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Colored Fluorescent Silk Made by Transgenic Silkworms

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Silk is a protein fiber used to weave fabrics and as a biomaterial in medical applications. Recently, genetically modified silks have been produced from transgenic silkworms. In the present study, transgenic silkworms for the mass production of three colors of fluorescent silks, (green, red, and orange) are generated using a vector originating from the fibroin H chain gene and a classical breeding method. The suitability of the recombinant silks for making fabrics is investigated by harvesting large amounts of the cocoons, obtained from rearing over 20 thousand silkworms. The application of low temperature and a weakly alkaline solution for cooking and reeling enables the production of silk fiber without loss of color. The maximum strain tolerated and Young's modulus of the fluorescent silks are similar to those of ordinary silk, although the maximum stress value of the recombinant silk is slightly lower than that of the control. Fabrics with fluorescent color are demonstrated using the recombinant silk, with the color persisting for over two years. The results indicate that large amounts of genetically modified silk can be made by transgenic silkworms, and the silk is applicable as functional silk fiber for making fabrics and for use in medical applications.

1. Introduction

Silk made by the domesticated silkworm *Bombyx mori* (*B. mori*) has been used for more than 5000 years for making fabrics. Silk fiber is also useful as a biomaterial in medical applications. For example, silk is used for long-term surgical sutures,[1] and an attempt to develop vascular grafts has been reported.[2] Furthermore, applications of silk within the fields of tissue engineering, wound dressing, implanted devices, and drug delivery have been reported.[3] As silk technologies advanced, processing technologies were developed extensively. However, there was no method for changing the silk protein itself, until technology was developed to produce a transgenic silkworm.[4] Genetically modified silkworms made by this method can carry and express the transgene in successive generations. Therefore, the number of these transgenic silkworms can easily be increased and maintained, once the silkworm strain is established. Additionally, the transgene inserted into the silkworm genome can be chosen to modify the silk protein for desirable characteristics.

The vector for the modification of silk targets the silk-expressing genes. The cocoon silk consists of sericin and fibroin proteins. Sericin is a glue-like protein covering the surface of the core silk fiber and is removed by the process of degumming. To date, three sericin genes, sericin1, 2, and 3, have been reported.[5–7] Fibroin is the protein that

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constitutes the core silk fiber and consists of three subunits, fibroin H chain, fibroin L chain, and fibrohexamerin.[8] Fibroin H chain, whose molecular weight is 350 to 400 kDa, consists of mainly repeats of $(Gly\text{-}Ser\text{-}Gly\text{-}Ala\text{-}Gly\text{-}Ala)_n$, and is the major protein determining the character of the silk fiber.[9,10] Fibroin L chain, whose molecular weight is around 25 kDa, forms hetero dimmers with the H chain via S–S bonds, and contributes to the secretion of fibroin from the posterior silk gland cell to the lumen.[11] Fibrohexamerin is also a small protein with a molecular weight of 25 kDa and contributes to the formation of the secreted hetero dimer complexes.[12] The many different types of transformation vectors for the modification of silk are constructed using these silk genes.[13,14] Among them, the vectors using the fibroin L chain gene and the fibroin H chain gene are useful for the production of modified silks. Many different functional silks are generated using the vector and have been shown to possess useful properties for fabrics and medicines. For example, the chimeric silkworm/spider recombinant silk made by the transgenic silkworm is tougher than ordinary silk fibers and as tough as native dragline.[15] Silk having a fusion protein of fibroin L chain and human fibroblast growth factor is reported to be biologically active and useful as a new biomaterial for tissue engineering.[16] The film made by silk containing fusion proteins of the fibroin L chain and a peptide of a partial collagen or fibronectin sequence was shown to have higher cell adhesion activity compared to unmodified silk.[17] To expand the utility of transgenic silkworm silks, it is important to establish a production method that yields large amounts of modified silk at low cost.

This article focuses on the production of fluorescent, colored silks because such silks have many uses as functional fibers, especially in the manufacture of textile and in medical applications.[18–20] The transgenic silkworms, adapted for mass production of recombinant silks having green, red, and orange fluorescent colors, can be created using the fibroin H chain gene vector and ordinary breeding methods. A large amount of silkworms, more than 20 000, were reared and the cocoons harvested. Using the cocoons, cooking and reeling methods were established for the production of recombinant silks without loss of the fluorescent color. The mechanical properties of the modified silk were similar to those of commercially available silks; modified silks can be used for making fabrics without loss of their fluorescent colors. We have established a method for producing fluorescent silks in large quantities using transgenic silkworms.

2. Results and Discussion

2.1. Construction of Transgenic Silkworms for Fluorescent, Colored Silk Production

The physical structure of the vector for making fluorescent colors is shown in **Figure 1**a. The fluorescent color protein, fused with N-terminal and C-terminal domains of the silkworm fibroin H chain, is expressed in the silkworm. For the production of green fluorescent silk, the silkworm strain reported previously was used as the original strain.[21] For the production of red and orange fluorescent silks, two transgenic silkworm strains were generated by injecting the vector DNA shown in Figure 1a with the helper plasmid into the preblastodermal eggs of the non-diapausing and white-eye strain w1-pnd. The use of non-diapausing strains as a host for making the transgenic silkworm is necessary, because the DNA must be injected immediately after egg-laying and the injected eggs must develop without diapausing.[4] However, the yield and robustness of the silks are poor in non-diapausing silkworm strains; therefore, they are not well suited to the production of large amounts of silk that can be used for creating textiles or biomaterials. The three original fluorescent, colored strains were bred

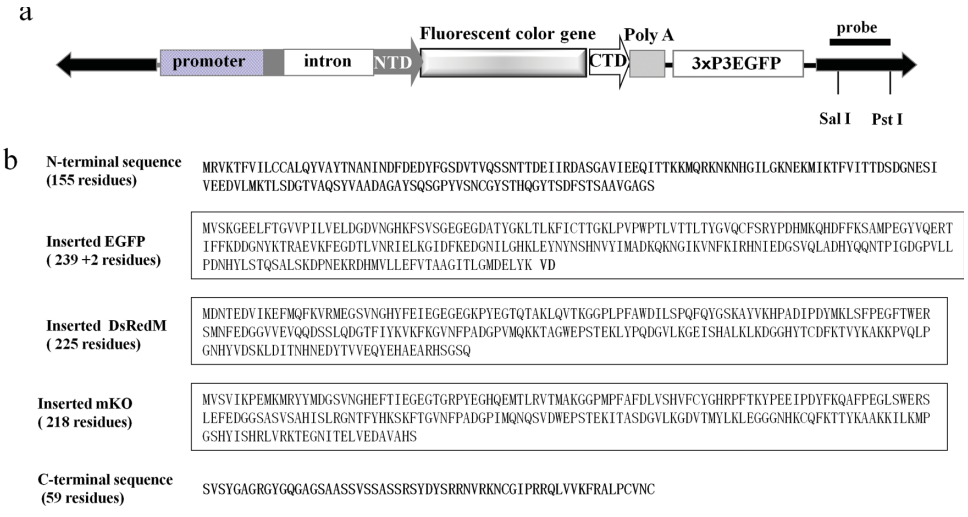

Figure 1. a) Vector structure for making green (EGFP), red (DsRedMonomer), and orange (mKO) colored fluorescent silk, and b) the amino acid sequence of fluorescent proteins fused with fibroin H chain N- and C-terminal regions. Arrows indicate right and left arms of the transposon *piggyBac*. promoter, the promoter region of *B. mori* fibroin H chain gene; NTD, N-terminal domain; CTD, C-terminal domain; 3 × P3GFP, marker gene for screening the transgenic silkworm. Recognition sites of SalI and PstI and the region used for the probe in Southern blotting (black bar) are also shown.

to increase their productivity; the Japanese and Chinese parent strains from the original strains were bred by repeated crossing with commercial parent strains, as described in the Experimental Section. Then, three hybrids, GFPGunma × GFP200 (EGFP), JSS1 × CSS1 (DsRed), and JSS2 × CSS2 (mKO), that spin the three different colored fluorescent silks, were made by mating the Japanese and Chinese parent strain of each color. Silk productivity and robustness of the hybrid silkworms were much higher than those of the original strain (Table S1, Supporting Information), and the constructed hybrids possessed one or two transgenes (Figure S1, Supporting Information) in their genomes.

2.2. Expression of Recombinant Silk Protein in the Transgenic Silkworm

The expression of enhanced green fluorescent protein (EGFP), DsRed monomer fluorescent protein (DsRed-M), and monomeric Kusabira orange (mKO) in the silk gland during larval development is shown in **Figure 2**. The fluorescence observed in each hybrid appeared only in the silk gland; the strong fluorescence is only observed in the middle and posterior parts of the silk gland on the final day of the 5th instar. It is known that the fibroin H chain, produced in the posterior region of silk gland, moves to the middle part of the silk gland and accumulates there. The observation indicates that each fluorescent protein is produced in the posterior region and accumulates in the middle region of the silk gland. The silk gland in the transgenic silkworms showed the green, red, or orange color, even when viewed under visible light (Figure 2). Furthermore, the fluorescent protein is equivalently expressed in the silk gland, suggesting that the silk contains the fluorescent protein homogeneously. The fibroin H chain mRNA occupied about 3.5% of the total RNA in the posterior region of the silk gland on the final day of the 5th instar.[22] The expression level of the transgene was 5 to 11% of the transcript when compared to that of the endogenous fibroin H chain in copy numbers (Figure S2, Supporting Information). Because the size of the fluorescent proteins with the fibroin H chain N- and C-terminal regions are about 1/6, the amount of mRNA is 0.8 to 2% of the fibroin H chain transcript. The production of fluorescent silk proteins, corresponding to the amount of the mRNA, was confirmed by SDS-PAGE and Western blot analysis of the antibodies of GFP, DsRed, and mKO (**Figure 3**). A protein with a molecular weight of 65 kDa appeared in the green fluorescent silk, producing race GFPGunma × GFP200, and reacted with the GFP antibody. A protein with a molecular weight of 70 kDa was detected in CSS1 × JSS1 and reacted with the DsRed antibody. A 60-kDa band was seen in CSS2 × JSS2 and reacted with the mKO antibody. The molecular weight of each protein was a slightly larger than the theoretical size calculated from the DNA sequence of the construct. This may be due to the abnormal mobility of the recombinant fibroin in SDS-polyaclylamide gel electrophoresis(SDS-PAGE) that has also been reported in other experiments.[15,21]

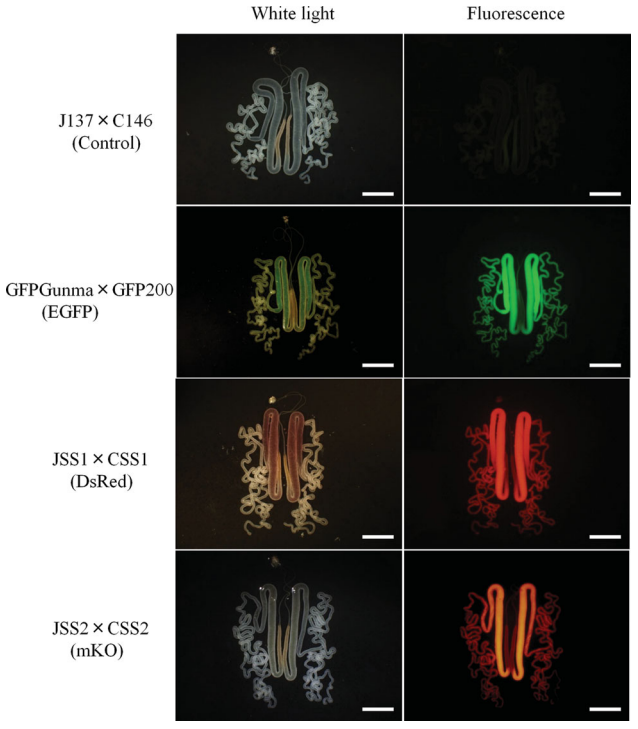

Figure 2. Silk glands of the transgenic silkworm with the fluorescent protein gene at the stage just prior to spinning. The silk gland of each of the hybrids is observed under white light and the green (EGFP), red (DsRed), and orange (mKO) fluorescence filter systems. The white scale bar is 1 cm.

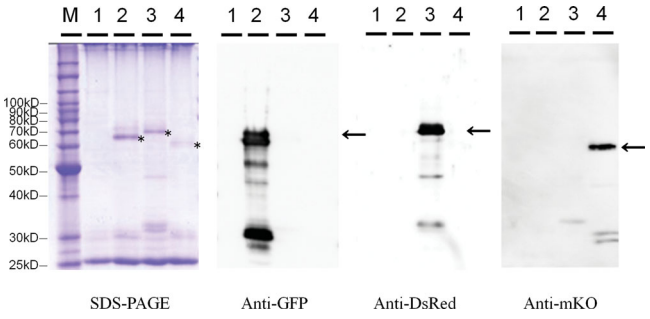

Figure 3. SDS-PAGE and Western blotting of the recombinant silk proteins produced in the transgenic silkworm with the EGFP, DsRed, and mKO genes. The fibroin proteins are separated by SDS-PAGE after being dissolved in 60% LiSCN solution and subjected to Western blotting with anti-GFP, DsRed, and mKO antibodies. 1, J137 × C146, (control); 2, GFPGunma × GFP200 (EGFP); 3, CSS1 × JSS1, (DsRed); 4, JSS2 × CSS2, (mKO). In SDS-PAGE, the bar indicates the fluorescent protein fused with fibroin H chain N- and C-terminal regions.

2.3. Mass Rearing of Transgenic Silkworms

To obtain large amounts of cocoons, more than 20 000 larvae of the three hybrid silkworms were reared on mulberry leaves, and more than 15 kg of cocoons were harvested. Because the three silkworm races producing the three different fluorescent colors are hybrids, their characteristics are almost the same as those of the original silkworms used on farms, and they can be reared on mulberry leaves. The harvested cocoons possessed strong

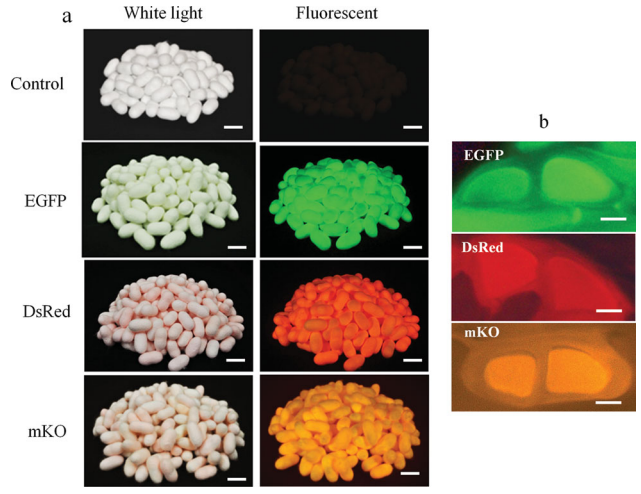

Figure 4. a) Colored fluorescent cocoons and b) cross section of the cocoon silk produced by the hybrid races. The cocoons are exposed with a blue LED, and their pictures are taken through a yellow filter. The pictures of the cross section are taken with a microscope equipped with a fluorescent filter system. EGFP, DsRed, and mKO represent the hybrids of GFPGunma × GFP200, JSS1 × CSS1, and JSS2 × CSS2, respectively. The white scale bars in the cocoon and in the cross section indicate 2 cm and 5 μm, respectively.

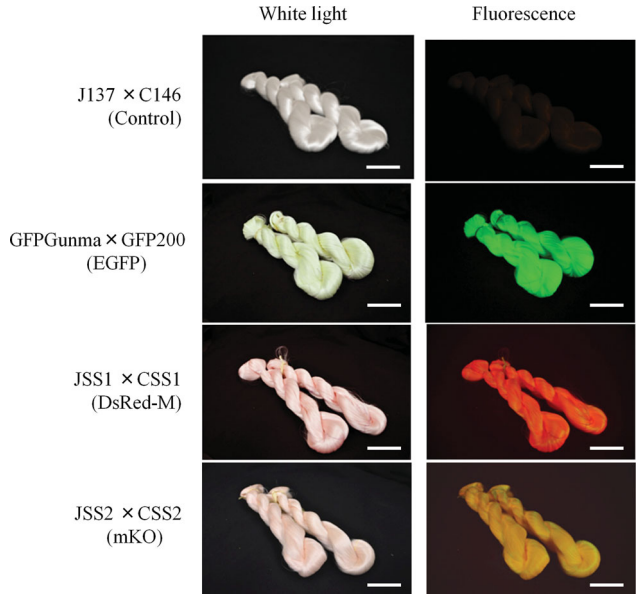

Figure 5. Fluorescent silks produced by the hybrid races. The pictures are taken through a yellow filter under a blue LED. The white scale bar indicates 5 cm.

fluorescent color, as shown in **Figure 4**a. When we observed the cross section of the cocoon silks, fluorescence was present throughout the fibroin but not in the sericin (Figure 4b), indicating that the protein was distributed equally in the fibroin core fiber region and was not secreted in the sericin layer. In fact, enzymatic degumming did not affect the color (Figure S3, Supporting Information). In a similar experiment using the fibrohexamerin gene and DsRed, it was shown that the recombinant protein was distributed in the surface of the silk.[23] In the present study, the colored fluorescent protein was designed to have N-terminal and C-terminal peptide sequences of fibroin H chain protein. Thus, the fluorescent protein was bound with the fibroin L chain by a disulfide bond, assembled to a mass elementary unit,[12] secreted into the lumen of the posterior silk gland. The fluorescent protein involved in the fibroin elementary unit was incorporated into the fibroin core region and is thought to be strongly bound with the endogenous fibroin protein by hydrogen bonding during the process of fibrillization. Therefore, the silk fiber did not lose the colored fluorescent protein during the processes of reeling and degumming.

2.4. Cooking and Reeling

The fluorescent proteins are generally denatured by heat treatment and thus lose their color. The normal reeling method includes processing by drying and cooking the cocoons near 100 °C before reeling. Therefore, the usual processing method cannot be used for making colored fluorescent silks. To develop a method that is applicable to producing transgenic silk without color loss, the cocoon drying conditions were first examined. Three different colored fluorescent cocoons were exposed to different temperatures (Figure S4, Supporting Information), and it was found that the cocoons lost their color when incubated at greater than 60 °C, although the thermal tolerance differed slightly for each fluorescent protein. Specifically, the disappearance of the fluorescent color of EGFP, DsRed, and mKO occurred at temperatures higher than 70 °C for EGFP and 80 °C for DsRed and mKO. For cocoons oven dried at 60 °C for 24 h (Figure S5, Supporting Information), no color loss occurred during the process (Figure S6, Supporting Information). The shape of the silkworm cocoon is fixed by partial attachments of the cocoon silks with sericin. In order to reel the silk, the sericin at the attachment sites must be partially dissolved, softening the cocoons. Generally, repeated treatments of boiling and cooling allow complete penetration of water, partially dissolving the sericin and softening the cocoons. However, this technique cannot be applied to the colored fluorescent silk without color loss. Therefore, a new cooking method to soften the fluorescent cocoons (Figure S7, Supporting Information) was developed: the cocoons were soaked in a solution of 0.1–0.4% sodium carbonate/0.2–0.4% nonionic surfactant at 60 °C and then placed under vacuum. The repeated vacuum treatments enabled complete penetration of the solution into the cocoons, and the cocoons were thus homogenously softened and ready for reeling. In this state, the cooked cocoons can be reeled by an automated reeling machine. The results of reeling are shown in Figure 5 and Table 1. The reelabilities of green and orange cocoons were lower than those of the controls of the w1 strain and C146 × J137. The reelability of the red cocoons was a slightly higher than those of the control cocoons. The length of the cocoon filament, raw silk percentage, and weight of cocoon filament of the three fluorescent cocoons were enhanced compared to the original strain, and the results for the red cocoon were similar to those for the commercial race C146 × J137. The three fluorescent cocoon filaments were

Table 1 Reeling test of the three colored fluorescent cocoons.

Race	Reelability Percentage [%]	Length of Cocoon Filament [m]	Raw Silk Percentage of Cocoon [%]	Weight of Cocoon Filament [g]	Size of Cocoon Filament [d]
w1 (Control)	77	474	10.05	0.089	1.72
GFPGunma × GFP200 (EGFP)	52	878	16.18	0.274	2.81
CSS1 × JSS1 (DsRed)	81	1003	20.49	0.282	2.53
CSS2 × JSS2 (mKO)	49	738	12.30	0.216	2.64
C146 × J137(Control)	77	1119	18.96	0.273	2.22

The colored fluorescent cocoons for the reeling test are harvested from the silkworms reared in the late autumn season on fresh mulberry leaves. Reeling is performed by a multi-ends reeling machine. The fiber size of the reeled silk was 27 denier and the reeling speed is 200 m min^{-1}. The w1 and C146 × J137 strains are used as controls to represent original and commercial races, respectively.

thicker than those of w1 and C146 × J137. Comparing the colors of the cocoons and silk, the lightness value of the fluorescent silks were slightly higher than those of the cocoons, indicating that a slight color change occurred during the cooking and reeling processes (**Table 2**).

2.5. Characterization of Colored Fluorescent Silks and their Applicability to Fabric Production

To evaluate the colored fluorescent silks as materials for making fabrics, stress tests were performed. The stress–strain curve of the colored fluorescent silks shows that the fluorescent silks were slightly weaker than the control silks (**Figure 6**). The maximum stress results for the green and orange silks were comparable of those for the original strain w1 but about 20% lower compared to the commercial race C146 × J137 (**Table 3**). The red fluorescent silk was significantly stronger than the w1 but about 10% weaker than the silk from the commercial race; the reasons for these differences are not clear. There is an possibility of that the production of fluorescent protein in the silk gland caused the difference; the presence of fluorescent protein fused with fibroin H chain N- and C-terminal regions may disturb the crystallization of the silk. However, the

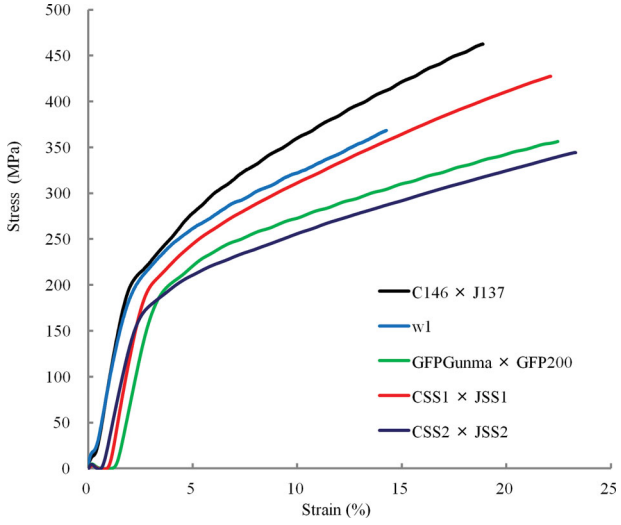

Figure 6. Stress–strain curve of the colored fluorescent silk.

Table 2. L*, a*, and b* values of the fluorescent cocoon and raw silk in Lab color space.

Strain		L* ± SD	a* ± SD	b* ±SD
J137×C146	cocoon	89.3 ± 1.0	−1.6 ± 0.6	6.0 ± 1.2
	raw silk	84.8 ± 0.8	0.5 ± 1.6	6.0 ± 1.0
GFPGunmaxGFP200 (EGFP)	cocoon	83.4 ± 3.3	−5.6 ± 1.8	19.4 ± 2.7
	raw silk	90.8 ± 1.2	−4.0 ± 1.3	16.6 ± 2.1
CSS1×JSS1(DsRed)	cocoon	79.3 ± 3.9	14.6 ± 2.5	4.9 ± 0.4
	raw silk	83.2 ± 0.9	13.5 ± 2.3	6.3 ± 2.1
JSS2×CSS2(mKO)	cocoon	82.2 ± 1.1	20.3 ± 2.8	5.7 ± 0.8
	raw Silk	84.1 ± 1.2	21.3 ± 1.0	4.2 ±1.0

The value is measured by a Color Reader CR-13 (Konica Minolta Optics, Tokyo, Japan), and the standard deviation (SD) is obtained from the data of 10 samples in each cocoon and silk color.

Table 3. Tensile strength and Young's modulus of three different colored fluorescent silks.

Strain or races	N	Maximum stress ± SD [MPa]	Maximum strain ± SD [%]	Young's modulus ± SD [GPa]
W1 (Control)	50	368.61 ± 41.88	14.03 ± 3.05	9.22 ± 0.96
GFP Gunma × GFP200	50	356.98 ± 35.51	21.34 ± 2.74	12.32 ± 1.31
CSS1 × JSS1 (DsRed)	50	419.97 ±20.04	21.02 ± 1.49	13.61 ± 0.80
CSS2 × JSS2 (mKO)	50	362.69 ± 27.63	23.13 ± 1.71	12.68 ± 1.28
C146 × J137 (Control)	50	461.85 ± 38.18	18.93 ± 1.94	10.38 ± 1.21

For the measurement of tensile strength, strain, and Young's modulus of raw silks, each silkworm is reared in the late autumn rearing season on fresh mulberry leaves. A thread of 100 assembled raw silk fibers is used for the test. The length of the sample is 100 mm, and the speed of elongation is 10 mm min^{-1}. The test is repeated 50 times, and the average and standard error are shown in the table.

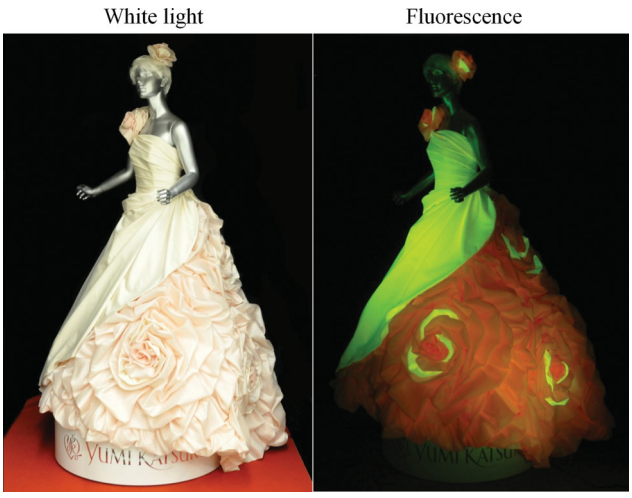

Figure 7. Wedding dress produced by the colored fluorescent silks. The pictures are taken under the same conditions described in Figure 5.

effect was small; thus, the produced raw silk having color and fluorescence possessed sufficient strength to make fabrics and biomaterials for medical applications. In fact, it is possible to weave various fabrics and textiles using the fluorescent silks; knitted dresses and suits were manufactured without problems. For example, wedding dresses were made using the textiles woven from the silks, as shown in **Figure 7**. The fabrics made from the silk retained fluorescence, even after one year, demonstrating that it is possible to produce new fabrics using the colored fluorescent silks. Because many different fluorescent and pigment color proteins are available,[24] many different fluorescent and colored silks can be made by the method developed in our work, and the silks are useful for making fabrics and materials for medical applications.

3. Conclusions

Recombinant silks with three different fluorescent colors, green, red and orange, can be produced in large amounts using transgenic silkworms. The transgenic strains can be stably maintained without changes in their characteristics; the transgene inserted into the silkworm genome can be transferred to successive generations, and the expression of the color protein in the progenies does not change during the breeding of silkworm strains. Large numbers of the transgenic silkworms can be reared by the same system used in the rearing of the commercial race in farms, and enough cocoons can be harvested to produce silks for making fabrics. Drying and cooking of the cocoons at low temperature enables the subsequent reeling of the colored fluorescent silks by an automatic reeling machine without color loss and can produce silks that can be used for making fabrics and biomaterials for medical applications. The silks produced are marginally weaker than ordinary silk but still have sufficient strength for use in fabrics. The method reported here can be applied to the construction of recombinant silks containing other fluorescent and pigment color proteins, and the cooking and reeling method is useful for making functional silks possessing protein sequences that confer unique biological activity in the produced biomaterials.

4. Experimental Section

Silkworm Strains: A non-diapausing strain, w1-pnd, with the characteristics of white eggs and eyes, was used to generate the transgenic silkworm. The diapausing strain w1 was mated with the transgenic silkworm to maintain it as a diapausing strain. To breed a silkworm race with green fluorescent colored silk, KH25[21] was used as the original strain. "Gunma" and "200" were used as Japanese and Chinese parent strains, respectively, having high silk productivity. For the breeding of red-colored silkworm races, the Japanese parent race "TN38" and Chinese parent race "TCS40" were used. For the breeding of the orange-colored silkworm race, the Japanese silkworm races "TN38", J137, and J603 were used. For the Chinese race, "TCS40", C146, and C604 were used. "Gunma" and "200" were maintained at the Gunma Sericultural Technology Center. The strains w1-pnd, w1, TN38, and TCS40, were preserved at the Transgenic Silkworm Research Unit, National Institute of Agrobiological Sciences. The silkworm races J137, J603, C146, and C604 were obtained from the Genetic Resources Center, National Institute of Agrobiological Sciences. The silkworms were either reared on an artificial diet (Nosan, Yokohama, Japan) or on fresh mulberry leaves at 25 °C.

Vector Construction: To construct the vector for producing the silkworm with red-colored fluorescent silk, the DsRed-Monomer sequence was amplified by PCR using pDsRed-Monomer N1 Vector DNA (Clontech, CA, USA) as a template and a pair of primers, DsRedM-5BamHI and DsRedM-3SalI. The amplified fragment was inserted into the *Bam*HI and *Sal*I sites of plasmid pHC-EGFP[21] after treatment with the same restriction enzymes. The resultant plasmid was digested with *Asc*I and *Fse* I and inserted into the *Asc*I and *Fse* I sites of the plasmid pBac[3xP3-EGFPafm][25] to produce the vector plasmid pBac[HC-DsRed Monomer –3xP3EGFP] (Figure 1). The vector for the production of orange-colored silk, pBac[HC-mKO Monomer –3xP3EGFP] (Figure 1), was constructed by the same method except for the use of a pair of primers, KS57 and KS59, and the template DNA of plasmid pmKO1-S1 (Amalgaam, Tokyo, Japan) instead of the pDsRed-Monomer Vector.

Generation of Transgenic Silkworm: Transgenic silkworms were constructed using a method reported elsewhere.[4] Briefly, the vector and plasmid DNA were injected into about 600 eggs at the preblastodermal stage. The hatched larvae were raised, and the adults were mated to each other. The obtained G_1 eggs were observed on the 6th and 7th days after egg-laying with a fluorescent microscope equipped with a GFP filter. To produce the transgenic silkworm with red-colored fluorescent silk, we injected the vector DNA solution of pBac[HC-DsRed Monomer –3xP3EGFP] and a helper plasmid DNA at a concentration of 0.2 mg mL^{-1} of each DNA into 661 eggs and obtained 130 G1 broods. Two of the 131 broods gave the transgenic silkworms. The silkworms having strong fluorescent color were mated with the diapausing w1 strain and confirmed as the transgenic line SS1. For the orange-colored silk, we injected pBac[HC-mKO monomer –3xP3EGFP] and helper plasmid into 667 eggs and obtained 144 G_1 broods. Many transgenic silkworms were obtained from the 32 G_1 broods. We selected the cocoons with strong fluorescent color from the two broods, mated the adults with the diapausing w1 strain, and confirmed two strains, SS2-4 and SS2-8, as the orange-colored fluorescent strain SS2.

Breeding of Japanese and Chinese Parent Strains for the Production of Colored Fluorescent Silks: For the production of green-colored fluorescent silk, the Japanese and Chinese parent strains GFPGunma and GFP200 from the KH25 strain were constructed.[18] A Japanese green-colored fluorescent strain GFPGunma by two backcrosses with the Japanese parent race Gunma was bread, mating with each other and repeatedly selecting the cocoons showing the strong GFP color and higher silk productivity for three generations. Similarly, a Chinese green-colored florescent strain GFP200 was made by three backcrosses

with the Chinese parent race 200, mating to each other and performing repeated selections for five generations to increase the silk productivity and strength of fluorescent color of the cocoon. A hybrid between GFPGunma and GFP200 strains was then made. For red-colored fluorescent silk, the Japanese and Chinese parent strains JSS1 and CSS1 were constructed from the original strain SS1. JSS1 was produced by three backcrosses with the Japanese parent race TN38 to SS1, mating to each other and performing repeated selections of the cocoons with the same criteria for three generations. Two more backcrosses with TN38 were performed, mating to each other and selecting for the desired characteristics. CSS1 was also bread by the same procedure used for JSS1, except using TN38. For CSS1, the Chinese parent strain TCS40 for TN38 was used. The final constructed hybrid was CSS1 × JSS1. For the orange-colored fluorescent silk, the Japanese and Chinese parent races, JSS2 and CSS2, were constructed. JSS2 was produced by two repeated backcrosses with TN38 to SS2-4, mating to each other and selecting the cocoons according to the above criteria. The backcross with the Japanese parent strain J137 was repeated, mating to each other and selecting the desired properties. The backcross with J137 two more times by crossing with J603 was repeated, mating to each other and selecting desired traits. For the construction of CSS2, the cross between SS2-8 and TCS40 was performed, mating to each other and selecting three generations to increase the productivity and strength of the orange color. They were crossed with the Chinese parent strain C146, mating to each other and selecting the cocoons. Two more backcrosses with C146 crossed with C604 were performed, mating to each other and selecting the cocoons.

Mass-Rearing: For the production of large amounts of colored fluorescent silks, mass rearing of the transgenic silkworms was performed. To ease the rearing and increase the size of each cocoon, the hybrids of each color strain were used. The F1 hybrids GFPGunma × GFP200, CSS1 × JSS1, and CSS2 × JSS2 were used for the production of green, red, and orange fluorescent cocoons, respectively. The silkworms were reared on artificial diets (Nosan, Yokohama, Japan) until the end of the 3rd instar and raised on fresh mulberry leaves during the 4th and 5th instars. The rearing was performed in the rearing room within the facility of P1A.

SDS-PAGE and Western Blotting: The silk protein was purified from the PSGs on the 4th or 5th day of the 5th instar larvae. The PSG dissected from the larvae were immersed in 30% ethanol overnight. It was then fixed with ethanol and manually separated from the silk gland cells. The separated silk protein was dehydrated with 100% ethanol and dried under air. The dried silk protein was dissolved in 60% LiSCN solution and dialyzed by 20 mM Tris-HCL (pH7.5)/5 M Urea. The dialyzed protein was analyzed by 10% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R250 or transferred to polyvinylidene fluoride (PVDF) membranes (Hybond-P; GE Healthcare, Buckinghamshire, England). The membranes were treated with anti-EGFP antibody (Abcam, Cambridge, UK), anti-DsRed antibody (Abcam, Cambridge, UK), anti-mKO antibody (Amalgaam, Tokyo, Japan), and fibroin L chain antibody (Inoue et al., 2005). The immunoreactive bands were visualized using ECL Plus (GE Healthcare, Bukinghamshire, UK) and an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan).

Cooking and Silk Reeling from the Colored Fluorescent Cocoons and Fabric Manufacture: Because the fluorescent color is removed by high-temperature treatment, the harvested cocoons were dried at 60 °C for 24 h and stored at room temperature until reeling. Cocoons were placed in a vacuum pan containing a cooking solution of 0.1–0.4% sodium carbonate and 0.2–0.4% nonionic detergent and treated with vacuum repeatedly to allow the solution to penetrate into the cocoons. The temperature was then increased to 60 °C. After cooking the cocoons, 27-denier raw silks were reeled using an FR-type automatic reeling machine (NISSAN, Tokyo, Japan) at a rate of 100 m min^{-1}. A water temperature of 50 °C was used for reeling and brushing. Re-reeling was performed using an NB-type re-reeling machine (SHINMASUZAWAKOGYO, Okaya, Japan) at a temperature of 40 °C and a rotary speed of 200 m min^{-1}. Textiles for dress making were woven by a weaving machine (TSUDAKOMA, Kanazawa, Japan).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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