

Transgenic silkworms (*Bombyx mori*) produce recombinant spider dragline silk in cocoons

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Abstract Spider dragline silk is a unique fibrous protein with a combination of tensile strength and elasticity, but the isolation of large amounts of silk from spiders is not feasible. In this study, we generated germline-transgenic silkworms (*Bombyx mori*) that spun cocoons containing recombinant spider silk. A *piggyBac*-based transformation vector was constructed that carried spider dragline silk (MaSp1) cDNA driven by the sericin 1 promoter. Silkworm eggs were injected with the vector, producing transgenic silkworms displaying DsRed fluorescence in their eyes. Genotyping analysis confirmed the integration of the MaSp1 gene into the genome of the transgenic silkworms, and silk protein analysis revealed its expression and secretion in the cocoon. Compared with wild-type silk, the recombinant silk displayed a higher tensile strength and elasticity. The results indicate the potential for producing recombinant spider silk in transgenic *B. mori*.

Keywords Spider dragline silk · Transgenic silkworm · *piggyBac* · Cocoon proteins · Mechanical characteristic

Introduction

Araneoid spiders are unique in their production and use of different silks throughout their lifetime. These silks are produced as soluble proteins in specialized glands and are then spun out as fibrous threads. Up to seven different protein-based silk fibers are produced by orbweb-weaving spiders, including major ampullate dragline, minor ampullate, flagelliform, aciniform, tubuliform, aggregate, and pyriform [1]. Among these silks, major ampullate dragline silk is of particular interest due to its unique combination of high tensile strength and high elasticity. On a weight-to-strength basis, it is stronger than steel and is referred to as “biosteel.” On the other hand, the elasticity of spider silk reaches up to 35%, which is much higher than that of steel [2–4]. These remarkable mechanical properties make it attractive for industrial, military, and medical applications.

The core constituents of major ampullate dragline silk are two types of fibrous proteins, major ampullate spidroin 1 (MaSp1) and 2 (MaSp2), and their ratio has been estimated to be about 3:2 [5–7]. Both proteins are large molecules of about 250–350 kDa, and their major parts are composed of approximately 100 tandem copies of a 30–40-amino-acid repeat sequence that always alternates between a Gly-rich domain and an Ala-rich domain. Ala-rich domains are considered to form crystallites that are responsible for the high tensile strength, whereas the Gly-rich domains are responsible for the elasticity.

To obtain large amounts of fibrous spider silk, researchers have attempted several methods, including isolating silks from spiders and synthesizing them via chemical methods. However, it is difficult to farm spiders due to their territorial nature and cannibalism; meanwhile, chemical synthesis methods depend on a clear knowledge

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of the polymerization process of spinning silk from liquid protein, which remains unknown. With the development of molecular biology, researchers have also turned to transgenic technology. Heterologous expression of spider silk proteins has been successfully achieved in *Escherichia coli*, yeast, mammalian cells, and higher plants [8–14]. However, the products only form soluble proteins, which must be purified and then spun into fibrous silk artificially [11, 12].

The silkworm *Bombyx mori* also synthesizes large amounts of silk proteins in silk glands and spins them out as a fibrous thread to form a cocoon. Insights into the sequences and molecular structures have shown that silkworm silk is very similar to that of spider dragline silk. For example, both of their genes have a high GC ratio, and the proteins are composed of tandem repeats that always contain Gly-rich and Ala-rich domains in their major parts [8, 15]. In addition, *B. mori* has been farmed on a large scale for several thousands of years, and the silk industry is still prevalent in China and other developing countries. Thus, it is reasonable to assume that the production of spider silk is feasible with *B. mori*.

To date, great progress has been made in research on silkworm transgenesis. In 2000, Tamura et al. [16] reported a method for stable germline transformation in silkworms by using a *piggyBac* transposon-derived vector, inaugurating a new era of silkworm transgenesis. Subsequently, many foreign genes have been transformed into the silkworm genome with successful expression [17–22]. These achievements suggest that it is technically possible to express a spider silk gene in *B. mori*.

Previously, we cloned and characterized several novel spider silk genes and observed their expression in *B. mori* cell lines and larval bodies [23–27]. In this report, we further attempted the expression of a spider *Nephila clavata* dragline gene, MaSp1, driven by a *B. mori* Ser1 promoter and found that it could be secreted into cocoons.

Materials and methods

Materials

The *Bombyx mori* N4 (white cocoon) and non-diapause pnd-w² strains were maintained in our laboratory. Larvae were reared on artificial diets at 25°C.

Cloning the sericin 1 promoter

The silkworm sericin 1 promoter (Ser1) was amplified from silkworm chromosomal DNA with the primers 5'-CCGCTCGAGGAAATTCTTAGCTACATCTAGCCCAG-3' (forward) and 5'-GTCCCTAGGGTGACCGAAAGCTTTTACGC-3' (reverse). The amplification program consisted of an

initial denaturing step (95°C for 5 min), followed by 25 cycles of denaturing (94°C for 1 min), annealing (58°C for 30 s), and extension (72°C for 4 min), and a final elongation step at 72°C for 5 min. PCR products were cut with the restriction enzymes *Xho*I and *Bln*I and then ligated into a pSLfa1180fa vector digested with *Xho*I and *Xba*I to achieve the plasmid pSL-Ser1.

Vector construction

The plasmid pSLfa1180fa (3.5 kb in size), which contains *Fse*I and *Asc*I sites flanking the multiple cloning site of pSL1180 [28], was used for gene cloning, and a *piggyBac*-based vector, pBac[3xP3-DsRedaf] [29], was used for transformation.

A 1.5-kb DNA fragment of the spider dragline silk gene (MaSp1) from *Nephila clavata* was characterized and obtained in our previous report [23]. This fragment was digested with *Spe*I and *Nhe*I and was then inserted into the plasmid pSL-Ser1 that had been digested with *Nhe*I and dephosphorylated. The resulting plasmid with a correct orientation of the MaSp1 gene was further digested with *Nhe*I, dephosphorylated, and inserted once again with the above-mentioned 1.5-kb MaSp1 fragment. In this way, the plasmid pSL-Ser1-3.0 kb containing the Ser1 promoter plus a 3.0-kb MaSp1 repetitive sequence was obtained.

A gene coding for the carboxyl terminus of the silkworm fibroin H-chain, named the L-chain binding site (LBS), was amplified from silkworm genomic DNA with a pair of primers, 5'-CTAGCTAGCAGTTACGGAGCTGGCAGGGGATACG-3' (forward) and 5'-CGGGATCCTAGTACATTCAAATAAAATGCATAC-3' (reverse). The PCR product was digested with *Nhe*I and *Bam*HI and then cloned into the plasmid pSL-Ser1-3.0 kb previously digested with the same enzymes. The resulting plasmid was then digested with *Asc*I, and two fragments were generated. The larger fragment was recovered from an agar gel after electrophoresis and was inserted into the vector pBac[3xP3-DsRedaf] at the *Asc*I site. The final vector was designated as pBac[3xP3-DsRedaf]-Ser1-MaSp1 (Fig. 1) and was used for embryo injection.

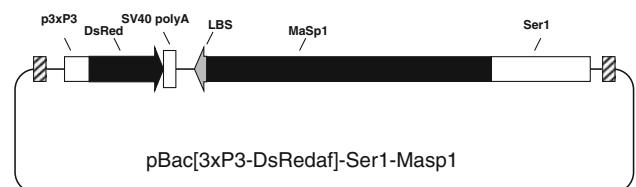


Fig. 1 Schematic of the transformation vector structure. The expression cassette consisting of a Ser1 promoter, a MaSp1 gene, and a LBS fragment was inserted into the pBac[3xP3-DsRedaf] vector. The DsRed gene driven by promoter p3xP3 was used as a transformation reporter. Boxes with oblique lines represent the *piggyBac* right and left arms

Transgenesis and screening of silkworms

Vector pBac[3xP3-DsRedaf]-Ser1-MaSp1 was purified with the QIAGEN Plasmid Midi kit (Qiagen K.K., Tokyo, Japan). A nonautonomous plasmid, pHA3PIG, was used as the helper for the production of transposase [16]. Embryo injection was performed as described by Tomita [17]. After injection, the embryos were allowed to develop at 25°C. G₀ moths were mated randomly, and the G₁ embryos were screened by detecting DsRed fluorescence under an Olympus SZX12 fluorescent stereomicroscope (Olympus, Tokyo, Japan). G₁ positive transgenic individuals were mated within the same family to generate the G₂ descendants.

Genotyping analysis of the transgenic silkworms

Chromosomal DNAs were extracted from the transgenic G₂ moths and wild-type moths, respectively, and completely digested with *Pst*I. The digested DNA samples were used as templates for the amplification of a 248-bp fragment of the MaSp1 gene. Vector DNA of pBac[3xP3-DsRedaf]-Ser1-MaSp1 was used as a positive control template. A forward primer, 5'-CTTCTCGCTTGCTCA GCTAG-3', and a reverse primer, 5'-CACCAGCTCCTT GTCCACTAAG-3', were used in this case. The PCR product was cloned into a pMD20-T vector (Takara Bio, Shiga, Japan), and the nucleotide sequences were determined by the dideoxy termination method using a DNA sequencer (Genetic Analyzer 3100, Applied Biosystems, Foster City, USA).

Analysis of cocoon proteins

Cocoons without floss were cut into small pieces and then washed with 50 mM Tris-HCl, pH 7.5. Proteins were extracted with saturated lithium thiocyanate (LiSCN) containing 2% 2-mercaptoethanol (100 ml per 1 g cocoon) at room temperature. The obtained proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) with an 8% gel and stained with CBB-R250. For immunoblotting analysis, proteins on the gels were transferred onto a polyvinylidene difluoride (PVDF) membrane and then reacted with an anti-MaSp1 antibody (1:2,000 dilution) and secondary antibody (1:10,000 dilution). Antibody-stained

protein bands were visualized with a Konica immunostaining HRP-1000 kit (Konica Minolta, Tokyo, Japan).

To determine the location of recombinant MaSp1 in the silk, cocoon samples were boiled in aqueous 0.05% Na₂CO₃ for 30 min to remove sericin, as described by Yamada et al [30]. The remaining fibroin was analyzed by SDS-PAGE and immunoblotting.

Mechanical characteristics of the silk

Twenty non-damaged cocoons from the wild-type and transgenic silkworms were chosen, respectively, and five pieces of silk threads from each cocoon were randomly selected. Silk samples were prepared as previously described [27]. Tests of mechanical characteristics were performed with a tensile strength tester (Tensilon UTM-II1-100, Toyo-Baldwin Co. LTD, Tokyo, Japan) according to the manufacturer's instructions.

Results

Generation of transgenic silkworms

We microinjected 2,575 eggs of the N4 strain and 2,320 eggs of the Nd strain, respectively, and the outcomes of the transgenesis are summarized in Table 1. In the G₁ embryos of the N4 strain, we obtained positive transgenic individuals with DsRed expression in 13 broods; while in those of the Nd strain, no positive transgenic individuals were achieved. Thus, we discarded the Nd strain and selected the N4 strain for further study. The reporter gene DsRed for the detection of positive transgenic silkworms was driven by an eye and nervous tissue-specific promoter, 3xP3, and its expression (in this case, we detected the eyes of the pupae and moths) results are shown in Fig. 2.

We then extracted genomic DNA from the G₂ moths for genotyping analysis. As a result, a 0.25-kb DNA fragment was amplified in all of the samples from the silkworms with DsRed expression, while such fragments were not found in the wild-type moths (Fig. 3). This fragment was ligated into a T-vector for DNA sequencing, and the results revealed that its sequence exactly matched a fragment between two *Pst*I sites of the 1.5-kb MaSp1 gene. This

Table 1 Results of transgenesis

Strain	Injected embryos	Number			Percent (%)
		Hatched embryos	G ₁ broods	Broods with DsRedpositive larvae	G ₁ broods with positive larvae
N4	2,575	221	161	13	8.07
Nd	2,320	198	112	0	0

Fig. 2 Fluorescence of DsRed in transgenic silkworms. Pupae (a) and moths (b) of wild-type (up panels) and transgenic (bottom panels) silkworms were illuminated. Photos in right panels showed fluorescent image; left panels were light image

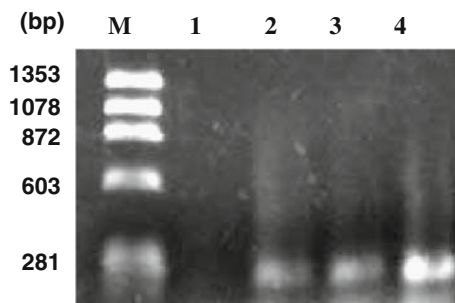
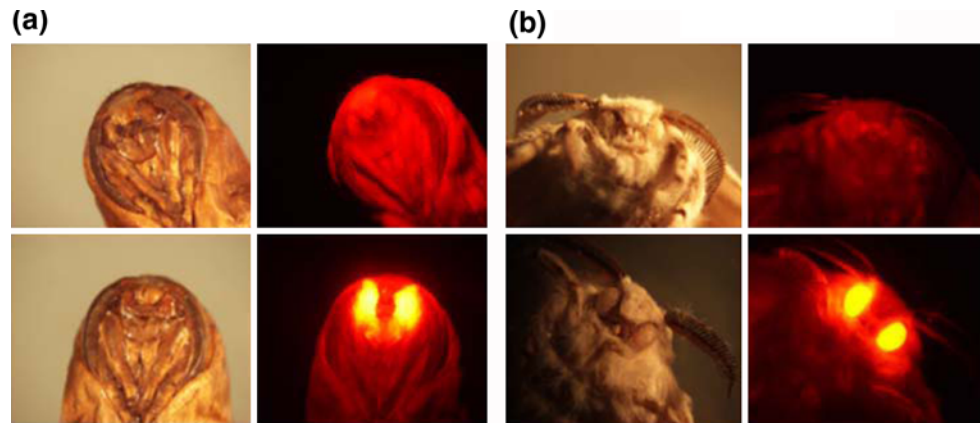


Fig. 3 Electrophoresis result of genotyping analysis. Lane M, DNA molecular weight maker; lane 1, PCR product of wide-type silkworm; lane 2 and 3, PCR products of transgenic silkworms; lane 4, PCR product of vector pBac[3xP3-DsRedaf]-Ser1-MaSp1

result confirmed that the MaSp1 gene had been integrated into the genome of the transgenic silkworms.

Analysis of cocoon proteins

Cocoon proteins from the transgenic and wild-type silkworms were obtained by LiSCN dissolution and reduction with 2-mercaptoethanol and were separated by SDS-PAGE. Comparative results revealed the existence of an exclusive 83-kDa protein in the transgenic silkworm (Fig. 4a), whose size agreed well with the calculated molecular mass of recombinant MaSp1. When analyzed with immunoblotting, a single band corresponding to the 83-kDa protein was found in the lane of the transgenic silkworm, but no band appeared in that of the wild-type (Fig. 4b). These results indicate the successful expression of MaSp1 in the cocoon. The silk from the transgenic silkworms was designated as recombinant silk.

We further detected the location of MaSp1 in the recombinant silk. The silks were boiled, and the MaSp1-specific band was difficult to observe in the sericin-removed samples when using either SDS-PAGE or immunoblotting analysis. This indicated that MaSp1 should be located in the sericin layer of the recombinant silk.

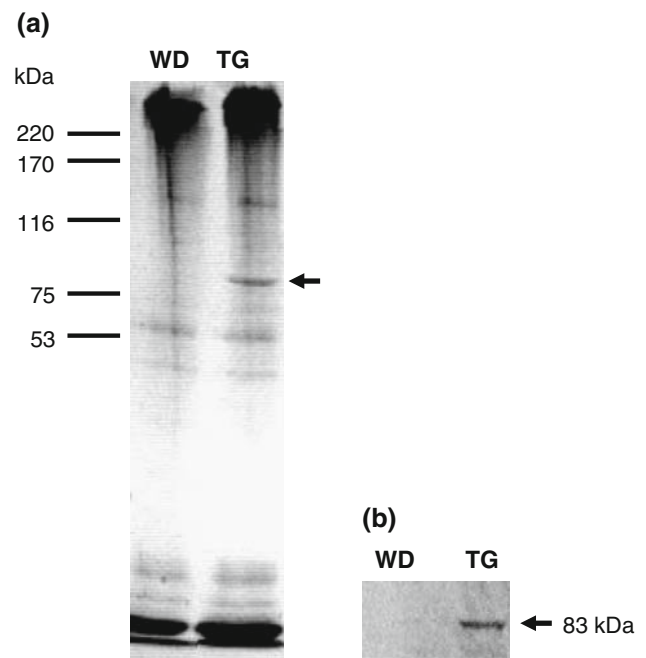
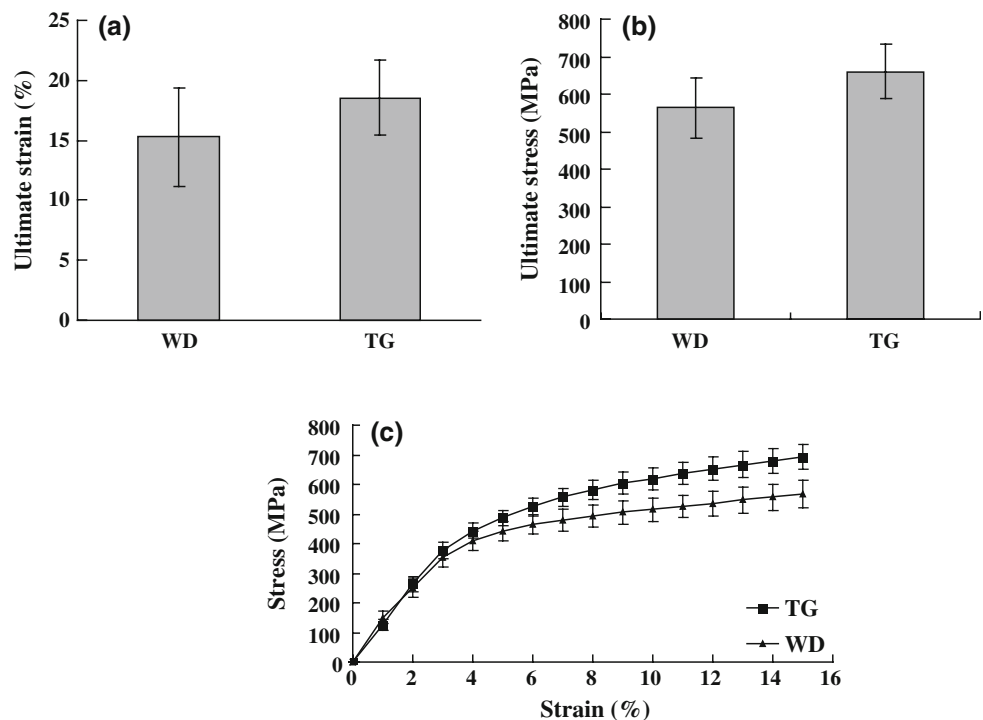


Fig. 4 Analysis of cocoon protein by SDS-PAGE. a CBB-R250 staining result; b Western blot result. Lane WD, proteins from wild-type silk; lane TG, proteins from recombinant silk. The position of the recombinant silk specific protein was indicated by an arrow

Analysis of the silk

Mechanical characteristics of the wild-type and recombinant silks were comparatively investigated on a tensile strength tester. We first compared their ultimate tensile strains (Fig. 5a). On average, the recombinant silk could be stretched up to 18.5%, showing a higher elasticity than the wild-type silk (in this case, it was 15.3%). We then detected their ultimate tensile stresses and found that the recombinant silk (660 MPa) could endure a stronger stress than the wild-type silk (564 MPa) before breaking (Fig. 5b). To further compare these two types of silks, we chose all of the samples with an ultimate strain between 15.0–20.0% and drew their strain-stress curves, using the

Fig. 5 Analysis of the tensile strain and stress of silks. **a** Ultimate tensile strain; **b** Ultimate tensile stress; **c** Strain-stress curves. WD, wild-type silk; TG, recombinant silk. The bars indicate standard deviations



average values (Fig. 5c). From the curves, we can see that a stronger stress is required for the recombinant silk to reach the same elongation. *T*-test analysis revealed that all of the data of the recombinant silk showed obvious differences from those of the wild-type silk ($P < 0.01$). These results definitely displayed a little increase in tensile strength and elasticity of the recombinant silk.

Discussion

The distinct properties of spider silks display their far-ranging applications. However, the manner in which to artificially produce spider silk on a large scale still presents a bottleneck. As part of an endeavor to overcome this barrier, we have studied the heterologous expression of spider silk in *B. mori* [23–27]. We anticipated that novel recombinant silks with the characteristics of spider silk could be spun as fibrous threads by transgenic *B. mori*. In this paper, we report the expression of the spider dragline silk (MaSp1) in transgenic silkworm cocoons. The MaSp1 gene was integrated into the *B. mori* genome via the *piggyBac* system and was driven by a Ser1 promoter. Recombinant MaSp1 protein was detected in the cocoon. Tests of the mechanical characteristics displayed a little increase in tensile strength and elasticity of the recombinant silk when compared with those of the wild-type. These results demonstrate progress towards our goal of producing spider silk with *B. mori*. To our knowledge, this is the first report of the expression of recombinant spider silk in transgenic *B. mori*.

The main components of the cocoon protein are the inner fibroin and the outer sericin, which account for about 75 and 25% of the weight, respectively. Their promoters are usually used for the expression of foreign genes in the silk glands [17, 18, 20, 21]. In this report, we showed that the sericin promoter Ser1 could be used to drive the expression of spider silk genes. In another research project, we found that MaSp1 could also be expressed and secreted into the cocoons when its gene was driven by the fibroin promoter (data unpublished). All of these findings indicate the feasibility of producing recombinant spider silks with transgenic *B. mori*.

We tested two silkworm strains, N4 and Nd, for transgenesis and obtained very different results. Although there were no significant differences between the two strains in the percentages of G_0 fertile moths in the microinjected eggs and G_1 broods in the G_0 fertile months, the percentages of G_1 broods with positive larvae in the total G_1 broods and in injected eggs were significantly different (Table 1). The transgenesis rate of the N4 strain was much higher than that of the Nd strain, and we failed to obtain any positive transgenic individuals with the latter. Zhong et al. compared the transgenesis efficiency of the *piggyBac* transposon among three different silkworm strains and reported similar results. They presumed that the transgenesis efficiency of the *piggyBac*-based system might vary with silkworm strains with different genetic backgrounds [31]. Our data seems to support their hypothesis.

The increase in tensile strength and elasticity of the recombinant silk should be attributed to the expression of

the spider silk protein MaSp1. However, the mechanical characteristics of the recombinant silk were still much lower when compared with those of the authentic spider dragline silk. In our previous report, we had determined that the ultimate strain and stress of authentic spider dragline silk were more than 30% and 1,300 MPa, respectively [27], much higher than those of the recombinant silk in this study. These differences may be due to the low ratio of recombinant MaSp1 in the total silk proteins, which was also confirmed by the SDS-PAGE analysis results (Fig. 4). We speculate that a higher level of spider silk protein in the recombinant silk will lead to a higher increase in tensile strength and elasticity.

To increase the amount and ratio of spider silk protein in the recombinant silk, some new strategies can be considered. (1) The addition of enhancer elements. It has been demonstrated that the biosynthesis of silk proteins is essentially controlled at the transcription level [18]. Tomita et al. reported that a baculovirus-derived enhancer *hr3* and a *trans*-regulator IE1 cooperatively increased the Ser1 promoter activity by more than 30-fold [21]. These reports indicate that we may improve the production of spider silk protein in the transgenic *B. mori* by adding enhancer elements to the promoter when constructing the expression vector. (2) Co-expression of MaSp1 and MaSp2 in transgenic silkworms. The major components of authentic spider dragline silk contain MaSp2 as well as MaSp1, so co-expression of both spidroins in the cocoon might increase their expression levels and change the mechanical properties of the recombinant silk to a higher extent. (3) The use of sericin and fibroin promoters to drive the expression of spider silk protein together. (4) A reduction of the endogenous expression of fibroin and sericin by RNAi or knock-out techniques. Attempts of these suggestions are now being performed, and we expect that novel transgenic silkworm germlines with a high production of recombinant spider silk will be generated.

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