was observed in all developmental stage. Chromosomal integration of the *piggBac* transposon was confirmed with inverse PCR (Table 2.).

In conclusion, we successfully produced high quality silks having ECFP fluorescent colors using transgenic silkworms (Fig. 3). Furthermore, we bred strains for mass-rearing of silkworms and harvesting large amounts of the cocoons with the ECFP fluorescence color. Accordingly, we suggest that this fibroin H-chain expression system will be useful for foreign gene production and for creation of novel functional silk material.

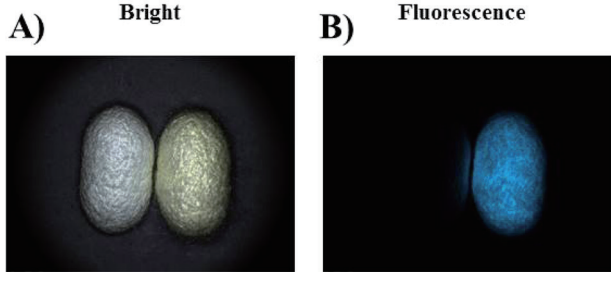

Fig. 3. Cyan fluorescent cocoons. The fluorescent cocoons produced by transgenic silkworms that have used ECFP fluorescent proteins. A) Normal cocoons. B) Cyan fluorescent cocoons. A) Cocoons were observed under the fluorescent light. C) Cocoons were observed under the GFP-excitation-wavelength light.

Acknowledgements

This study was carried out with the support of "Research Program for Agricultural Science & Technology Development (Project No. PJ0100612016)", National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea.

References

Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen JS, Lu H, Richmond J, Kaplan DL (2003) Silk-based biomaterials. Biomaterials 24, 401-416.

Hino R, Tomita M, Yoshizato K (2006) The generation of germline transgenic silkworms for the production of biologically active recombinant fusion proteins of fibroin and human basic fibroblast growth factor. Biomaterials 27, 5715-5724.

Inoue S, Kanda T, Imamura M, Quan GX, Kojima K, Tanaka H, Tomita M, Hino R, Yoshizato K, Mizuno S, Tamura T (2005) A fibroin secretion-deficient silkworm mutant, Nd-sD, provides an efficient

system for producing recombinant proteins. Insect biochemistry and molecular biology 35, 51-59.

Inoue S, Tanaka K, Arisaka F, Kimura S, Ohtomo K, Mizuno S (2000) Silk fibroin of Bombyx mori is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. J Biol Chem 275, 40517-40528.

Kim SW, Kim SR, Park SW, Choi Kh, Goo TW (2015) Production of the BmCecB1 antimicrobial peptide in transgenic silkworm. Int J Indust Entomol 31, 85-89

Kurihara H, Sezutsu H, Tamura T, Yamada K (2007) Production of an active feline interferon in the cocoon of transgenic silkworms using the fibroin H-chain expression system. Biochemical and biophysical research communications 355, 976-980.

Ogawa S, Tomita M, Shimizu K, Yoshizato K (2007) Generation of a transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: production of recombinant human serum albumin. Journal of Biotechnology 128, 531-544.

Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000) Germline transformation of the silkworm Bombyx mori L. using a piggyBac transposon-derived vector. Nature biotechnology 18, 81-84.

Tanaka K, Kajiyama N, Ishikura K, Waga S, Kikuchi A, Ohtomo K, Takagi T, Mizuno S (1999) Determination of the site of disulfide linkage between heavy and light chains of silk fibroin produced by Bombix mori. Bba-Protein Struct M 1432, 92-103.

Tatemastu K, Sezutsu H, Tamura T (2012) Utilization of transgenic silkworms for recombinant protein production. Journal of Biotechnology & Biomaterials S9, 1-8.

Thomas JL, Da Rocha M, Besse A, Mauchamp B, Chavancy G (2002) 3xP3-EGFP marker facilitates screening for transgenic silkworm Bombyx mori L. from the embryonic stage onwards. Insect biochemistry and molecular biology 32, 247-253.

Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura T, Yoshizato K (2003) Transgenic silkworms produce recombinant human type III procollagen in cocoons. Nature biotechnology 21, 52-56.

Yamada H, Nakao H, Takasu Y, Tsubouchi K (2001) Preparation of undegraded native molecular fibroin solution from silkworm cocoons. Mat Sci Eng C-Bio S 14, 41-46.

Zhao Y, Li X, Cao G, Xue R, Gong C (2009) Expression of hIGF-I in the silk glands of transgenic silkworms and in transformed silkworm cells. Sci China C Life Sci 52, 1131-1139.

Zhou CZ, Confalonieri F, Esnault C, Zivanovic Y, Jacquet M, Janin J, Perasso R, Li ZG, Duguet M (2003) The 62-kb upstream region of Bombyx mori fibroin heavy chain gene is clustered of repetitive elements and candidate matrix association regions. Gene 312, 189-195.

Zhou CZ, Confalonieri F, Medina N, Zivanovic Y, Esnault C, Yang T, Jacquet M, Janin J, Duguet M, Perasso R, Li ZG (2000) Fine organization of Bombyx mori fibroin heavy chain gene. Nucleic Acids Res 28, 2413-2419.