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Development of an Injection Method for the Genetic Engineering of Diapause Silkworm Egg Using Dimethyl Sulfoxide

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Abstract

Genetic engineering of the domesticated silkworm *Bombyx mori* is a useful tool for developing sericulture. New races that produce recombinant silk with fluorescent color and spider silk protein have been constructed. Strains resistant to *B. mori* nuclear polyhedrosis virus have also been constructed and started to rear in the field. In this genetic engineering method, the use of nondiapause eggs that do not arrest embryonic development is indispensable as it includes the injection process of vector DNA into the eggs. However, most silkworm strains are bivoltine or univoltine and lay diapause eggs that arrest their embryonic development. Thus, applying this method to these silkworm strains is impossible. To enable the application, whether dimethyl sulfoxide (DMSO), which can prevent the diapause of eggs, can be used to develop an injection method for the genetic engineering of diapause strains was investigated. The DMSO treatment worked well for this purpose, and an injection method for the genetic engineering of diapause eggs was successfully developed. This method gave high hatchability in the injection of diapause eggs. Genetically modified silkworms were efficiently created in three different diapause silkworms: hybrid race and Japanese and Chinese strains. Thus, DMSO treatment is useful for creating genetically modified silkworms.

Discipline: Sericulture
Additional key words: transgenesis, Bombyx, injection, DMSO, bivoltine, univoltine

Introduction

The domesticated silkworm *Bombyx mori* is an economically important insect that has been used for sericulture in the silk industry for > 5,000 years. Sericulture was conducted in > 50 countries, and the total annual production of silk was ~90,000 tons in 2020. Many silkworm races that adapt to the local climate in each country have been used to increase the productivity of silk and avoid the damage of the disease. Genetic engineering of the silkworm was established in 2000 (Tamura et al. 2000) and used for the development of new innovative breeds, for example, silkworm races that produce silk with fluorescent colors or that spin the spider silk (Iizuka et al. 2013, Iizuka 2016, Shimizu 2018, Teule et al. 2012, Kuwana et al. 2014). The creation of strain resistant to *B. mori* nuclear polyhedrosis virus is another example (Isobe et al. 2004, Subbaiah et al. 2013, Dong et al. 2020). Some resistant strains have been reared in the field to produce commercial silk. Thus, the genetic engineering method is useful for breeding new silkworm strains for sericulture. However, the method highly depends on the injection of vectors into nondiapause eggs that do not arrest their embryonic development (Tamura et al. 2000, 2007). Therefore, it has been mainly applied to silkworm strains that lay nondiapause eggs. In contrast, commercial silkworm races reared by farmers are mostly bivoltine or univoltine that lay diapause eggs. Although commercial races possess higher productivity and quality of silk than nondiapause strains, genetic

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engineering cannot be directly applied to them. To create genetically modified silkworm races adapted to rearing by farmers, breeding of genetically engineered nondiapause silkworms has been performed through repeated backcrosses with commercial strains and selections (Iizuka et al. 2007, Iizuka 2016, Subbaiah et al. 2013). The process is time-consuming and requires labor-intensive works.

To solve this problem, the injection method for the genetic engineering that can be directly applied to diapause strains has been investigated (Inoue et al. 2005, Zhao et al. 2012, Shiomi et al. 2015). However, no reliable method has been established yet. Dimethyl sulfoxide (DMSO) is a cryoprotectant for cultured cells and living tissues and is used for the cryopreservation of ovaries, testes, sperm, and cultured cells in the silkworm (Takemura et al. 2008, Sahara & Takemura 2003). Because DMSO can prevent the diapause of silkworm eggs (Yamamoto et al. 2013) and is less toxic than hydrochloric acid (HCl), which is routinely used to prevent the diapause of eggs, it might be useful for develop an injection method for diapause silkworm strains.

This study evaluated the application of DMSO by constructing transgenic silkworms of diapause strains and found that it is practically applicable to the injection. This study also successfully created transgenic silkworms of commercial hybrid race and Japanese and Chinese strains, indicating that it can be applied for most other diapause strains.

Materials and methods

1. Silkworm strains

The diapause hybrid silkworm race Ariake, Japanese bivoltine strain J137, and Chinese bivoltine strain C146 were used for the experiments. These strains were obtained from the Institute of Agrobiological Sciences, National Agriculture and Food Research Organization. Eggs were incubated at 25°C under dark conditions. The silkworms were reared on an artificial diet (Nihon Nosan, Yokohama, Japan) at 25°C-28°C.

2. DMSO treatment

DMSO was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). To obtain eggs at the same developmental stage, female and male moths that emerged in the morning were mated and kept for 3-5 h at 25°C. The mated moths were kept for 1-3 days in the cold room at 5°C. To obtain synchronized eggs just after oviposition, female moths were separated from the males, transferred on the glued paper in a dark box, and kept for 2 h at 25°C. The eggs laid on the paper were washed with tap water and immersed in distilled water for 5 min. Then, the eggs were detached from the paper with fingers. The detached and floated eggs were collected using a stainless-steel mesh. The collected eggs were kept in a Petri dish with a 5-cm diameter, and the remaining water was thoroughly removed using a Pasteur pipette and filter paper. Then, 100% DMSO (15 mL) was added to the dish and kept in an air incubator at 25°C during treatment, and the eggs were collected using the mesh. The DMSO-treated eggs were washed with tap water for > 5 min, transferred to a Petri dish, and air-dried. The eggs for injection were transferred to a glass slide from the Petri dish immediately after being washed with tap water, fixed with instant glue, and dried. The eggs fixed on the glass slide were used for injection, as previously reported (Tamura et al. 2007, Takasu et al. 2014). To examine the effect of the DMSO treatment time on hatchability, the eggs were kept in a moisturized plastic box at 25°C for 14 days, and the number of hatched eggs was counted.

3. Injection of vector plasmids into DMSO-treated eggs

Vector plasmid DNA was injected using the previously reported method (Tamura et al. 2000, 2007; Takasu et al. 2007). Helper plasmid pHA3PIG (Tamura et al. 2000) and two vector plasmids pBac[3xP3-DsRedafm] and pBac[3xP3-EGFPafm] (Horn & Wimmer 2000) were purified using the Qiagen Plasmid Hispeed Midi Kit (Qiagen, Hilden, Germany) and used for the injection of DMSO-treated eggs. The helper and vector plasmids were dissolved in 0.5-mM phosphate buffer (pH 7.0) containing 5-mM KCl with a concentration of 200 µg/mL of each plasmid DNA and used for the injection of the eggs at the stage from 4 to 6 h after oviposition. The injected eggs were incubated at 25°C in a moisturized plastic box until hatching. The hatched larvae were transferred to a plastic box, including an artificial diet, and reared at 25°C-27°C until they reached the adult stage. The developed G_0 adults were mated with each other or their host strains for > 1 h, and the females were transferred onto an egg-laying paper, covered with a lid, and left overnight at 25°C to lay their eggs. The G_1 eggs obtained the next day were treated with 6 N HCl for 1 h at 25°C to terminate the diapause and washed with running tap water for > 30 min. The HCl-treated eggs were incubated in a moisturized box at 25°C and used to detect the fluorescence of DsRed and enhanced green fluorescent protein (EGFP). The fluorescence of the eggs at the stage just before hatching was observed under a microscope equipped with DsRed or GFP filter. The eggs with the fluorescence were transferred to a Petri dish.

The hatched larvae from the transferred eggs were reared and used for the establishment of transgenic lines.

4. Southern blotting

Genomic DNA was purified from the G_1 moths of the established transgenic lines using the sodium dodecyl sulfate–phenol method (Tamura et al. 2000). Purified DNA (10 μg) was digested with the restriction enzyme *Eco*RI or *Msp*I, and the digested DNA was charged in each lane of a 1% agarose gel. After electrophoresis, the gel was carried onto a positively charged nylon filter (Hybond N+, Cytiva, Marlborough, MA, USA). The DNA was transferred using the VacuGene XL Vacuum Blotting System (GE Healthcare Life Sciences, Chicago, IL, USA) from the gel to the membrane and fixed by ultraviolet cross-linking. Hybridization and detection were performed using the previously reported method (Sumitani et al. 2015). The *piggyBac* right-arm DNA was amplified using M13 forward and reverse primers and pGE-pigRarm (Kojima et al. 2007) as a template. The plasmid possessed the fragment of 691 bp in the transposon *piggyBac* right-arm region amplified using the primer set 5′-TGTTTTATCGGTCTGTATATCGAGG-3′ and 5′-GGTGGCCTATGGCATTATTGTACGG-3′ and plasmid DNA of pBac[3xP3-DsRedafm]. The amplified DNA was labeled with Amersham AlkPhos Direct Labeling Reagents (GE Healthcare Life Sciences) and used as a probe. The signal of the transposon sequence was detected using the Amersham CDPStar Detection Reagent (GE Healthcare Life Sciences).

Results

1. Diapause prevention effect of DMSO on the eggs just after oviposition

The egg diapause prevention effect of DMSO was comparable to that of the HCl treatment (Yamamoto et al. 2013). The highest effect appeared when the eggs at 12 h after oviposition were treated with 100% DMSO for 45 min at 25°C. The shorter and longer treatment times as well as the earlier and later egg stages reduced the effect. For the genetic engineering in silkworms, a vector solution has to be injected into the eggs whose embryonic developmental stage is < 8 h after oviposition (Tamura et al. 2000, 2007). DMSO treatment of the eggs after injection was predicted to be impossible as this allows the penetration of the chemicals through the hole of the chorion created by the injection. Therefore, the prevention effect of the DMSO treatment on diapause eggs just after oviposition was first investigated. The synchronized eggs at 0-2 h after oviposition was collected using the previously reported method (Tamura et al. 1990) and treated with DMSO. Three different diapause silkworms, namely, Ariake, J137, and C146, were used for the experiment, and the hatchability of the eggs treated with different times of 100% DMSO was examined. As presented in Figure 1, no hatched larva appeared in the experiment without treatment. The unhatched eggs can be categorized into two groups: fertilized and unfertilized eggs. They can be discriminated by the color of the serosal membrane. The dark-brown color indicated fertilized and diapause eggs, and the faint yellow color indicated unfertilized eggs. The ratio of fertilized eggs in the hybrid race Ariake was relatively higher than those in the other two strains (J137 and C146). Hatched larvae only appeared in the DMSO-treated eggs. The eggs treated for 15-60 min gave high hatchability in all three strains, and the treatment of > 60 min reduced it. In the hybrid race Ariake, the high hatchability of > 80% was observed in the treatment times of 30 and 60 min. In the Japanese bivoltine race J137, the high hatchability of > 70% was observed in the treatment times between 15 and 60 min; on the contrary, less or more than those times reduced the hatchability. In the Chinese bivoltine race C146, the high hatchability of > 90% was observed at 15-, 30-, 45-, and 60-min treatments. Good hatchability was observed in the 7.5-min treatment, whereas it declined in the 90- and 120-min treatment. The DMSO treatment times of 45 min for Ariake and 15 min for J137 and C146 would be most suitable for the eggs just after oviposition and were determined to be used for the injection for genetic engineering.

2. Creation of transgenic silkworms using diapause eggs treated with DMSO

To investigate how the transgenic silkworm can be efficiently created, a DNA solution containing the vector, pBac[3xP3DsRedafm] or pBac[3xP3EGFPafm], and the helper plasmid pHA3PIG DNA was injected into DMSO-treated diapause eggs of one hybrid race and two parent strains. The vector possessed a marker gene that expressed red or green fluorescence in the stemmata of the embryos and complex eyes of adult transgenic silkworms. The helper plasmid was for the supply of transposase of transposon *piggyBac* in the injected eggs. Four different experiments were conducted to create transgenic silkworms to evaluate the applicability of the injection methods (Table 1).

Two different vectors were first injected into diapause eggs of Ariake. Diapause eggs of the hybrid race Ariake were used to inject the vector pBac[3xP3DsRedafm] and helper pHA3PIG plasmids in Experiment 1 and pBac[3xP3EGFPafm] and pHA3PIG

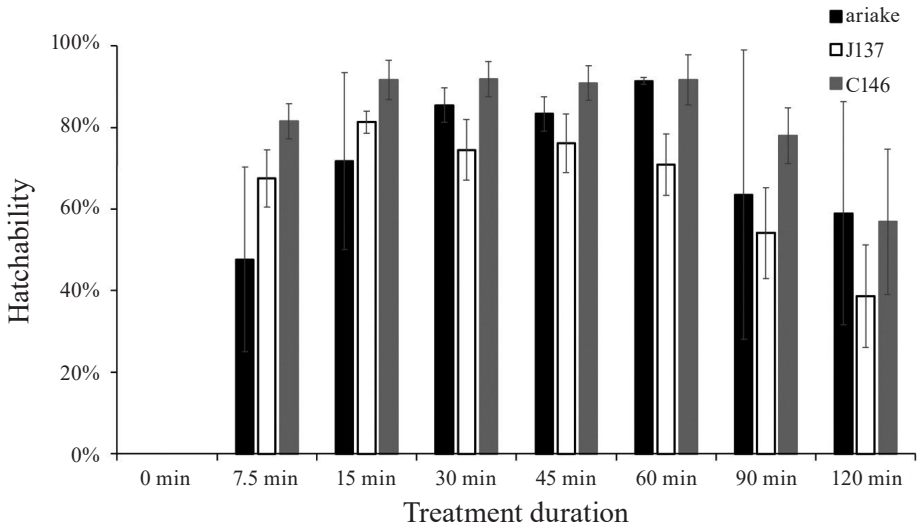

Fig. 1. Hatchability of diapause eggs of the hybrid race Ariake (■), Japanese parent strain J137 (□), and Chinese parent strain C146 (■) after the DMSO treatment
The eggs laid after 0-2 h were collected and immersed in DMSO. After washing the eggs with tap water for 5 min, they were incubated at 25°C for 15 days, and the number of hatched eggs was counted. The bar represents the average of three independent measurements of hatchability in the samples obtained for each strain. The vertical bars indicate the standard deviation measured from the three measurements of each treatment and strain.

Table 1. Construction of transgenic silkworms using diapause eggs treated with DMSO

Strain	Experiment no.	Marker	DMSO treatment time (min)	No. eggs injected	No. hatched eggs (%)	No. adults obtained (%)	Viability of hatched larvae*	No. broods obtained	No. broods containing transgenic eggs (%)
Ariake	1	DsRed	45	288	70 (24.3)	48 (16.7)	68.6%	17	7 (41.1)
	2	EGFP	45	288	87 (30.2)	41 (14.2)	47.1%	12	2 (16.6)
J137	3	DsRed	15	284	178 (62.7)	139 (48.9)	78.1%	50	17 (34.0)
C146	4	DsRed	15	282	41 (14.5)	28 (9.9)	68.3%	7	3 (42.8)

*The viability of hatched larvae was calculated by dividing the number of adults by the number of hatched larvae.

plasmids in Experiment 2. The hatchability of the eggs injected with the DsRed or EGFP vector was 24.3% and 30.2%, respectively. Thus, there was not much difference in the hatchability due to the difference in the vectors. However, the values obtained were slightly smaller than those in the experiments using nondiapause eggs (Tamura et al. 2000, 2007). The viability of the hatched larvae were 47.1% and 68.6% in the DsRed vector DNA and EGFP, respectively. The hatchability of the eggs injected with the EGFP vector was slightly lower than that in the DsRed, and the viability in EGFP was much higher than that in DsRed. Although the reason for such differences could not be examined, it might be due to the conditions of injection and rearing in each experiment. Then, female and male adults were mated with each other or with their host strain, and the next generation of the mated adults was obtained as broods of eggs laid by the females. Because the obtained G_1 eggs would diapause, the HCl treatment was applied to prevent it. The DsRed or EGFP fluorescence expression in the stemmata of the embryos was investigated using the eggs whose diapause was prevented by the HCl treatment. The frequency of the appearance of broods containing the embryos with the DsRed or EGFP expression is presented in Table 1. Seven and two positive broods with injected DsRed or EGFP vectors using Ariake, respectively, were obtained. Although the efficiency of positive brood appearance was slightly lower than that of nondiapause eggs (Tamura et al. 2000, 2007), transgenic silkworms using diapause eggs of the hybrid race Ariake in both vectors was created.

Next, it was investigated whether the method works

in diapause eggs of other strains. Japanese and Chinese bivoltine strains are used to create hybrid races generally reared by farmers. Therefore, the applicability of the practical Japanese strain J137 and Chinese strain C146 was studied in Experiments 3 and 4. In the injection of DMSO-treated eggs, the high hatchability of 62.7% was obtained in Experiment 3 using the Japanese strain J137, and a low value of 14.5% was obtained in Experiment 4 using the Chinese strain C146. The differences in the hatchability between the strains depended on the tolerance of the injected eggs against the DMSO treatment. However, the viabilities of the hatched larvae were not much different in the two strains (Table 1). In J137 and C146, 78.1% and 68.3% of the hatched larvae developed to adults, respectively. Thus, a good viability of the hatched larvae in Experiments 3 and 4 was achieved using the Japanese and Chinese parent strains. The frequency of the appearance of positive eggs in the broods of the parent strains was comparable to those in previous experiments using nondiapause eggs (Tamura et al. 2000, 2007; Imamura et al. 2003). Positive brood was obtained at frequencies of 34.0% and 42.8% in J137 and C146, respectively. Therefore, creating transgenic silkworms is possible even in diapause eggs of the practical Japanese and Chinese strains.

How frequently positive embryos appeared in positive brood is another important evaluation criterion to judge the efficacy of the genetic engineering method in the silkworm. The number of embryos expressing fluorescence in each brood was counted, and it was found that the number of positive embryos significantly differed by the brood. Table 2 presents the numbers of eggs expressing DsRed or GFP in the stemmata of the embryos in each brood used to construct the transgenic lines. The frequency varied from 0.8 to 16.9% in each brood, indicating that the efficiency of the appearance of positive G_1 eggs in each brood was not much different from experiments using nondiapause eggs (Tamura et al. 2000, 2007; Imamura et al. 2003). Then, seven transgenic lines were constructed from positive broods with relatively larger numbers of positive embryos.

3. Characterization of the established transgenic lines

To characterize the transgenic silkworms, three, one, two, and one line from Experiments 1-4, respectively, were established. Because these lines laid diapause eggs, they were treated with HCl, and the DsRed or GFP expression in the stemmata of the embryos was examined. The fluorescence expression in the stemmata of the established lines was much weaker (Fig. 2) than that of the transgenic silkworm using the nondiapause strain w1-pnd characterized by white and nondiapause eggs. The weak fluorescence in the established lines was due to the dark-brown pigmented serosal membrane, whereas it was a white membrane in the w-1pnd strain. The difficulty of screening G_1 eggs was apparently due to the weak fluorescence in diapause eggs. The DsRed and EGFP expression in the complex eyes of adults of the established lines was also investigated (Fig. 2). The expression in the adults was much stronger than that in the embryos due to the strong fluorescence expression in the complex eyes of adults. The discrimination between nontransgenic and transgenic moths was easily conducted, although there were some differences in the strengths among the lines.

To confirm the insertion of the marker gene into the genome of transgenic silkworms *via* biochemical analysis, Southern blotting of genomic DNA extracted from these transgenic silkworms of the seven established lines was performed. In the transgenesis using the transposon *piggyBac*, the region between the transposon

Table 2. Numbers of eggs expressing DsRed or GFP in the stemmata of the embryos

Host strain	Marker gene	Experiment no.	Positive G_1 brood no.	No. eggs examined	No. positive embryos (%)
Ariake	3xP3DsRed	1	1	310	5 (1.6)
			2	290	42 (14.5)
			3	255	13 (5.1)
	3xP3EGFP	2	1	301	9 (3.0)
			2	288	7 (2.4)
J137	3xP3DsRed	3	1	218	33 (15.1)
			2	201	34 (16.9)
			3	279	3 (1.1)
C146	3xP3DsRed	4	1	267	10 (3.7)
			2	171	11 (6.4)
			3	254	2 (0.8)

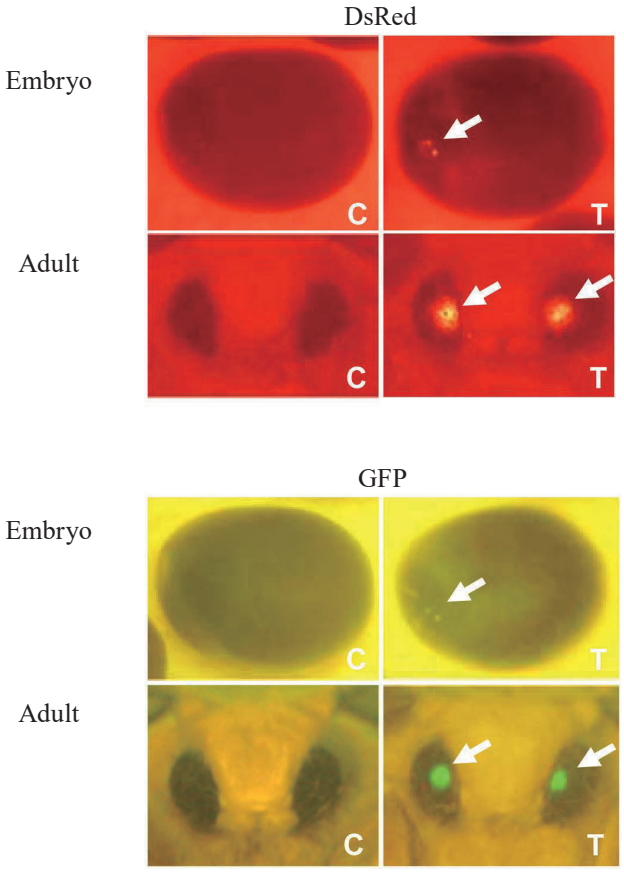

Fig. 2. DsRed and EGFP expression in the stemmata of the embryos and complex eyes of adult in the transgenic silkworm lines (T)
Control (C) is a nontransgenic silkworm. The arrows indicate the position of the DsRed and EGFP expression. The pictures were taken under a stereomicroscope equipped with a DsRed or GFP filter. The transgenic strains with DsRed and EGFP were obtained from Experiments 1 and 2 (see Table 1).

piggyBac left and right arms (Fig. 3a, b) was inserted into the target sequence TTAA arbitrarily distributed in the silkworm genome every 256 bp. The *piggyBac* right-arm region was used as a probe, and DNA was digested using *Eco*RI or *Msp*I. Therefore, the band larger than the size between the end of the right-arm and the first *Eco*RI site of the vector (> 2.3 kb) must appear in the blotting of the *Eco*RI digested DNA, as the other restriction site depended on the *Eco*RI recognition site arbitrarily present in the genomic sequence of the transgenic silkworm inserted with the transgene. In the digestion of *Msp*I, the band with a size of > 0.85 kb must appear for the same reason. Because both vectors have the same *piggyBac* right-arm sequences, the shortest bands could be detected in either. The difference in the sizes of bands detected in the blotting indicated the variation of the insertion site. As presented in Fig. 3c, blotting using *Eco*RI digested DNA showed bands with a size of > 2.3 kb, and one line on lane 2 possessed three bands with different sizes. Silkworms on lanes 1, 4, 5, and 7 possessed two bands, and the lines of lanes 3 and 6 had one band. The same result was observed in the blotting of *Msp*I. Thus, one line possessed three transgenes, four lines had two insertions, and two lines had one insertion.

Discussion

The injection method for the genetic engineering of practical diapause strains can be categorized into two types. One is to produce nondiapause eggs from diapause silkworm strains, and the other is to prevent the diapause of the eggs. For the first method, adults that lay nondiapause eggs were produced by injecting an antibody against the diapause hormone or incubating the parent eggs at low temperatures (Grenier et al. 2004, Zhao et al. 2013, Zabelina et al. 2015). The diapause of silkworm eggs was determined by the diapause hormone synthesized at a subesophageal ganglion and secreted from the corpus cardiacum into the hemolymph of the larva and pupa (Hasegawa 1957, Ichikawa et al. 1995). Diapause eggs formed under the presence of the hormone during the pupal stage. The nondiapause eggs of diapause strains can be produced by injecting the diapause hormone antibody into the pupae or larvae (Shiomi et al. 1995). The nondiapause eggs produced through antibody injection can be used for the genetic engineering of the silkworm (Grenier et al. 2004, Zabelina et al. 2015). However, it required the production of a highly efficient antibody and adjustment of injection conditions to silkworms. To date, no successful result of transgenic silkworm creation using nondiapause eggs produced by injecting the diapause hormone antibody has been reported. Low-temperature incubation of the parent eggs is another method for producing nondiapause eggs in diapause strains. The moths in some bivoltine strains lay nondiapause eggs when their embryonic development occurs at low temperatures (Umeya & Harada 1955, Kosegawa et al. 2000). The nondiapause eggs produced using the method can be used for the genetic engineering of diapause strains. Construction of transgenic silkworms and genome editing by CRISPR/Cas9 using low-temperature incubation of the parent eggs was reported, and the method worked well (Zhao et al. 2012, Iizuka 2016, Tomihara et al. 2021). However, this method is only applicable to some bivoltine strains. In most other diapause strains, especially in most Japanese and European strains, the method cannot be employed because the low-temperature incubation of their parent eggs does not change the diapause character, and only

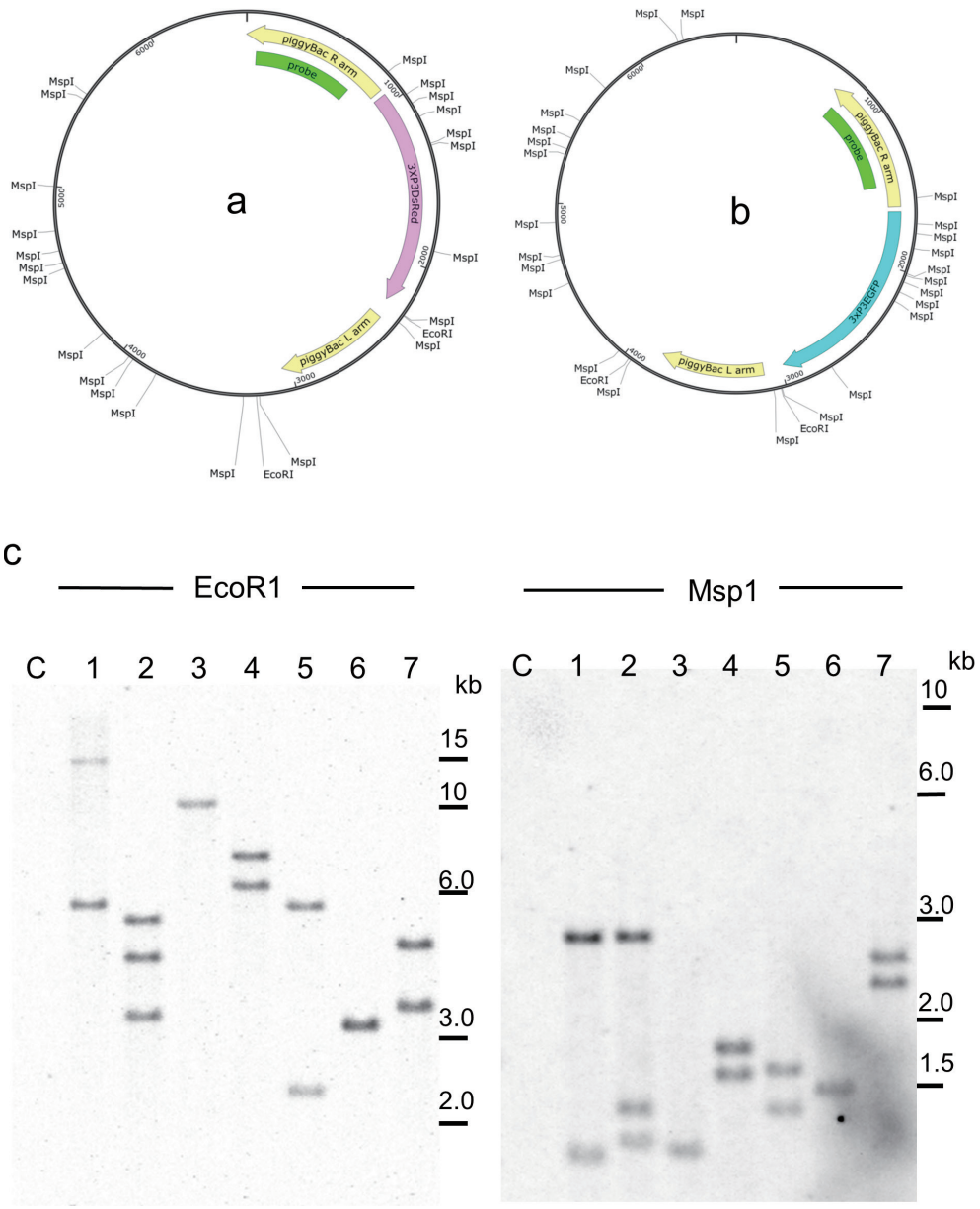

Fig. 3. Physical maps of pBac[3xP3-DsRedafm] (a) and pBac[3xP3EGFPafm] (b), vectors used for the experiments and Southern blotting of transgenic silkworm lines (c)
(a and b) The region of the right and left arms of transposon *piggyBac* are indicated by yellow arrows (*piggyBac*R and *piggyBac*L, respectively). The marker genes *3xP3DsRed* and *3xP3EGFP* are indicated by pink and blue arrows, respectively. The sequence used as a probe for Southern blotting is shown in the green bar. These figures were made using the SnapGene software. (c) Numbers 1-5 are DNA extracted from one G_1 moth of DsRed lines 1-5, and numbers 6 and 7 are from GFP lines 1 and 2. DsRed lines 1-3, 4, and 5 are derived from Experiments 1, 4, and 5, respectively. GFP lines 1 and 2 are from Experiment 2 in Table 1.

diapause eggs are produced (Kosegawa et al. 2000). As the second method that prevents egg diapause, the application of HCl was studied (Inoue et al. 2005, Zhao et al. 2013). HCl treatment is a reliable method for preventing the diapause of silkworm eggs and a standard method for preventing diapause eggs in sericulture. The standard treatment is to immerse the eggs at 24-h development after oviposition in 5 N HCl for 5 min at 46°C or 6 N HCl for 1 h at 25°C. However, the suitable injection time for genetic engineering is 0-6 h after oviposition because the blastoderm is formed at the embryonic developmental stage at 12-16 h (Tamura et al. 1900, 2000, 2007). The injection into the eggs must be performed much earlier than the standard HCl treatment

time. The requirement of earlier time treatment causes difficulty as the eggs just after oviposition are very weak for the treatment. Therefore, the eggs prevented by HCl gave significant low hatchability in the injection for genetic engineering (Zhao et al. 2012). Thus, many efforts have been exerted to develop the injection method for diapause strains, but no authorized method applicable to most strains has been established yet.

In this experiment, the injection method for genetic engineering that can be applied to all practical diapause silkworm strains was investigated, and the DMSO treatment of diapause eggs can be used for this purpose. The details of the method established in this study are presented in Figure 4. In the silkworm, the fertilization, the fusion of female and male nuclei, occurs in the anterior center of the eggs at 2 h after oviposition. The fused nuclei start cleavage every 1 h, move to the surface of the eggs, and form blastoderms (Nagy et al. 1994). To engineer the silkworm, an injection into the egg has to be performed before the formation of the blastoderm. Otherwise, the injected DNA does not reach the nuclei and interacts with genome DNA (Tamura et al. 1990, Nagaraju et al. 1996). To obtain the eggs at an early stage of embryogenesis, the mated male and female moths were maintained for 3-5 h at 25°C and kept at 5°C for 1-3 days. Then, the female moths separated from the males were placed on an egg-laying paper (the surface covered with starch) in a dark box at 25°C and kept for 1-2 h to lay the eggs. The female moths treated in the process are known to lay eggs after transfer to the dark box, and the eggs just after oviposition can be obtained without fail (Tamura et al. 1990). To remove the eggs from the paper, the paper was immersed in water for 5-10 min. Because the starch on the paper surface is dissolved in water, the eggs can be

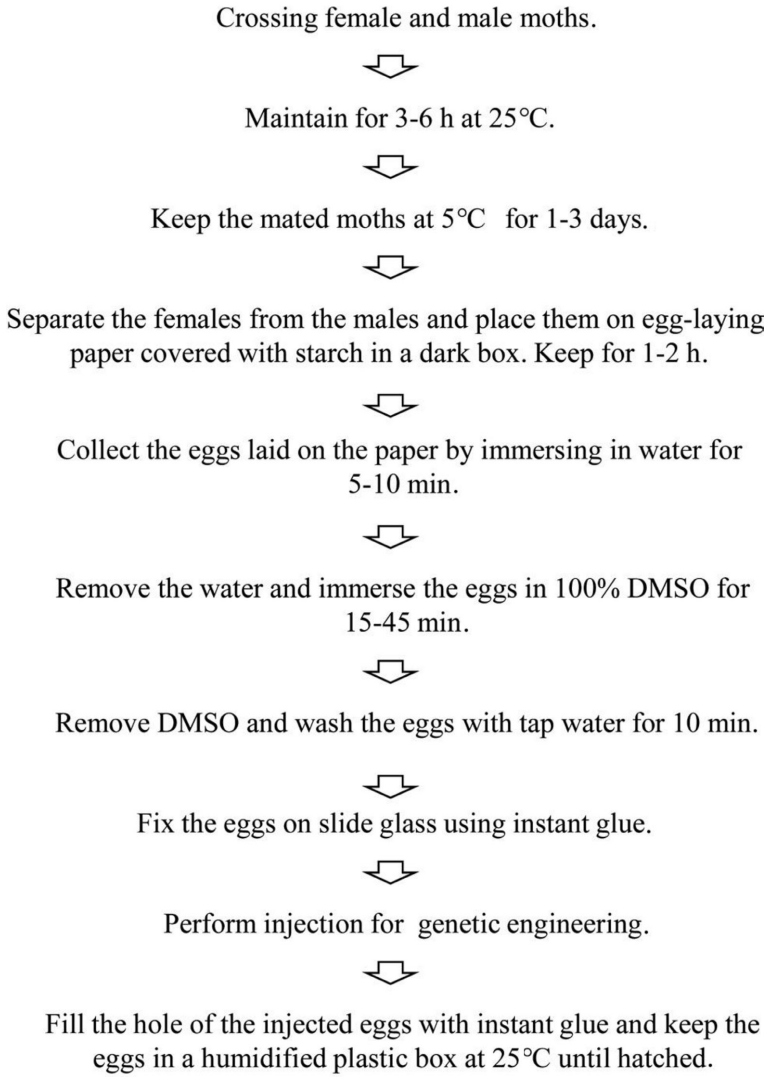

Fig. 4. Established protocol for injection into silkworm diapause eggs using DMSO

removed from it and floated in water. The eggs were collected using a stainless mesh, and they were transferred into a small Petri dish and then immersed in DMSO. After washing with tap water and sterile distilled water, the eggs were fixed on the glass slide using instant glue, and the injection was carried out. After injection, the egg hole created by the process was filled with instant glue and kept in a humidified plastic box at 25°C until hatching. This method achieved hatchability values of 24.3% and 30.2% in Ariake and 62.7% and 14.5% in J137 and C146, respectively. The efficiencies in the creation of transgenic silkworms using DMSO were comparable to that of nondiapause eggs. In addition, the method was applied to three different practical diapause strains, indicating that it can be used with most other diapause strains.

Based on these results, the injection method for diapause strains using DMSO was successfully developed. The method is easy to perform and reliable. It can be applied to different diapause silkworms, including hybrid variety and Japanese, Chinese, European, and mutant bivoltine and univoltine strains. The development of this method will accelerate the construction of new breeds of silkworms with high economic importance in sericulture and contribute to the analysis of gene functions of the silkworm as a model organism for life science.

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