# Silkworms transformed with chimeric silkworm/spider silk genes spin composite silk fibers with improved mechanical properties

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**The development of a spider silk-manufacturing process is of great interest. However, there are serious problems with natural manufacturing through spider farming, and standard recombinant protein production platforms have provided limited progress due to their inability to assemble spider silk proteins into fibers. Thus, we used *piggyBac* vectors to create transgenic silkworms encoding chimeric silkworm/spider silk proteins. The silk fibers produced by these animals were composite materials that included chimeric silkworm/spider silk proteins integrated in an extremely stable manner. Furthermore, these composite fibers were, on average, tougher than the parental silkworm silk fibers and as tough as native dragline spider silk fibers. These results demonstrate that silkworms can be engineered to manufacture composite silk fibers containing stably integrated spider silk protein sequences, which significantly improve the overall mechanical properties of the parental silkworm silk fibers.**

biomaterials | biotechnology | transgenic animals

In addition to being used as sutures, silk fibers hold great potential as biomaterials for wound dressings, artificial ligaments, tendons, tissue scaffolds, microcapsules, and other applications (1–3). Silkworms are the current biological source of silk sutures, but spider silk fibers have superior mechanical properties that are ideal for procedures requiring finer sutures, such as ocular, neurological, and cosmetic surgeries (2, 4, 5). Silkworms can be cultivated en masse, but territorialism and cannibalism preclude spider farming as a viable manufacturing approach (2). Thus, there is great interest in developing an inexpensive, convenient, and reliable biotechnological approach that can be used to manufacture spider silk fibers as biomaterials (3, 6).

A major step in this direction was taken with the cloning and sequencing of cDNAs encoding spider silk proteins, including the major ampullate spidroin-1 and spidroin-2 and flagelliform silk proteins of *Nephila clavipes* (7–9). These genes and their products are highly repetitive, with correlations between certain short, repetitive amino acid sequence motifs in spider silk proteins and the mechanical properties of spider silk fibers (5, 10). These correlations suggest that one could custom-design unique, synthetic spider silks with mechanical properties ideally suited for specific medical applications. Recent reports describe efforts to customize spider silks by assembling DNA sequences encoding synthetic spider silk proteins with mixed motifs (11, 12). In this study, we used a similar DNA sequence encoding a unique spider silk protein, $A2S8_{14}$, with both elastic [$(GPGGA)_8$] and strength (linker-alanine$_8$) motifs.

The isolation of spider silk gene sequences enabled efforts to produce recombinant spider silk proteins in heterologous systems. Many different hosts, including bacteria (13, 14), yeast (15), baculovirus/insect systems (16–18), mammalian cells (19), transgenic plants (20), and transgenic animals were used to produce spider silk proteins. These efforts were successful, as each yielded spider silk proteins, but none of these hosts provided high yields; most were expensive to scale up, and none was naturally equipped to spin silk fibers. One could use post-production spinning technologies, such as extrusion, to try to spin fibers from recombinant silk proteins (12), but these efforts have not yet yielded an efficient and reliable manufacturing process. These results underscore a major limitation of mainstream biotechnological approaches for spider silk production, which is the inability of heterologous protein production systems to assemble spider silk proteins into fibers.

One approach that might overcome this limitation is to use silkworms as surrogate hosts for spider silk production. Although silkworms are not recognized as a major recombinant protein production platform (21–23), they might be the perfect host for spider silk fiber production because transgenic silkworms can be efficiently produced using *piggyBac* vectors (24–26), recombinant protein production can be targeted to the silk gland with tissue-specific promoters (27–32), and the silk gland is naturally equipped to assemble silk proteins into fibers (33). One caveat is that silkworms produce endogenous silk proteins. Nevertheless, we engineered transgenic silkworms to express the synthetic $A2S8_{14}$ spider silk gene in an effort to produce composite fibers consisting, at least in part, of the synthetic spider silk protein. We expected that these composite fibers might have improved mechanical properties due to incorporation of the spider silk sequences. Our results showed that transgenic silkworms encoding synthetic spider silk proteins can, indeed, spin composite silk fibers with improved mechanical properties, relative to the fibers produced by the parental animals.

## Results

***piggyBac* Vector Design.** *piggyBac* was the vector of choice for this project because it can be used to efficiently transform silkworms (24–26). The specific *piggyBac* vectors used in this project were designed to carry genes with several crucial features (Fig. 1). These included the *Bombyx mori* fibroin heavy chain (fhc) promoter to target expression of the foreign spider silk protein to

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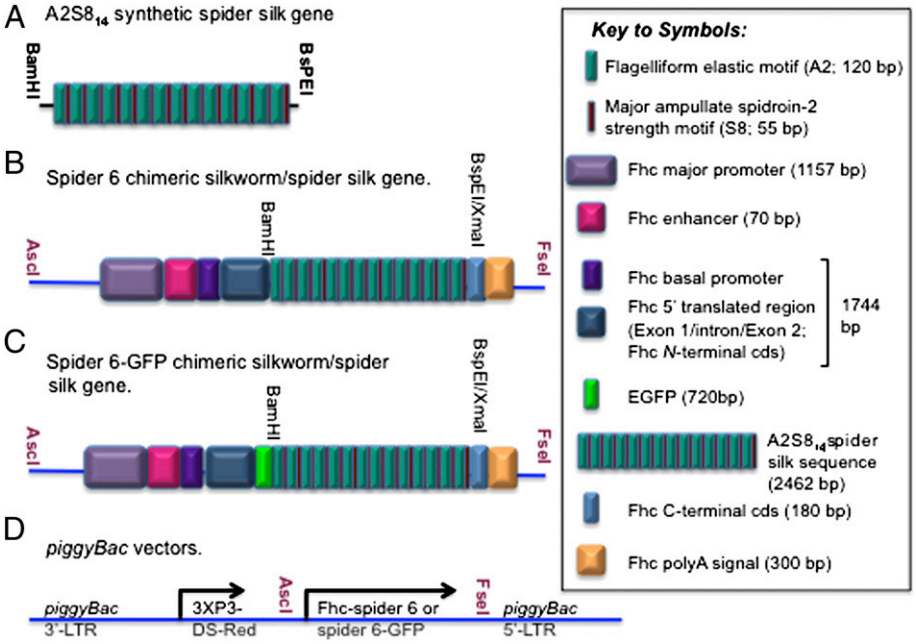


**Fig. 1.** *piggyBac* vector designs.

the posterior silk gland (29, 30) and an fhc enhancer to increase expression levels to facilitate assembly of the foreign silk protein into fibers (31). Our *piggyBac* vectors also encoded A2S8$_{14}$ (Fig. 1*A*), a relatively large (78 kDa) synthetic spider silk protein with both elastic (GPGGA)$_8$ and strength (linker-alanine$_8$) motifs. Importantly, the synthetic spider silk protein was flanked by N- and C-terminal domains of the *B. mori* fhc protein (Fig. 1 *B* and *C*). This chimeric silkworm/spider silk design was used previously to direct foreign proteins into nascent, endogenous silk fibers in the *B. mori* silk gland to produce composite silk fibers (29, 30). One of the *piggyBac* vectors constructed in this study encoded the chimeric silkworm/spider silk protein alone (Fig. 1*B*) and another encoded the same protein with an enhanced green fluorescent protein (EGFP) tag (Fig. 1*C*). The latter construct facilitated analysis of silk fibers produced by transformed offspring and was used for ex vivo silk-gland bombardment assays to examine chimeric spider silk protein expression in silk glands, as described in *Materials and Methods*.

The ex vivo assay results showed that the *piggyBac* vector encoding the GFP-tagged chimeric silkworm/spider silk protein induced green fluorescence in the posterior silk gland region (Fig. S1). Immunoblotting assays with a GFP-specific antibody demonstrated that the bombarded silk glands contained an immunoreactive protein with an apparent molecular weight ($M_r$) of ~116 kDa, about the expected size of the fusion protein (106 kDa), and two smaller immunoreactive proteins (Fig. S2). These results validated the basic design of our *piggyBac* vectors and prompted us to proceed to isolate transgenic silkworms using these constructs.

**Transgenic Silkworm Isolation.** Each *piggyBac* vector was mixed with a plasmid encoding the *piggyBac* transposase and microinjected into eggs isolated from *B. mori* pnd-w1 (24). We used this silkworm strain because it has a melanization deficiency and clear cuticle phenotype, which facilitated detection of the EGFP-tagged chimeric silkworm/spider silk protein in transformants. Putative F1 transformants were identified by a red-eye phenotype resulting from expression of Ds-Red under the control of the neural-specific 3XP3 promoter (34) in each *piggyBac* vector (Fig. 1*D*). These animals were used to establish homozygous transgenic silkworm lineages, as described in *Materials and Methods*, which were designated spider 6 and spider 6-GFP, denoting the *piggyBac* vectors used for transformation.

Even by visual inspection under white light, without excitation, we observed EGFP in cocoons from the spider 6-GFP transformants (Fig. 2*A*). We also observed strong EGFP signals when their silk glands (Fig. 2 *B* and *C*) and cocoons (Fig. 2*D*) were examined under a fluorescence microscope. At least some silk fibers in the cocoons appeared to contain integrated EGFP signals. Expression of the EGFP-tagged chimeric silkworm/spider silk proteins in the spider 6-GFP silk glands and cocoons was confirmed by immunoblotting cocoon extracts with spider silk protein-specific antisera (Fig. 3). Similar results were obtained with spider 6 cocoon extracts (Fig. 3). These results indicated that we had isolated transgenic silkworms encoding EGFP-tagged or untagged forms of the chimeric silkworm/spider silk protein and that these proteins were associated with the silk fibers produced by those transgenic animals.

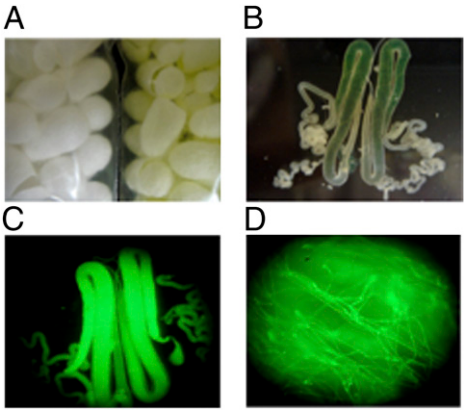


**Fig. 2.** Expression of the chimeric silkworm/spider silk/EGFP protein in (*A*) cocoons, (*B* and *C*) silk glands, and (*D*) silk fibers from spider 6-GFP silkworms.

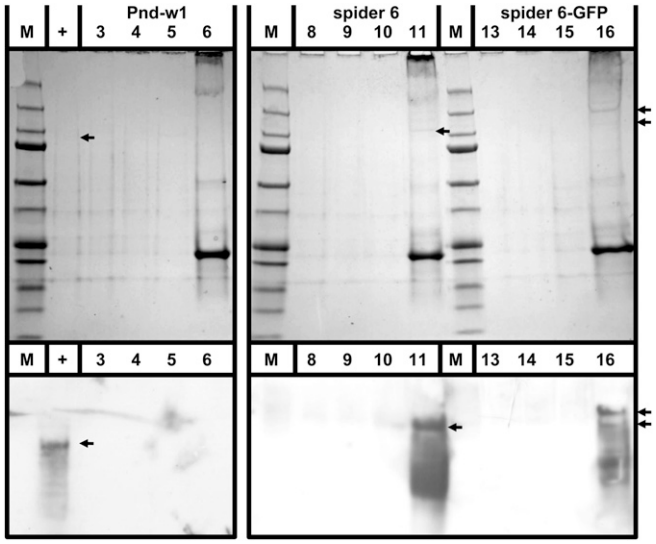


**Fig. 3.** Sequential extraction of silk fibers. Cocoons produced by pnd-w1 (lanes 3–6), spider 6 (lanes 8–11), or spider 6-GFP (lanes 13–16) silkworms were degummed and subjected to a sequential extraction protocol, as described in *Materials and Methods*. Proteins solubilized in each extraction step were analyzed by SDS/PAGE and (*Upper* panels) Coomassie blue staining or (*Lower* panels) immunoblotting with a spider silk protein-specific antiserum. M, molecular weight markers; +, $A2S8_{14}$ spider silk protein expressed and purified in *E. coli*. Lanes 3, 8, and 13: saline extracts. Lanes 4, 9, and 14: SDS extracts. Lanes 5, 10, and 15: 8 M LiSCN/2% mercaptoethanol extracts. Lanes 6, 11, and 16: 16 M LiSCN/5% mercaptoethanol extracts. The arrows mark the chimeric spider silk proteins. The apparent molecular weights were ~75 kDa for $A2S8_{14}$ from *E. coli*, ~106 kDa for spider 6, and ~130 and ~110 kDa for spider 6-GFP.

**Analysis of the Composite Silk Fibers.** A sequential protein extraction approach was used to analyze the association of the chimeric silkworm/spider silk proteins with the composite silk fibers produced by the transgenic silkworms. After removing the loosely associated sericin layer, the degummed silk fibers were subjected to a series of increasingly harsh extractions, as described in *Materials and Methods*. After each step, the soluble and insoluble fractions were separated, the soluble fraction was held for immunoblotting, and the insoluble fraction was used for the next extraction. The final extraction solvent completely dissolved the remaining silk fibers. Immunoblotting controls verified that the spider silk protein-specific antiserum recognized no proteins in pnd-w1 silk fibers (Fig. 3, lanes 3–6), but recognized the chimeric silkworm/$A2S8_{14}$ spider silk protein from *Escherichia coli* (Fig. 3, lane 2). Sequential extraction of degummed cocoons from the transgenic animals using saline (Fig. 3, lanes 8 and 13), SDS (Fig. 3, lanes 9 and 14), and 8 M lithium isothiocyanate LiSCN/2% β-mercaptoethanol (Fig. 3, lanes 10 and 15) released no detectable immunoreactive proteins. However, subsequent extraction of the residual silk fibers with 16 M LiSCN/5% β-mercaptoethanol released an immunoreactive protein with a $M_r$ of ~106 kDa from the residual spider 6 (Fig. 3, lane 11) and two immunoreactive proteins with $M_r$'s of ~130 and ~110 kDa from the residual spider 6-GFP fibers (Fig. 3, lane 16). These proteins were all larger than expected (78 and 106 kDa for spider 6 and spider 6-GFP, respectively). Potential explanations for these differences include transcriptional-translational "stuttering" due to the highly repetitive nature of the spider silk sequences, anomalous migration of the protein products on SDS/PAGE, and/or posttranslational modifications. Notably, the $M_r$ of the chimeric silkworm/$A2S8_{14}$ spider silk protein produced in *E. coli*, which was the positive immunoblotting control, also was larger than expected (~75 vs. 60 kDa). Irrespective of the sizes of the transgene products or the reasons for their appearance, the sequential extraction results clearly demonstrated that at least some chimeric silkworm/spider silk proteins expressed by our transgenic silkworms were extremely stably incorporated into composite silk fibers. The chimeric silkworm/$A2S8_{14}$ spider silk protein content of the composite fibers was determined to be about 2–5% in additional immunoblots with known amounts of the *E. coli* product as quantification standards (Table S1).

**Mechanical Properties of the Composite Silk Fibers.** Finally, we compared the mechanical properties of the composite silk fibers produced by the transgenic silkworms in parallel under precisely matched conditions. The results (Table 1 and Table S2) demonstrated that the degummed composite fibers containing either EGFP-tagged or untagged chimeric silkworm/spider silk proteins were, on average, significantly tougher than parental silkworm fibers and as tough as native dragline spider silk fibers tested under the same conditions ($P < 0.00001$). In addition, the composite fibers from spider 6 and spider 6-GFP line 4 were, on average, stronger than the parental fibers, but none of the composite fibers was as strong as native dragline spider silk ($P < 0.00001$). Finally, the composite silk fibers from all three transgenic silkworms were more extensible than the parental silkworm and native dragline spider silks ($P < 0.00001$). The mechanical properties of the composite silks from the transgenic animals were more variable than those of the parental fibers, and the composite fibers from two different spider 6-GFP lines had similar extensibilities, but different tensile strengths. This variation in the mechanical properties of composite silk fibers within an individual transgenic line and among different lines probably reflects heterogeneity in the fibers due to differences in chimeric silkworm/spider silk protein ratios and/or the localization of these proteins along the fiber. One can see evidence of heterogeneity in the composite fibers in Fig. 2*D*.

A comparison of the best mechanical performances observed for the composite fibers from the transgenic silkworms, the fibers from the parental silkworm, and a representative native (dragline) spider silk fiber is shown in Fig. 4, where toughness is defined by the area under the stress/strain curves. These data showed that all of the composite, transgenic silkworm silk fibers were tougher than the parental fibers, with values of 86.3 (spider 6, line 7), 98.2 (spider 6-GFP, line 1), and 167.2 (spider 6-GFP, line 4) compared with 43.9 $MJ/m^3$ (pnd-w1). Furthermore, these best-case measurements showed that the composite fiber from spider 6-GFP, line 4, was tougher than the native spider dragline silk fiber tested under identical conditions. Thus, these results demonstrate that the incorporation of chimeric silkworm/spider silk proteins can significantly improve the mechanical properties of composite silk fibers produced using the transgenic silkworm platform.

## Discussion

Spider silks have enormous potential as biomaterials for various applications, but serious obstacles to spider farming preclude the natural manufacturing approach. Thus, there is a need to develop an effective biotechnological approach for spider silk fiber production. Efforts to use the standard repertoire of recombinant protein production platforms have been only partially successful. Many different platforms have been used to produce recombinant spider silk proteins, but it has been difficult, at best, to efficiently process these proteins into useful fibers. The requirement to manufacture fibers, not just proteins, makes the silkworm a uniquely qualified platform for this particular biotechnological application.

Previous studies have shown that the silkworm can be used to produce recombinant proteins (21, 23). Silkworms can be efficiently transformed with *piggyBac* vectors (25, 26), and *piggyBac* vectors can be engineered to target foreign gene expression in a silk gland-specific fashion (27–29) and to direct the incor-

**Table 1. Mechanical properties of degummed native and composite silk fibers**

| | pnd-w1 | | Spider 6 | | Spider 6-GFP (line 1) | | Spider 6-GFP (line 4) | | Dragline (spider) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *n* = 11 | | *n* = 11 | | *n* = 10 | | *n* = 19 | | *n* = 7 | |
| | Average | SD | Average | SD | Average | SD | Average | SD | Average | SD |
| Average diameters (μm) | 21.8 | 1.6 | 21.1 | 1.4 | 19.8 | 2.7 | 20.6 | 1.3 | 8.1 | 0.4 |
| Mechanical properties | | | | | | | | | | |
| Maximum stress (MPa) | 198.0 | 28.1 | 315.3 | 65.8 | 281.9 | 57.7 | 338.4 | 87.0 | 664.6 | 60.5 |
| Maximum strain (%) | 22.0 | 5.8 | 31.8 | 5.2 | 32.5 | 4.3 | 31.1 | 4.5 | 19.7 | 4.8 |
| Break stress (MPa) | 197.0 | 28.0 | 314.5 | 65.6 | 281.0 | 57.5 | 336.3 | 87.3 | 658.1 | 59.2 |
| Toughness (MJ/m$^3$) | 32.0 | 10.0 | 71.7 | 13.9 | 68.9 | 16.2 | 77.2 | 29.5 | 79.6 | 25.4 |
| Young's modulus (MPa) | 3,705.0 | 999.6 | 5,266.8 | 1,656.5 | 4,860.9 | 1,269.2 | 5,498.1 | 1,181.2 | 8,949.2 | 2,096.2 |

The mechanical properties of silk fibers produced by parental and transgenic silkworms were measured under precisely matched conditions. The average mechanical properties of spider (*N. clavipes*) dragline silk fibers determined in parallel are included for comparison. The table includes the numbers (*n*) of fibers tested in each group, isolated from cocoons spun by two (spider 6 and spider 6-GFP, line 4) or three (spider 6-GFP, line 1) individual animals.

poration of foreign proteins into composite silk fibers (27–30, 32). The use of *piggyBac* vectors with these features has yielded transgenic silkworm lines that produced composite fibers containing a variety of different recombinant proteins, including procollagen (28), GFP (27, 29), and feline IFN (30). In addition, while our work was in progress, others isolated transgenic silkworms that can produce spider silk proteins (35, 36).

A transgenic silkworm engineered to produce a spider silk protein was isolated using a *piggyBac* vector encoding a native *N. clavipes* major ampullate spidroin-1 silk protein under the control of a *B. mori* sericin (Ser1) promoter (35). The spidroin sequence was fused to a downstream sequence encoding a C-terminal fhc peptide. The transgenic silkworm isolated using this *piggyBac* construct produced cocoons containing the chimeric silkworm/spider silk protein, but it was only loosely associated in the sericin layer. In contrast, the chimeric silkworm/spider silk protein produced by our transgenic silkworms is an integral component of composite fibers. The relatively loose association of the chimeric silkworm/spider silk protein designed by Wen and coworkers (35) might reflect the absence of an N-terminal silkworm fhc domain. Alternatively, the use of the Ser1 promoter in their *piggyBac* vector might not support fiber assembly, as this promoter is transcriptionally active in the middle silk gland, whereas the fhc, fibroin light chain, and fibrohexamerin promoters are all active in the posterior silk gland (37). The assembly of silkworm silk proteins into fibers requires tight spatial and temporal regulation of silk gene expression. Thus, we engineered our vectors with the fhc promoter to express the chimeric silkworm/spider silk protein in the same place and at the same time as the native silk proteins and facilitate stable integration of the chimeric protein into newly assembled, composite silk fibers. Wen et al. (35) detected minor increases in the elasticity and tensile strength of fibers in cocoons from their transgenic silkworms. However, they did not remove the sericin layer before mechanical testing, which is an essential cocoon step in commercial silk fiber production. If they had processed their cocoons in conventional fashion, the recombinant spider silk/silkworm protein would have been removed and the resulting silk fibers would not be expected to have improved mechanical properties.

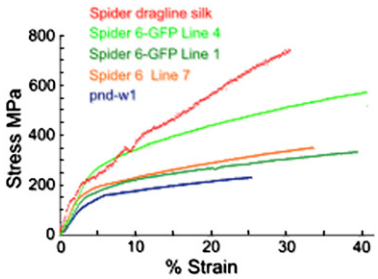


**Fig. 4.** Mechanical properties of degummed silk fibers. The best mechanical performances measured for the parental silkworm (pnd-w1) and representative spider (*N. clavipes* dragline) silk fibers are compared with those obtained with composite silk fibers produced by transgenic silkworms. All fibers were tested under the same conditions. The toughest fibers have the following energy to break values: silkworm pnd-w1 (blue line, 43.9 MJ/m$^3$); spider 6, line 7 (orange line, 86.3 MJ/m$^3$); spider 6-GFP, line 1 (dark green line, 98.2 MJ/m$^3$); spider 6-GFP, line 4 (light green line, 167.2 MJ/m$^3$); and *N. clavipes* dragline (red line, 138.7 MJ/m$^3$).

Transgenic silkworms producing spider silk proteins were reported as an aside in another recent study, which focused on the regeneration of fibers from silk proteins dissolved in hexafluoro solvents (36). This study described two transgenic silkworms produced with *piggyBac* vectors encoding extremely short (~5–15 kDa), synthetic, "silk-like" peptides from *N. clavipes* major ampullate spidroin-1 or flagelliform silk proteins (36). Both silk-like peptides were flanked by N- and C-terminal fhc domains. Although the authors did not directly address this issue, this *piggyBac* vector design should have driven stable incorporation of the chimeric silkworm/spider silk-like products into composite silk fibers. Our speculation is supported by the authors' statement that the silk fibers from these animals included 1–6% silk-like proteins, assuming that they degummed the cocoons before the compositional analysis. Mechanical testing showed that the silk fibers produced by these transgenic animals had slightly greater tensile strength, but no change in elasticity. Our data suggest that the small increases in tensile strength, which ranged from 41 to 73 MPa, were probably within the SD of the measurements. The authors concluded that the relatively small changes observed in the mechanical properties of their composite fibers reflected a low level of recombinant protein incorporation. However, because we observed a low level of incorporation with a larger impact on mechanical properties, perhaps the specific spider silk-like peptide sequences used in their constructs and/or their small sizes accounted for the relatively small changes in the mechanical properties of the composite fibers produced by these transgenic silkworms.

In summary, the present study yielded transgenic silkworm lines that produce composite silk fibers containing stably integrated chimeric silkworm/spider silk proteins that significantly improve their mechanical properties, despite low incorporation levels. On average, the composite fibers produced by our transgenic silkworm lines were significantly tougher than those produced by the parental animals and as tough as a native dragline spider silk fiber. In best-case measurements, the composite fiber produced by one of our transgenic silkworms was even tougher than the native dragline spider silk fiber. We believe that there

are several reasons for this. One is the use of the 2.4-kbp $A2S8_{14}$ synthetic spider silk sequence encoding repetitive flagelliform-like $(GPGGA)_8$ elastic and major ampullate spidroin-2 (linker-$alanine_8$) crystalline motifs. Preliminary data had shown that this relatively large synthetic spider silk protein could be spun into fibers by extrusion after being produced in *E. coli*, indicating that it had the inherent ability to assemble into fibers. However, we also recognized that this protein would be expressed in concert and would have to interact with the endogenous silkworm fhc, fibroin light chain, and fibrohexamerin proteins to be incorporated into silk fibers. Thus, we embedded the $A2S8_{14}$ spider silk sequence within N- and C-terminal fhc domains to direct the assembly process, as originally described by Kojima and coworkers (29). Together with the ability of the fhc promoter to drive their expression in spatial and temporal proximity to the endogenous silkworm silk proteins, we believe that these design features accounted for the ability of the chimeric silkworm/spider silk proteins to participate in the assembly of composite silk fibers and contribute significantly to their mechanical properties.

## Materials and Methods

***piggybac* Vector Constructions.** DNA fragments encoding key regulatory elements and/or protein sequences were produced by PCR with genomic DNA isolated from the silk glands of *B. mori* strain P50/Daizo and the gene-specific primers shown in Table S3. These included fragments encoding the fhc major promoter and upstream enhancer element, two versions of the fhc basal promoter and N-terminal domain (exon 1/intron 1/exon 2) with different 5′ and 3′ restriction sites, the fhc C-terminal domain [3′ coding sequence and poly(A) signal], and EGFP. Each amplification product was gel-purified, recovered, and cloned into plasmid vectors, and bacterial transformants containing error-free inserts were identified. The regulatory elements and protein coding sequences were then assembled to create functional cassettes in two intermediate plasmids, as shown in Fig. 1 *B* and *C*. Finally, these cassettes were excised and subcloned into pBAC[3XP3-DsRedaf] (38) to produce the two *piggyBac* vectors (Fig. 1*D*) used for ex vivo silk-gland bombardment assays and silkworm transgenesis in this study. These *piggyBac* vectors were designated spider 6 and spider 6-EGFP to denote the absence or presence of the EGFP marker. A more detailed description of the molecular cloning scheme used for their construction is given in *SI Materials and Methods*.

**Ex Vivo Silk-Gland Bombardment Assays.** *B. mori* strain *pnd-w1* silkworms entering the third day of fifth instar were sterilized with 70% ethanol and placed in 0.7% wt/vol NaCl. The entire silk glands were then aseptically dissected and held in petri dishes containing Grace's medium plus antibiotics before DNA bombardment. In parallel, 3-mg aliquots of tungsten microparticles (1.7-μm M-25 microcarriers; Bio-Rad Laboratories) that had been pretreated according to the manufacturer's instructions and held in 3 mg/50 μL aliquots in 50% glycerol at −20 °C were coated with 5 μg of the relevant *piggyBac* DNA in a maximum volume of 5 μL, according to the manufacturer's instructions, with aliquots coated with distilled water as DNA-negative controls. Each bombardment experiment included six replicates and each individual bombardment included one pair of intact silk glands. The glands were transferred from the Grace's medium onto 90-mm petri dishes containing 1% wt/vol sterile agar, and the petri dishes were placed in the Bio-Rad Biolistic PDS-1000/He Particle Delivery System chamber. The chamber was evacuated to 20–22 in Hg, and silk glands were bombarded with precoated tungsten microparticles using 1,100 psi helium at a distance of 6 cm from particle source to target tissue, as described previously (39). After bombardment, the silk glands were placed in fresh petri plates containing Grace's medium with 2× antibiotics and incubated at 28 °C. Transient expression of the EGFP marker in the spider 6-GFP *piggyBac* vector was assessed by fluorescence microscopy at 48 and 72 h post bombardment. Images were taken with an Olympus FSX100 microscope at a magnification of 4.2×, a phase of 1/120 s, and green fluorescence of 1/110 s (capture). Transient expression of the EGFP-tagged and untagged chimeric silkworm/spider silk proteins also was assessed by immunoblotting bombarded silk-gland extracts with EGFP- or spider silk-specific antisera, as described below.

**Silkworm Transformation.** Eggs were collected 1 h after being laid by pnd-w1 moths and arranged on a microscope slide. Preblastoderm embryos were then injected with 1–5 nL of vector and helper plasmid DNA mixtures dissolved in injection buffer (0.1 mM sodium phosphate, 5 mM KCl, pH 6.8) at a final concentration of 0.2 μg/uL, each using a system consisting of a World Precision Instruments PV820 pressure regulator, a Suruga Seiki M331 micromanipulator, and a Narishige HD-21 double-pipette holder. The punctured eggs were sealed with Helping Hand Super Glue gel (The Faucet Queens) and then incubated in a growth chamber at 25 °C with 70% humidity. After hatching, the larvae were reared on an artificial diet (Nihon Nosan Company), and subsequent generations were obtained by mating siblings within the same line. Transgenic progeny were tentatively identified by the presence of the DsRed fluorescent eye marker using an Olympus SXZ12 microscope with filters between 550 and 700 nm.

**Sequential Extraction of Silkworm Cocoon Proteins.** Parental and transgenic silkworm cocoons were harvested, and the sericin layer was removed by gentle stirring in 0.05% (wt/vol) $Na_2CO_3$ for 15 min at 85 °C with a material: solvent ratio of 1:50 (wt/vol) (40). The degummed silk was removed and washed twice with hot (50–60 °C) water with careful stirring and the same material:solvent ratio. The degummed silk fibers were then lyophilized and weighed to estimate the efficiency of sericin-layer removal. The degummed fibers were used for a sequential protein extraction protocol, with rotation on a mixing wheel to ensure constant agitation, as follows. Thirty milligrams of the degummed silk fibers from cocoons produced by two individuals in each line were treated with 1 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2PO_4$, 1.8 mM $KH_2PO_4$) for 16 h at 4 °C. The material was separated into insoluble and soluble fractions by centrifugation, the supernatant was removed and held at −20 °C as the PBS-soluble fraction, and the pellet was subjected to the next extraction. This pellet was resuspended in 1 mL of 2% (wt/vol) SDS and incubated for 16 h at room temperature. Again, the material was separated into insoluble and soluble fractions by centrifugation, the supernatant was removed and held at −20 °C as the SDS-soluble fraction, and the pellet was subjected to the next extraction. This pellet was resuspended in 1 mL of 9 M LiSCN containing 2% (vol/vol) β-mercaptoethanol (BME) and incubated for 16–48 h at room temperature. After centrifugation, the supernatant was held at −20 °C as the 9 M LiSCN/BME-soluble fraction. The final pellet obtained at this step was resuspended in 1 mL of 16 M LiSCN containing 5% (vol/vol) BME and incubated for about 1 h at room temperature. This resulted in complete dissolution and produced the final extract, which was held as the 16 M LiSCN/BME-soluble fraction at −20 °C until the immunoblotting assays were performed.

**Analysis of Silk Proteins.** Silk glands were homogenized on ice in sodium phosphate buffer (30 mM $Na_2PO_4$, pH 7.4) containing 1% (wt/vol) SDS and 5 M urea and then clarified for 5 min at 13,500 × *g* in a microcentrifuge at 4 °C. The supernatants were harvested as silk gland extracts, and these extracts, as well as the sequential cocoon extracts described above, were diluted 4× with 10 mM Tris·HCl/2% SDS/5% BME buffer, and samples containing ∼90 μg of total protein were mixed 1:1 with SDS/PAGE loading buffer, boiled at 95 °C for 5 min, and loaded onto 4–20% gradient gels (Pierce Protein Products). After separation, proteins were transferred from the gels to PVDF membranes (Immobilon, Millipore) using a Bio-Rad transfer cell, according to the manufacturers' instructions. Primary antibodies for immunodetection were a spider silk protein-specific polyclonal rabbit antiserum produced against the *N. clavipes* flagelliform silk-like A2 peptide (GenScript) or a commercial EGFP-specific mouse monoclonal antibody (Living Colors GFP, Clontech Laboratories). Secondary antibodies were goat anti-rabbit IgG-HRP (Promega) or goat anti-mouse IgG H + L HRP conjugate (EMD Chemicals), respectively. All antibodies were used at 1:10,000 in blocking buffer (1× phosphate-buffered saline containing 0.05% Tween-20 and 0.05% nonfat dry milk), and antibody–antigen reactions were visualized by chemiluminescence with a commercial kit (ECL Western Blotting Detection Reagents; GE Healthcare). Reactions were quantified using a Bio-Rad GelDoc EZ Imager (Bio-Rad Laboratories), with known amounts of the chimeric silkworm/$A2S8_{14}$ spider silk protein from *E. coli* as standards.

**Mechanical Testing of Silk Fibers.** The degummed silkworm and spider silk fibers used for mechanical testing had initial lengths ($L_0$) of 19 mm. The average cross-sectional diameters were measured across two brins comprising the degummed silkworm silk and dragline spider silk fibers. Thus, fiber diameters and cross-sectional values were consistently overestimated, whereas strength (stress) and toughness were underestimated. Single-fiber testing was performed at ambient conditions (20–22 °C and 19–22% humidity) using an MTS Synergie 100 system (MTS Systems) mounted with both a standard 50-N cell and a custom-made 10-g load cell (Transducer Techniques). The mechanical data (load and elongation) were recorded from both load cells with TestWorks 4.05 software (MTS Systems) at a strain rate of 5 mm/min and frequency of 250 MHz, which allowed for the calculation of stress and strain values.

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