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Construction of a Transgenic Silkworm Carrying the Fibroin Gene of the Japanese Oak Silkworm, *Antheraea yamamai*

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We describe the generation of transgenic silkworm that carrying the chimeric fibroin light chain (L-chain) gene. Previously, we have cloned the complete fibroin L-chain gene from the silkworm Baekok-Jam, *Bombyx mori*, and the complete fibroin gene from the oak silkworm, *Antheraea yamamai*. The 444 bp repetitive sequence of *A. yamamai* fibroin gene was inserted into the exon 6 of *B. mori* fibroin L-chain gene to produce chimeric fibroin L-chain gene. The chimeric fibroin L-chain gene was cloned into the polyhedrin gene site of *Autographa californica* nuclear polyhedrosis virus (AcNPV) to yield a recombinant baculovirus as a fibroin gene targeting vector. One-day-old fifth instar female silkworm larvae were injected with the recombinant baculovirus and then mated with normal male moths. Genomic DNA from their progenies was extracted and screened for the desired targeting event by using PCR and Southern blot analysis. The analysis showed that the chimeric fibroin gene had intergrated into the L-chain gene on the genome by homologous recombination and was transmitted through generations. The transgenic silkworm carrying the chimeric fibroin gene were approximately 43.2% in F_2 generation, and the silkworms synthesized the fusion protein in cocoons layer.

Key words: fibroin L-chain gene, AcNPV, transgenic silkworm, chimeric fibroin

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Introduction

The silk fibroin produced by the domestic silkworm is one of the most abundant natural fibers and can be obtained easily and inexpensively (Tanaka *et al.*, 1993). In recent years, the interest in morphology and structure of the silk fibroin increased due to its attractiveness for bio-related application such as sutures in the surgical field (Mori and Tsukada, 2000). The silkworm itself has considerable importance as a producer of recombinant silk proteins by using the genetic transformation technologies such as baculovirus system (Maeda, 1989). For the stable gene transformation, the germ cells are the targets of all efforts at DNA introduction (Inoue *et al.*, 2000). A number of methods for delivering DNA to developing insect germ cells have been used, including microinjection, biolostics, and electroporation (Baldarelli and Lengyel, 1990; Leopoid *et al.*, 1996; Mialhe and Miller, 1994; Rubin and Spradling, 1982). Microinjection remains the best option for penetrating insect chorion and delivering DNA vector to the germ cells, and typically, techniques used for handling and microinjecting insect embryos are based on those that have developed for *D. melanogaster* (Rubin and Spradling, 1982).

Recently, *P*-transposable elements and gene targeting are common methods for gene transformation into insects (Inoue *et al.*, 2000). Unfortunately the *P*-transposable element, while highly successful in *Drosophila*, ultimately proved to be useless for those wishing to genetically transform non-drosophilid insect (Atkinson *et al.*, 2001). Furthermore, all of the insect transposable element gene vectors currently capable of transforming non-drosophilid insects have transformation rates of ~5%, and have little or no information concerning whether they can be easily remobilized after integration (Atkinson, 2002). Gene tar-

geting by homologous recombination has become an effective approach towards the transformation of several lower eukaryotes as well as plant and vertebrate systems (Bollag *et al.*, 1989). It has the major advantage of gene replacements or "knock-out". Homologous recombination has been reported for drosophila (Cherbas and Cherbas, 1997) and mosquito cell line studies (Eggleston and Zhao, 2000), but only recently has it been demonstrated *in vivo* where it was mediated by a baculovirus in the silkmoth, *B. mori* (Yamao *et al.*, 1999) and used in an experimental system in *Drosophila* (Rong and Golic, 2000). A recent attempt at the homologous recombination in drosophila was based on the promise that a linear extra chromosomal molecule could be recombinogenic with chromosomal DNA having homologous sequences (Rong and Golic, 2000). The baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is being used by many researchers as a model system for studies of viral gene expression, regulation *in vivo* and *in vitro* (Luckow and summers, 1989; Yamao *et al.*, 1999). Although AcNPV can replicate in the silkworm, the larvae survive and grow without any symptoms of nuclear polyhedrosis (Mori *et al.*, 1995).

Transgenesis in silkworms was not reproducible or of sufficient efficiency. However, two groups have been recently successful for the transgenesis of the silkworm. One is attempting to generate transgenic silkworms using the *piggyBac* transposon. The other is the gene targeting into the fibroin light chain gene by homologous recombination using AcNPV (Yamao *et al.*, 1999). Gene targeting in the silkworm is more useful for other purposes. Targeted disruption of an endogenous gene would permit analysis of gene function through the production of gene knockouts in the silkworm and modified fibroin H-chain, L-chain and sericin synthesized within the silk glands would permit the modification of the physicochemical character of the silk.

Previously, we have cloned and characterized the complete fibroin L-chain gene from the silkworm Baekok-Jam, *Bombyx mori*, and the complete fibroin gene from the *A. yamamai* (Choi *et al.*, 2002; Hwang *et al.*, 2001). We decided to use the fibroin L-chain gene as a homologous region, and the oak silkworm fibroin gene was introduced into the exon 6 of L-chain gene. In the present study, we produced transgenic silkworm with recombinant baculovirus vector carrying the chimeric fibroin L-chain gene fused with the Japanese oak silkworm fibroin gene.

Materials and Methods

Animal

Baekok-Jam, *Bombyx mori*, a recommended variety in Korea was used in this experiment. Recombinant AcNPV-injected fifth instar female larvae reared by fresh mulberry leaves at 25°C.

Long template PCR and vector construction

Genomic DNA of silkworm was extracted from posterior silk gland of fifth instar larvae using a Wizard™ Genomic DNA Purification kit, according to the manufacturer's instructions (Promega). The rear region of fibroin L-chain gene (SFL, nt 7342 ~ nt 14604) was PCR-amplified from the genomic DNA with the primer, SFL-F (5'-GTACCGG GATTGCGCGTGTCAGGATGACTCAGA-3') and SFL-R (5'-CCATGTACCCACTGTCAATCCACCGTCT TTGG G-3') synthesized according to the published sequence data for the L-chain gene (GenBank No. M76430; Kikuchi *et al.*, 1992). PCR reaction was carried out with a long template PCR system (Boehringer Mannheim) with 10 pmol of each primer and 500 ng of genomic DNA. After heating the reaction mixture (50 ul) at 94°C for 10 min, amplification was carried out for 30 cycles of denaturation (30 sec at 94°C) and annealing extension (10 min at 68°C). A final 12 min step at 72°C was performed at the complementation of these cycles. The amplified SFL gene was cloned into pGem-T-easy plasmid vector (Promega) to produce pGem-T-SFL. An outline of the baculovirus transfer vector construction is shown in Figure 1. pGem-T-SFL was digested with *Apa* I and filled with klenow fragment, and digested with *Pst* I. The SFL gene flagment was inserted into the *Sma* I site of pBacPAK9 (Clontech) to yield a plasmid vector pBacPAK-SFL (12.7 kb). Subsequently, to construct the recom- binant baculovirus transfer vector pBacPAK-SFL-AYF, pGem-T-AYF clone (Hwang *et al.*, 2001) was digested with *Sna*B I and *Hin*c II, and the 444 bp AYF gene inserted into the *Pvu* II site on exon 6 of the L-chain gene in pBacPAK-SFL.

Cell line and recombinant AcNPV

Spodoptera frugiperda (Sf9) cells were maintained at 27°C in TC-100 medium (Sigma) supplemeted with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, Gibco). Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summer and Smith, 1987). Thirty-five millimeter cell culture dishes were seeded with 10^6 Sf9 cells and incubated at 27°C for 1 hr to allow cell attachment. One microgram of pBacPAK6 viral DNA (Clontech), five microgram of pBacPAK-SFL-AYF in 20 mM HEPES buffer and sterile water to make a total volume of 50 μl were mixed in a polystyrene tube. Fifty microliters of 100 ug/m*l* Lipofectin (Gibco) were gently mixed with the DNA solution and the mixture was incubated at room temperature for 20 min. The cells were washed three

times with 2 m*l* serum free TC-100 medium and fed with 1.5 m*l* serum-free TC-100 medium. The Lipofectin-DNA complexes were added drop-wise to the medium covering the cells while the dish was gently swirled. After incubation at 27°C for 5 hrs, TC-100 medium containing 10% FBS (Gibco) was added to each dish, and incubation at 27°C was continued. At 5 days post-inoculation (p.i.), the supernatant was harvested, clarified by centrifugation at 2,000 rpm for 5 min. Titer of the recombinant baculovirus (vSFL-AYF) was expressed as plaque forming units (PFU) per m*l* (O'Reilly *et al.*, 1992).

Injection and screening methods

One-day-old fifth instar female larvae of the Baekok-Jam were individually injected with 50 μl inoculum (vSFL-AYF, 5×10^5 PFU) by a disposable syringe. In this experiment, 4,600 female larvae were inoculated. After the injection, the female larvae were reared to moths, and mated with normal male moths. The primers were designed to amplify a part of the SFL gene; primer 1 (5'-GTAGCAGTTACGGATCAGGCTCGTC-3') and primer 2 (5'-CGTCCGCCCATACCGCCAATGCCAG-3'), and primer 3 (5'-ACTAACCTGTCTACTCGAATAGCGGC-3') and primer 4 (5'-CAGATTGTACAACTCTCCCAACAGCC-3'). Genomic DNA was extracted from 100 embryos of each female moths, and used as the template for PCR screening. The larvae from the remaining PCR-positive siblings were reared, whereas the PCR-negative siblings were discarded. In larval screening, the genomic DNA was extracted separately from each fifth instar larvae, and used as the template for PCR screening. PCR screening was conducted for 100 ng of genomic DNA and 10 pM each primers in a final volume of 20 μl using a Pre-Mix Top^{TM} (Bioneer) with the DNA Thermal Cycler (TaKaRa). The PCR amplification condition consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, and a final extension step of 72°C for 6 min. The amplified products were analyzed by 1.0% agarose gel electrophoresis as described by Maniatis *et al.* (1989). Male and female moths (F_1) remaining in PCR-positive screening were mated with normal moths, and their progenies (F_2) were reared.

Southern hybridization was performed on the PCR products of F_2 larvae. Electrophoresed PCR products were transferred onto Hybond-N membrane (Amersham), and hybridized at 65°C for 16 hrs with the probe in a hybridization buffer containing $5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate), 50% formamide, 0.1% (W/V) *N*-lauroylsarcosine, 0.02% SDS and 2% blocking agent (Boehringer mannheim). To detect the chimeric fibroin gene, the AYF gene (444 bp) was excised from pGem-T-AYF clone with *Sna*B I and *Hin*c II, and then labeled with (α-^{32}P) dATP (Amersham) using the Prime-It II Random Primer Labeling Kit, according to the manufacturers instructions (Stratagene). After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and $0.2 \times$ SSC at 65°C, and finally exposed to X-ray film. For the detection of hybrids, the color reaction was done by NBT and X-phosphate solution. The reaction was quenched with stop buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

SDS-PAGE of cocoon protein

Cocoon protein was analyzed by SDS-PAGE (Laemmli, 1970). To dissolve the cocoon protein, 150 m*l* of 70% LiSCN was added to 5 mg of the cocoon layer, and 40 m*l* of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol was added to 10 m*l* of the dissolved silk protein as described by Inoue *et al.* (2000). The prepared cocoon solutions were then subjected to 12.5% polyacrylamide slab gel, and visualized by staining gel with CBB R-250 (Sigma).

Results and Discussion

Recombinant baculovirus vSFL-AYF and Inoculation

We constructed a recombinant baculovirus for the purpose of silkworm fibroin L-chain gene targeting. As shown in Figure 1, the 7.2 kb rear region of fibroin L-chain (SFL) gene was cloned, and then a Japanese oak silkworm, *A. yamamai*, fibroin gene (AYF) was introduced as a foreign gene transmitted into the *Pvu* II site on exon 6 of the SFL gene. Therefore, the AYF gene was fused between 6 kb upstream region and 1.2 kb downstream region on the SFL gene which serve for a homologous regions. The *A. yamamai* fibroin gene structure was very similar to *B. mori* fibroin gene structure, and consists of an initial exon and a long second exon (Hwang *et al.*, 2001; Yamaguchi *et al.*, 1989). The AYF gene named in this study indicates one polyalanine motif (444 bp) of the exon 2.

To construct recombinant baculovirus vSFL-AYF, pBac PAK-SFL-AYF and BacPAK6 viral DNA were co-transfected into Sf9 cell. The strategy was to create a recombinant baculovirus was described in Figure 1. The polyhedrin gene of AcNPV was replaced with the chimeric L-chain gene. Homologous recombination was expected to occur via the 6 kb upstream and 1.2 kb downstream. ORF of the chimeric fibroin L-chain gene was terminated by the stop codon of the 3'-flanking region of the SFL gene. The chimeric fibroin gene was supposed to be expressed as a fusion protein of SFL and AYF under the control of the silkworm fibroin L-chain promoter.

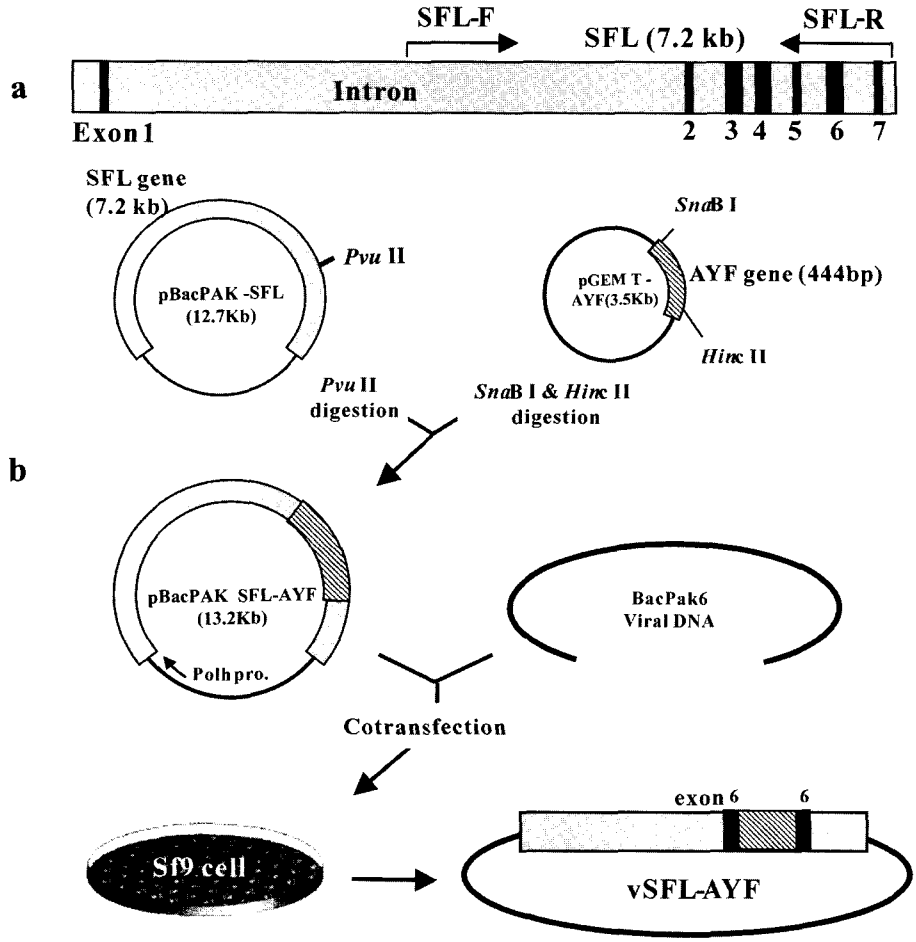

Fig. 1. Structures of rear region of fibroin L-chain (SFL) gene and recombinant baculovirus. (a) 7.2 kb long DNA of the SFL gene was composed of six exons and seven introns. (b) Strategy for construction of recombinant baculovirus vSFL-AYF. One polyalanine motif (AYF, 444 bp) of *A. yamamai* fibroin gene was cloned into the *Pvu* II site on exon 6 of the SFL gene.

One-day-old fifth instar female larvae (F_0) were injected with 50 μl of recombinant virus containing 5×10^5 PFU per individual by percutaneous inoculation using disposable syringe (Yamao *et al.*, 1999). In the experiment, 4,600 Baekok-Jam larvae were inoculated with recombinant baculovirus vSFL-AYF. An average durations of the larval and pupal stages in the control larvae were about 12 days, but the duration of the pupal stage of infected larvae was a few days longer than in the control larvae. This phenomenon suggested that the *ecdysteroid UDP-glucosyltransferase* (egt) gene of AcNPV was expressed, and the secreted EGT altered growth of the infected silkworms (Smith and Goodale, 1998). The egt gene extends the feeding period by inactivating the ecdysteroids using sugar conjugation reaction (Shikata, 1998).

In the present study, the silkworm Jam 123 (Japanese race) and Jam 124 (Chinese race) were used in the first experiment. These races showed very different susceptibility toward the recombinant baculovirus infection. In Jam 123, the larvae inoculated ($< 5 \times 10^5$ PFU per a larva) with recombinant baculovirus have stopped growth in the pre-pupa stage and died at the rate of high than 90%. Contrary, Jam 124 was not susceptible toward recombinant baculovirus. The phenomenon indicates that the two races were not good for this experiment using baculovirus system. Therefore, hybrid silkworm Baekok-Jam that has adequate baculovirus susceptibility (> 70% survival rate) was used in this study. Baekok-Jam is a variety generated by single cross F_1 hybrid between Jam 123 (Japanese race) and Jam 124 (Chinese race).

Screening of transgenic silkworm

Homologous recombination event was expected to occur within the loci of fibroin L-chain on the genome during nuclear fision (Rong and Golic, 2000). The vSFL-AYF inoculated female larvae were mated with normal male moths, and then allowed dispersed on egg-papers. Genomic DNA (F_0) was extracted from respectively 100

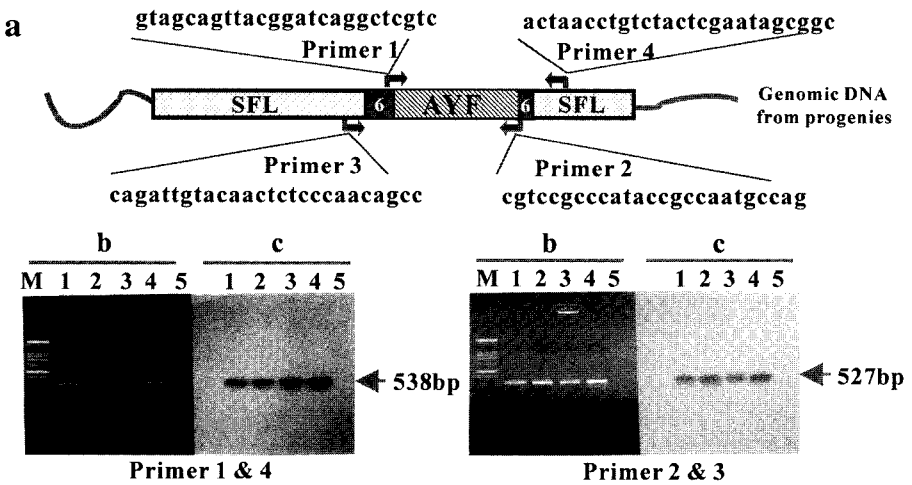

Fig. 2. PCR and southern blot analysis for the screening events. (a) Localization of the primers used for PCR-screening of chimeric fibroin gene. PCR (b) and southern blot (c) analysis of transgenic silkworms. Southern blot hybridization was performed with the AYF gene as a probe.

Table 1. Frequency of gene targeting events in the silkworm by using recombinant baculovirus vSFL-AYF

Variety (No. of inoculated F_0 larvae)[a]	No. of F_1 siblings containing AYF gene (% positive)[b]	No. of F_1 larvae containing AYF gene containing AYF gene (% positive)[c]	No. of F_2 larvae containing AYF gene (% positive)[c]
Baekok (4,600)	5/2,340 (1.5%)	6/265 (2.7%)	57/132 (43.2%)

[a]Female fifth instar larvae were inoculated with vSFL-AYF and mated with normal moths.
[b]Genomic DNAs were prepared from 100 embryos of each inoculated F_0 larvae.
[c]Genomic DNAs from larval hemolymphs were analyzed by PCR and Southern blot analysis.

embryos and used as the template for PCR screening with primer 1 and 4, and primer 2 and 3 to screen for presence of the chimeric fibroin gene (Fig. 2a). Approximately 1.5% (5 positive/2,340 tested) of the inoculated larvae gave rise to F_0 embryos that were positive by PCR screening (Table 1). The larvae from the remaining PCR-negative siblings were discarded. About 150 – 200 individuals larvae (F_1) were collected, and hemocytes were harvested from each individual separately, and their genomic DNAs were tested by PCR analysis. Approximately 2.7% of all the F_1 individuals tested (265 animals) were positive (6 animals) for the chimeric fibroin gene presence (Table 1).

F_1 male and female moths were mated with normal moths, and their progenies (F_2) were reared. About 300 offsprings were produced from each cross, and hemocytes were harvested separately from each fifth instar larvae. The genomic DNAs were assayed for the chimeric fibroin gene transmigration by PCR and southern blot analysis that specifically detects a chimeric fibroin gene. As shown in Figure 2b, the vSFL-AYF was correctly recombined with the intact fibroin L-chain genome. Southern hybridization was performed on the PCR products with the AYF gene as a probe. A single band was detected that scored positive for the 527 bp and 538 bp of the chimeric fibroin gene, respectively (Fig. 2c). To confirm further, nucleotide sequence of the chimeric fibroin gene as PCR products was determined by direct sequencing with primers 1, 2, 3 and 4, demonstrating that the 527 bp and 538 bp PCR products were identified as the chimeric fibroin gene consisted of SFL and AYF gene (Fig. 3). The result indicated that the chimeric fibroin gene had been introduced into the fibroin L-chain on the genome of transgenic silkworms by homologous recombination. The frequency of transgenic silkworm harboring the chimeric fibroin gene is summarized in Table 1. After inoculation of 4,600 animals of fifth instar larvae (F_0) with the vSFL-AYF, the gene targeting events were ultimately detected in fifty-seven individuals (F_2) that derived distinct fibroin L-chain gene targeting events that occurred at a frequency of approximately 43.2% (57 positive/132 tested).

SDS-PAGE analysis of cocoon protein

To verify the targeting event occurred by homologous recombination, SDS-PAGE analysis was performed with the cocoon produced from transgenic silkworms. The prepared cocoon layer samples were separated on 12.5% polyacrylamide gel (Fig. 4). The results revealed a novel protein with a molecular mass of 39 kDa and native form

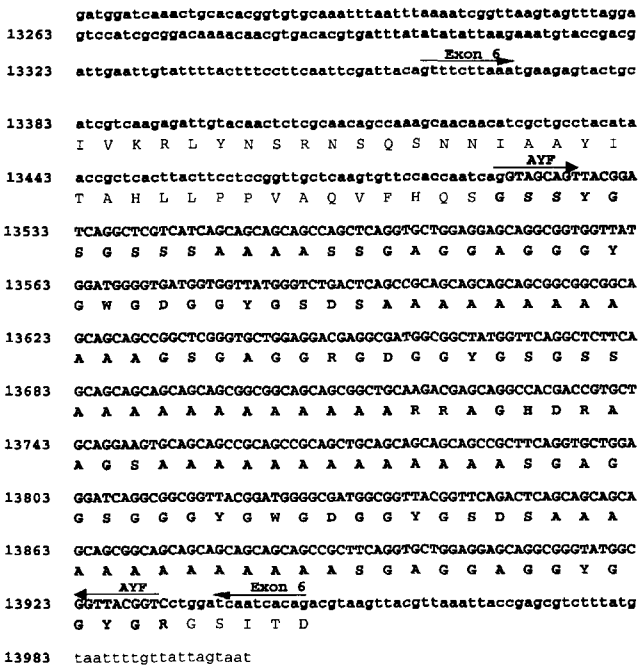

Fig. 3. Nucleotide and deduced amino acid sequences of the chimeric fibroin gene. The AYF gene was fused with exon 6 of the SFL gene. The deduced amino acid sequences are represented under nucleotides sequences.

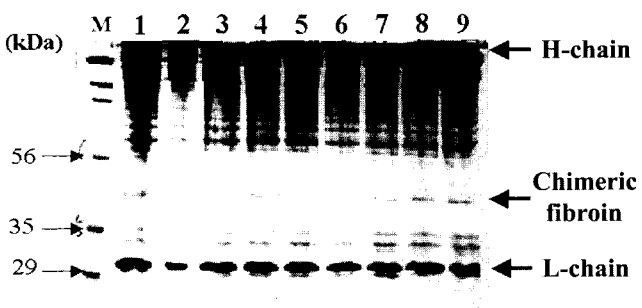

Fig. 4. SDS-PAGE analysis of chimeric cocoon proteins. The molecular mass of the novel protein correlated to that of chimeric protein (39 kDa) consisting of native fibroin L-chain (25 kDa) and AYF (14 kDa). Transgenic cocoon proteins (lane 1, 4, 7, 8 and 9); Normal cocoon proteins (lanes 2, 3, 5 and 9); Molecular weight standards are shown in lane M.

of fibroin L-chain (25 kDa) in the silk protein of transgenic silkworms as compared with the normal. The molecular mass of the novel silk protein correlated to with that of the chimeric protein consisting of a fibroin L-chain (25 kDa) and *A. yamamai* fibroin (AYF, 14 kDa), and also suggesting that the chimeric fibroin gene was present per haploid genome of each individual. However, some of transgenic silkworms did not produced any novel silk protein. The phenomenon suggested that the AYF gene inserted in fibroin L-chain gene on the genome does not has transcriptional stability yet, and the AYF gene was segregated in the F_2 generation. Therefore, it is necessary to study the high efficiency of transovarian transmisional stability of the targeted gene generations (Mori *et al.*, 1995).

Recombinant baculovirus-mediated transgenesis of silkworm allowed specific alterations in a target sequence. Targeted disruption of an endogenous gene would permit analysis of gene function through the production of gene knockouts in the silkworms (Mori *et al.*, 1995). In addition, homologous recombination of a foreign gene downstream from a powerful promoter, such as fibroin promoter, would allow large scale and constitutive production of a useful chimeric protein in the silkworm (Marshall, 1998). This experiment was presented on the possibility of production of chimeric silk proteins by developing gene-targeting system to modify silk fibroin L-chain gene and using baculovirus. The next problem must be overcome prior to producing new biomaterials effectively and its stability. However, a useful selective marker for screening of gene targeting events and utilization of a recombinase activity would lead to successful production of useful transgenic silkworm.

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