Transgenic silkworms produce recombinant human type III procollagen in cocoons

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We describe the generation of transgenic silkworms that produce cocoons containing recombinant human collagen. A fusion cDNA was constructed encoding a protein that incorporated a human type III procollagen mini-chain with C-propeptide deleted, a fibroin light chain (L-chain), and an enhanced green fluorescent protein (EGFP). This cDNA was ligated downstream of the fibroin L-chain promoter and inserted into a *piggyBac* vector. Silkworm eggs were injected with the vectors, producing worms displaying EGFP fluorescence in their silk glands. The cocoons emitted EGFP fluorescence, indicating that the promoter and fibroin L-chain cDNAs directed the synthesized products to be secreted into cocoons. The presence of fusion proteins in cocoons was demonstrated by immunoblotting, collagenase-sensitivity tests, and amino acid sequencing. The fusion proteins from cocoons were purified to a single electrophoretic band. This study demonstrates the viability of transgenic silkworms as a tool for producing useful proteins in bulk.

Collagen is used in many medical applications, including tissue engineering[1] and drug delivery materials[2], because of its strength and stability as well as its general compatibility with living tissues. Currently, the main source of collagen is cow skin. This source carries a high risk of contamination and can also cause allergic reactions[3]. Thus, there is a need for alternative sources of collagen to produce large quantities.

The domestic silkworm, *Bombyx mori*, synthesizes vast amounts of silk protein in its silk glands and spins it into cocoons during the last larval instar. Recently, a method for stable germline transformation in *B. mori* was developed using a *piggyBac* transposon–derived vector[4]. *B. mori* is therefore a good candidate host for the production of recombinant proteins at an industrial scale. The cDNA of type III collagen is an appropriate choice for a transgene because of its simple gene composition. To avoid a possible problem with the large size of collagen molecules, we decided to use cDNA of the type III procollagen mini-chain as the actual transgene. Lees and Bulleid originally designed the procollagen mini-chain which is composed of an N-propeptide, one-fifth of a triple-helix domain, and a C-propeptide[5]. In the present study, we produced transgenic silkworms with *piggyBac* vectors carrying the cDNAs of a fusion protein containing human type III procollagen mini-chains with C-propeptide deleted. The silkworms synthesized the fusion protein in silk glands and secreted it into cocoons. The fusion proteins were purified to a single band on electrophoretic gels.

Results and discussion

Generation of transgenic silkworms. We constructed a cDNA encoding a fusion protein comprising a human type III procollagen mini-chain[5], a fibroin L-chain, and EGFP under the control of a fibroin L-chain promoter sequence. This cDNA was incorporated into a *piggyBac* vector prepared as follows. Preliminary experiments on the expression of this vector in isolated silk glands were performed by transfecting the vector into the glands with a particle gun. These experiments showed that the C-propeptide of the mini-chain strongly suppresses the expression of fusion proteins (data not shown). In the present study we constructed cDNAs containing the sequences of N-propeptide and one-fifth of the triple-helix domain of human type III procollagen (human type III procollagen mini-chain with C-propeptide deleted).

We prepared three fusion cDNAs—LE, MOSRA-7, and MOSRA-8—encoding, respectively, fibroin L-chain/EGFP, fibroin L-chain/N-telopeptide/the triple-helix domain of the procollagen mini-chain/C-telopeptide/EGFP, and fibroin L-chain/the C-propeptide-deleted procollagen mini-chain/EGFP (Fig. 1). These cDNAs were inserted between the fibroin L-chain gene 5′-flanking and 3′-flanking sequences; the resulting constructs will be referred to as expression units. The expression units were inserted into pBac[3xP3-DsRed], in which the gene for the red fluorescent protein (*DsRed*) was introduced as a marker gene under the eye and nervous tissue–specific promoter 3xP3 in place of the *EGFP* gene of the original *piggyBac*-derived vector pBac[3xP3-EGFPafm][6]. These three vectors were designated pLE, pMOSRA-7, and pMOSRA-8, respectively (Fig. 1).

Each of the vectors, pLE, pMOSRA-7, and pMOSRA-8, was mixed with the helper plasmid pHA3PIG[4] and injected into pre-blastoderm silkworm embryos. Hatched larvae (G0) were allowed to develop to moths. Moths were mated within the same family. The resulting G1 broods were screened for DsRed fluorescence. The number of G1 broods containing DsRed-positive larvae is summarized in Table 1.

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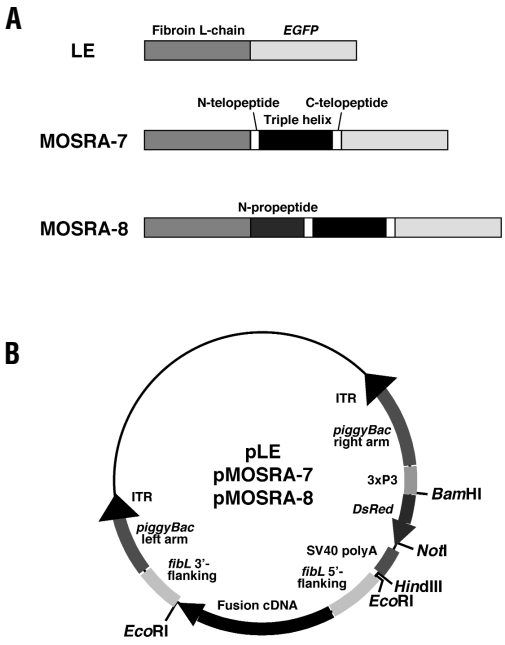

Figure 1. Structures of fusion cDNAs of LE, MOSRA-7, and MOSRA-8 and of the vectors pLE, pMOSRA-7, and pMOSRA-8. (A) Structures of fusion cDNAs. LE comprises cDNAs of fibroin L-chain and EGFP. MOSRA-7 was made by inserting N-telopeptide/the triple-helix domain of type III procollagen mini-chain/C-telopeptide between fibroin L-chain and EGFP of LE. MOSRA-8 has the structure that also includes N-propeptide between fibroin L-chain and N-telopeptide of MOSRA-7. (B) Structures of *piggyBac*-based vectors. Each of the above three fusion cDNAs was placed between the 5′-flanking (*fibL* 5′-flanking) and the 3′-flanking sequence (*fibL* 3′-flanking) of the fibroin L-chain gene. Thus, we made three *piggyBac*-based vectors, pLE, pMOSRA-7, and pMOSRA-8 that contained cDNA of LE, MOSRA-7, and MOSRA-8, respectively. The restriction enzyme sites are indicated for *Bam*HI, *Not*I, *Eco*RI, and *Hin*dIII.

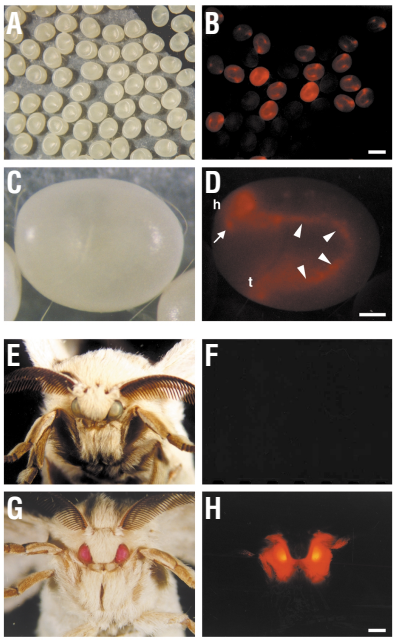

Figure 2. Fluorescence of DsRed in transgenic silkworms bearing pMOSRA-7. G1 broods with DsRed-positive embryos at the fifth day of embryonic development were viewed under white light (A) and light of the DsRed excitation wavelength (B). Panel C and D show a magnification of a DsRed-positive embryo in panel A and B. An arrow and arrowheads in panel D point to the ocelli and the abdominal nervous system, respectively. h, head; t, tail. Adults of wild-type (E, F) and transgenic (G, H) silkworms were also illuminated under white light (E, G) and DsRed-excitation-wavelength light (F, H). Scale bars, B and H, 1 mm; D, 0.2 mm.

The rate of successful transgenesis for G1 broods was 25.8%, 18.3%, and 27.6% for pLE, pMOSRA-7, and pMOSRA-8, respectively (Table 1). These transformation frequencies were comparable to those with *P*-element-mediated transformation in *Drosophila melanogaster*[7], showing the effectiveness of these vectors in transgenesis of *B. mori*. The proportion of DsRed-positive individuals in G1 broods containing at least one DsRed-positive embryo varied from 0.5% to 35.0%. As an example, photos of DsRed-positive embryos are shown for pMOSRA-7 transgenesis (Fig. 2A, B). The DsRed fluorescence became visible in the ocelli and in the central and peripheral nervous system on the fifth day of embryonic development (Fig. 2C, D). The fluorescence in the ocelli was observed throughout the larval stages (Fig. 4A, B, panel b) and strong fluorescence was also observed in the compound eyes of the pupae and moths (Fig. 2E–H). The red fluorescence from the compound eyes of the moths could be observed even under white light (Fig. 2G). Thus, the use of the 3xP3-driven *DsRed* cDNA as a marker allowed us to rapidly distinguish transgenic from wild-type worms through all developmental stages except the early embryonic stages.

Southern blot analysis. Genomic DNA was extracted from all the DsRed-positive moths, digested with *Hin*dIII, and analyzed by Southern blotting. Results are shown for five DsRed-positive individuals picked at random from each of the three pLE-, pMOSRA-7-, and pMOSRA-8-bearing broods (Fig. 3). All individuals showed bands of variable size that were hybridized with the *EGFP*-specific probe (Fig. 3A). When hybridized with the collagen probe, the moths carrying pMOSRA-7 and pMOSRA-8 showed identical bands as in the case of the *EGFP* probe (Fig. 3B, lanes 2–11), whereas the collagen probe did not produce any signal for pLE (Fig. 3B, lanes 12–16). The control animals did not show a positive signal with either probe (Fig. 3A, B, lane 1). These results indicate that all three cDNAs encoding fusion proteins, pLE, pMOSRA-7, and pMOSRA-8, were integrated into *B. mori* chromosomes. Inverse PCR analysis[4] further confirmed integration of the cDNA into all silkworms tested (data not shown). Consistent with previous studies on transgenesis of insects with *piggyBac*-derived vectors[4,8–10], most of the positive broods comprised several sublines with different insertions. Analysis of all positive G1 animals allowed grouping of the pLE-, pMOSRA-7-, and pMOSRA-8-bearing animals into 42,

Table 1. Outcome of transgenesis

	Number				Percent
Vector	Injected embryos[a]	Hatched embryos[b]	G1 broods[c]	Broods with DsRed-positive larvae[d]	G1 broods with positive larvae
PLE	1,900	589	163	42	25.8
PMOSRA-7	2,597	855	131	24	18.3
PMOSRA-8	2,757	562	127	35	27.6

[a]*B. mori* embryos were injected with a mixture of pHA3PIG and pLE, pMOSRA-7, or pMOSRA-8.
[b]Hatched larvae (G0) were allowed to develop to moths.
[c]The moths were intercrossed and the resulting G1 broods were counted.
[d]G1 broods of embryos thus obtained were screened for DsRed fluorescence.

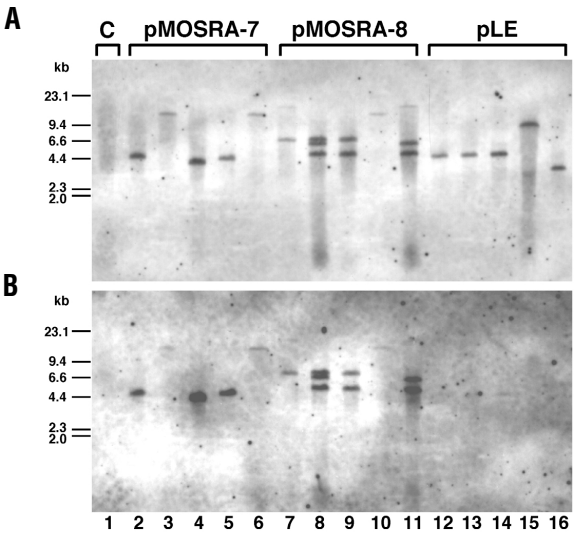

Figure 3. Genomic Southern blot hybridization. Five positive individuals were picked up from each of the pLE, pMOSRA-7 and pMOSRA-8 broods. Genomic DNAs extracted from a control animal (C, lane 1) and transgenic animals (pMOSRA-7, lanes 2–6; pMOSRA-8, lanes 7–11; pLE, lanes 12–16) were digested with *Hin*dIII, and used for hybridization with *EGFP* probes (A) and collagen probes (B).

21, and 28 sublines, respectively. Furthermore, two or three insertions were occasionally present in a single animal, as shown in lanes 7–9 and 11. Sublines with multiple insertions in a single animal were determined for all the pLE, pMOSRA-7, and pMOSRA-8 positive moths, which showed that frequencies of two and three insertions were 20.9% and 2.2%, respectively.

Synthesis and secretion of transgene products. We surveyed EGFP fluorescence in the DsRed-positive individuals under light at the excitation wavelength of EGFP. In a living first-instar, pMOSRA-7 transgenic silkworm larva, the ocelli and the ganglia of the abdominal nervous system emitted DsRed fluorescence (Fig. 4A, panel b). EGFP fluorescence was observed in the silk glands (Fig. 4A, panel c). pMOSRA-7 transgenic silkworm larvae at the fifth instar, when the weight of silk glands is about 40% of body weight[11], displayed strong EGFP fluorescence in the silk glands (Fig. 4B, panel c). Dissection of silk glands from a fifth-instar larva revealed that EGFP was localized in the anterior through posterior silk glands (Fig. 4C, panel d). EGFP fluorescence was very high in the lumen of the middle silk glands as a result of accumulation of the secreted fusion proteins. Fluorescence was not observed in any other tissues, indicating that the fibroin L-chain 5′-flanking sequence used in this study determined the tissue-specific expression of the gene constructs very efficiently. The cocoon spun by the silkworm displayed strong green fluorescence (Fig. 4D, panel d), proving that the fusion protein was present in the cocoon. Identical results were obtained with the pMOSRA-8 and pLE transgenic silkworms and their cocoons (data not shown).

To further assess the presence of fusion proteins in cocoons, we extracted proteins, separated them by SDS-PAGE, and immunoblotted them with antibodies against EGFP and fibroin L-chains. The SDS-PAGE gels from silkworms carrying pLE, pMOSRA-7, or pMOSRA-8 contained proteins that were stained with Coomassie Brilliant Blue (CBB) and migrated at 53, 75, and 88 kDa, respectively (Fig. 5A, lanes 1–4). These are the predicted sizes for the fusion protein of the three transgenes. These proteins were not present in wild-type cocoons. On immunoblotting, the three bands reacted with antibodies against EGFP (Fig. 5A, lanes 6–8) and the fibroin L-chain (Fig. 5A, lanes 10–12). To demonstrate the presence of the collagen-derived peptide sequences in the fusion proteins, the proteins were treated with nonspecific protease–free bacterial collagenase that recognizes the -Gly-X-Y- repeats within the triple-helix domain of collagen and digests them into smaller fragments. The digests were immunoblotted with anti-EGFP antibodies (Fig. 5B). In contrast to the EGFP-immunoreactive 75 and 88 kDa bands from pMOSRA-7 and pMOSRA-8 transgenic worms, respectively, only a 30 kDa immunoreactive band (arrowhead) resulted from the treated materials (Fig. 5B, lanes 6, 8). This band was equivalent in size to the region comprising type III collagen C-telopeptide and EGFP. This shift in molecular size of the immunoreactive bands shows that the fusion proteins contain collagen-derived sequences (the -Gly-X-Y- repeats). The molecular size of the immunoreactive band in the pLE cocoon was unaltered by the treatment, which can be explained by the absence of a collagen-derived sequence in the fusion protein (Fig. 5B, lane 4). Furthermore, we determined the internal amino acid sequence of the 75 kDa protein in the pMOSRA-7 cocoons using a quadrupole time-of-flight mass spectrometer, which revealed the presence of fibroin L-chain, the procollagen mini-chain, and EGFP sequences in the 75 kDa proteins. We concluded that the 53, 75, and 88 kDa proteins were recombinant fusion proteins synthesized from the fusion cDNAs in pLE, pMOSRA-7-, and pMOSRA-8-bearing worms, respectively.

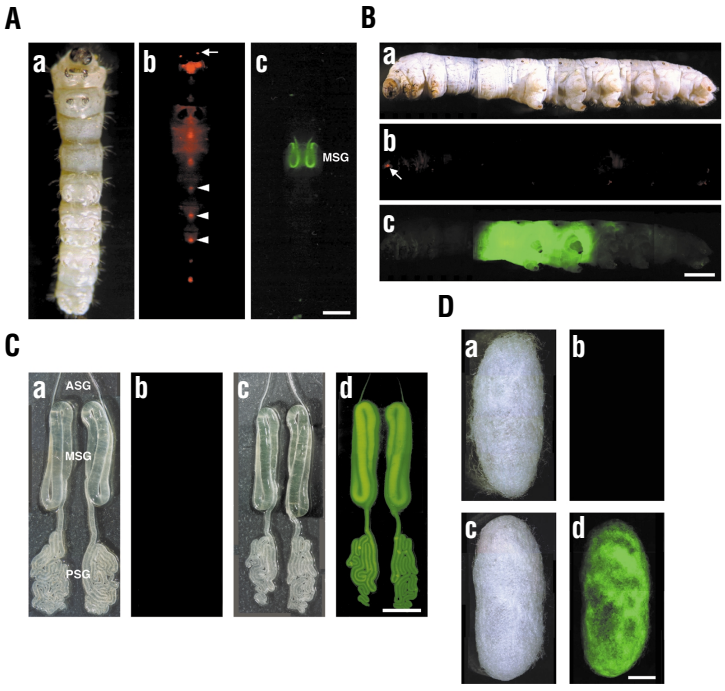

Figure 4. The fluorescence of EGFP in pMOSRA-7-bearing transgenic silkworms and their cocoons. (A, B) Larvae of the transgenic silkworms at (A) the first-instar stage and (B) the fifth-instar stage. Animals were illuminated under white light (a), DsRed-excitation-wavelength light (b), and EGFP-excitation-wavelength light (c). An arrow in A and B and arrowheads in A point to the ocelli and the abdominal ganglia, respectively. MSG, middle silk glands. (C) Silk glands were dissected from wild-type (a, b) and transgenic (c, d) silkworms at day 3 of the fifth instar, and were observed under white light (a, c) and EGFP-excitation-wavelength light (b, d). (D) Cocoons produced by wild-type (a, b) and transgenic (c, d) silkworms at the fifth instar were similarly observed under white light (a, c) and EGFP-excitation-wavelength light (b, d). ASG, anterior silk glands; PSG, posterior silk glands. Scale bars, A, 0.5 mm; B, C and D, 5 mm.

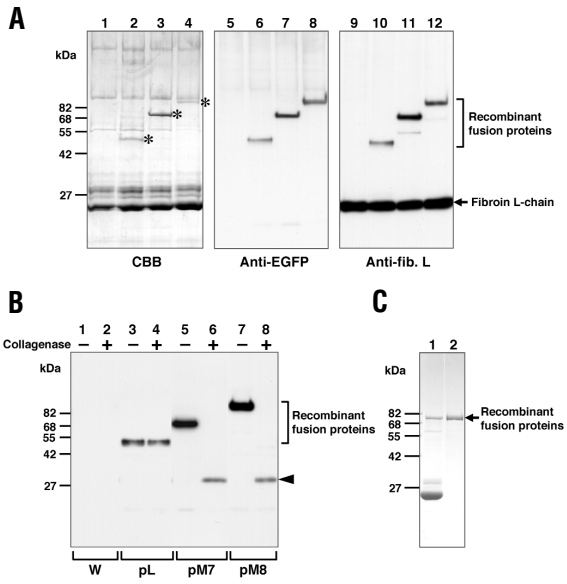

Figure 5. Analysis of cocoon proteins. (A) Proteins were extracted from cocoons of wild-type (lanes 1, 5, 9), pLE (lanes 2, 6, 10), pMOSRA-7 (lanes 3, 7, 11), and pMOSRA-8 silkworms (lanes 4, 8, 12), separated by SDS-PAGE, and then stained with CBB (lanes 1–4) or immunoblotted with either anti-EGFP (lanes 5–8) or anti-fibroin L-chain (anti-fib.L) antibodies (lanes 9–12). Asterisks in lanes 2–4 indicate CBB-stained recombinant fusion proteins. (B) Proteins were extracted from cocoons of wild-type (W, lanes 1, 2), pLE (pL, lanes 3, 4), pMOSRA-7 (pM7, lanes 5, 6), and pMOSRA-8 animals (pM8, lanes 7, 8), treated with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) collagenase, and immunoblotted with anti-EGFP antibodies. The arrowhead at the right side of the gel points to the 30 kDa immunoreactive band referred to in the text. (C) Fusion proteins were extracted from pMOSRA-7 cocoons, precipitated with $(NH_4)_2SO_4$, and purified by gel filtration. The $(NH_4)_2SO_4$ precipitates (lane 1) and purified proteins (lane 2) were electrophoresed and visualized by CBB. The Arabic numerals at the left sides of the gels of A, B, and C are molecular masses in kDa.

We determined the protein concentration in one subline of the pMOSRA-7 transgenic silkworms by densitometry on a CBB-stained gel. The concentration of the fusion protein was 36.7 μg/mg of total extracted protein or 8.4 μg/mg of dried cocoon. Next we purified the recombinant fusion protein from cocoons of transgenic silkworms. After trypsinization to remove sericins, cocoons of one subline of the pMOSRA-7-transgenic silkworms were dissolved in 6 M guanidine thiocyanate containing 5% (vol/vol) β-mercaptoethanol (β-ME). The extracted fusion proteins were concentrated by $(NH_4)_2SO_4$ precipitation and purified by gel filtration. This simple method was sufficient to purify the recombinant proteins to homogeneity (Fig. 5C).

We then investigated the conformation of the triple-helix region in the fusion protein. Proteins extracted from cocoons of pMOSRA-7 or pMOSRA-8 silkworms were treated with pepsin and subjected to SDS-PAGE, which showed the absence of pepsin-resistant peptides (data not shown). Most likely the proline residues in the helix region were poorly hydroxylated and, as a result, the region was not in a triple-helical conformation. In fact, *B. mori* silk glands showed a very low activity of prolyl 4-hydroxylase (data not shown) the enzyme responsible for triple-helix nucleation in the early stages of collagen synthesis[12] and for stabilizing the folded triple helix[13].

The present technology to produce recombinant collagens in transgenic silkworms has several advantages over previously reported expression systems[14–18]. Silk glands are highly active in protein synthesis[19] and the protein content of cocoons is very high (more than 95%). The pnd-w1 strain used in this study produces about 70 mg protein (dry weight) per cocoon. Protein production can be easily scaled up by transforming typical silkworm strains such as Kinshu × Showa, a hybrid strain of Kinshu crossed with Showa. A single cocoon of this strain was found to contain 0.3–0.5 g protein (dry weight). In addition, improvement of the regulatory sequences for gene expression could increase protein production. Another advantage lies in the simplicity of the silk protein components in cocoons, which is shown in the electrophoretic separation pattern of cocoon proteins (Fig. 5A). Major protein components of the cocoon are fibroins (H-chain, L-chain, and p25) and sericins. The simplicity of protein components facilitates the purification of recombinant collagens from cocoon proteins. In fact, we could purify the recombinant fusion proteins from the cocoon proteins by a single chromatographic step.

Silkworms have been successfully used for the production of silk. It is a reasonable assumption that other proteins expressed in this system by transgenesis, as described in the present study, could attain bulk-quantity yields approaching those of silk. The establishment of a recombinant source for pharmaceutical proteins such as human albumin would eliminate some of the perceived or real risks associated with products derived from human tissue. The present study offers experimental evidence for the possibility of using transgenic silkworms as a viable tool to produce recombinant human collagens at an industrial scale and without the risk of disease-causing contaminants. Our calculations shows that it is possible to produce 5 kg of collagen per year in a facility with a floor surface of about 300 m^2 and five workers caring for a total of about 1.5 million silkworms. These worms produce a total of about 600 kg of cocoon material, which translates into the predicted 5 kg of total collagen production.

Experimental protocol

Animals. *Bombyx mori*, strain pnd-w1, was obtained from the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan). DNA-injected embryos were maintained at 25 °C in moist chambers until hatching. The hatched larvae were transferred to an artificial diet (Mukin Yosan System Lab., Kyoto, Japan) and reared on it at 25 °C.

Vector construction. *DsRed* cDNA was excised from pDsRed2-1 vectors (Clontech, Palo Alto, CA) by treating with *Bam*HI and *Not*I. pBac[3xP3-DsRed] was made by replacing the *EGFP* cDNA located between the *Bam*HI and *Not*I sites of pBac[3xP3-EGFPafm][6] with *DsRed* cDNA. We PCR-amplified the fibroin L-chain gene 3′-flanking sequence[20] (nt 13114–nt 13597 including the putative polyadenylation signal) from genomic DNA isolated from *B. mori* adults. The PCR product was inserted into the *Bam*HI site of pBac[3xP3-DsRed] to generate pBac[3xP3-DsRed/pA].

The fibroin L-chain cDNA sequence[21] (nt 28–nt 767) was PCR-amplified from the cDNAs of posterior silk glands with the primers, FL-F (5′-CTGCAGTAACAGACCACTAAAATGAAG-3′) and FL-R (5′-GGATCCGCGTCATTACCGTTGCCAAC-3′) which contain a *Bam*HI site (underlined). The amplified cDNA fragments were treated with *Bam*HI and ligated to *EGFP* cDNAs that had been excised from the pEGFP vector (Clontech) with *Bam*HI and *Apa*I. The resulting fusion cDNA was placed downstream of the fibroin L-chain gene 5′-flanking sequences[20] (nt 600–nt 34) that had been amplified by PCR from genomic DNA. The DNA fragments containing the fibroin L-chain gene 5′-flanking sequence and the fusion cDNA were inserted into the *Eco*RI site of pBac[3xP3-DsRed/pA] to generate pLE.

cDNA of human type III procollagen mini-chain was constructed according to Lees and Bulleid[5]. Two mini-chain–derived cDNA fragments, the mini-chain triple-helix domain and the C-propeptide–deleted procollagen mini-chain, were amplified by PCR using the procollagen mini-chain cDNAs as templates. The mini-chain triple-helix domain and the C-propeptide–deleted procollagen mini-chain were amplified using N-F (5′-GGATCCCCAGGAAGCTGTTGAAGGAGGA-3′) and H-R (5′-GGATCCGCTCCATAATACGGGGCAAAACC-3′), and H-F (5′-GGATCCCCAGTATGATTCATATGATGTCAAG-3′) and H-R as primers, respectively. The resulting two cDNA fragments were inserted into the *Bam*HI site of pLE located between the fibroin L-chain and *EGFP* cDNAs, giving rise to pMOSRA-7 and pMOSRA-8, respectively.

Transgenesis and screening of silkworms. Each of the vectors (pLE, pMOSRA-7, and pMOSRA-8) was dissolved in 5 mM KCl and 0.5 mM phosphate buffer, pH 7.0, at a concentration of 0.2 μg/μl, and was mixed with the helper plasmid pHA3PIG dissolved in the same buffer and at the same concentration as the vectors. About 15–20 nl of this mixture was injected individually into pre-blastoderm embryos at 2–8 h after oviposition as described previously[4]. After the injection, the embryos were allowed to develop at 25 °C. G1 embryos were screened under a fluorescence stereomicroscope (MZ FLIII; Leica, Heerbrugg, Switzerland) equipped with appropriate filter sets for the detection of DsRed and EGFP fluorescence.

Southern blot analysis. Genomic DNA was extracted from G1 adults and digested with *Hin*dIII. The digested DNA (10 μg per lane) was separated on a 0.6% (wt/vol) agarose gel and transferred onto a nylon membrane (Hybond N+; Amersham Biosciences, Piscataway, NJ) under vacuum. The membranes were hybridized with digoxigenin-labeled EGFP or procollagen mini-chain probes at 65 °C and immersed in solution containing alkaline phosphatase–conjugated anti-digoxigenin antibodies (Roche Diagnostics, Basel, Switzerland). The hybridization signals were visualized using CDP-*Star* (Amersham Biosciences).

Analysis of cocoon proteins. Proteins were extracted from cocoons with 60% (wt/vol) lithium thiocyanate, dialyzed against 5 M urea in 20 mM Tris-HCl, pH 8.0, electrophoresed on 0.1% (wt/vol) SDS/5–20% (wt/vol) polyacrylamide gradient gels (Atto, Tokyo, Japan), and visualized by staining gels with CBB R250. For immunoblot analysis, proteins on the gels were transferred onto nitrocellulose membranes (BA85; Schleicher and Schuell, Dassell, Germany), reacted with anti-EGFP (Clontech) or anti-fibroin L-chain antibodies, and visualized with the ECL Western Blotting Detection System (Amersham Biosciences).

Proteins were extracted from cocoons with 60% (wt/vol) lithium thiocyanate, and dialyzed against 2 mM $CaCl_2$ and 150 mM HEPES, pH 7.2. The proteins were treated with 200 units/ml of highly purified bacterial collagenase (Advance Biofactures, Lynbrook, NY) at 37 °C for 16 h. After dialysis against 5 M urea and 20 mM Tris-HCl, pH 8.0, the digested proteins were separated with SDS-PAGE and immunoblotted with anti-EGFP antibodies as above.

Fusion proteins were extracted from cocoons that had been treated with 1 mg/ml of trypsin to remove sericins, and dissolved in 6 M guanidine thiocyanate and 5% (vol/vol) β-ME. The soluble proteins were dialyzed against 2 M urea, 5% (vol/vol) β-ME, and Tris-HCl, pH 8.0, and precipitated with 19% saturated $(NH_4)_2SO_4$. After centrifugation, proteins were dissolved in 6 M guanidine thiocyanate and β-ME, and separated on a column of Superdex 200 (Amersham Biosciences).

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Competing interests statement

The authors declare that they have no competing financial interests.

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