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Editing of the heavy chain gene of *Bombyx mori* using transcription activator like effector nucleases

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ABSTRACT

The silk gland of *Bombyx mori* represents an established in vivo system for producing recombinant proteins. However, low yields of recombinant proteins have limited the system's further development because endogenous silk proteins were present. Transcription activator-like effector nucleases (TALENs) tool which work in pairs to bind and cleave DNA at specific sites, have recently been shown to be effective for genome editing in various organisms, including silkworms. To improve the yield of recombinant proteins synthesized in the silkworm by eliminated competition with endogenous fibroin synthesis, the *heavy chain* (*H-chain*) gene was knocked out using transcription activator-like effector nucleases (TALENs). A pair of TALENs that targets the 1st exon in the *H-chain* gene was synthesized and microinjected into silkworm embryos; the injected silkworms were screened for *H-chain* gene knock out (H-KO) based on their sericin cocoon-making characteristics. Sequence analysis revealed that the *H-chain* of the mutation was successfully edited. The TALENs was very efficient in editing the genome DNA of silkworm. By being eliminated competition with the H-chain, the production of recombinant proteins would be expected to increase markedly if this H-KO system is used.

1. Introduction

In addition to its use in the silk industry, *Bombyx mori* is used to produce recombinant proteins due to the efficient protein biosynthetic activity present in its silk gland [1,2]. Pharmacologically important proteins or other value-added products can usually be independently produced in the soluble sericin layer because of its purification convenience [3]; the recombinant proteins, for example spider silk protein, can be produced in the silk core by fusing with fibroin to improve the physical properties of silk [4]. Great progress has been made over the past ten years in developing a silk gland expression system. However, the low yield of recombinant proteins is still the biggest limitation when using this system. Even when using the strongest *H-chain* promoter, the ratio of recombinant protein never exceeds 10% [5]. One primary reason is that the recombinant proteins are always at a competitive disadvantage compared with the endogenous silk proteins. The idea of knocking out or silencing the endogenous silk genes to improve the ratio of recombinant proteins is very attractive. Although many attempts have been made, there is still no stable system available to date.

The recently developed transcription activator-like effector nucleases (TALENs) work in pairs to bind and cleave DNA at specific sites, and this system represents a great improvement in genome editing. TALEN applications have been reported in a number of organisms, since the first success in human cells [6–8]; TALENs also have been shown to work effectively in silkworms [9,10]. Therefore, using custom TALENs, we attempted to knock-out the *H-chain* gene of *B. mori*, which accounts for 92% of silk fibroin translation, to develop a highly efficient system for producing recombinant proteins. A pair of TALENs that target the 1st exon of the *H-chain* gene, which encodes the main fibroin molecule, was directly synthesized. Microinjection of the TALENs induced the sericin cocoon-making mutations. To our knowledge, this is the first report of a hereditary and stable H-KO system in silkworms, and we hope that this system will greatly improve the expression of recombinant proteins in *B. mori.*

2. Materials and methods

2.1. Silkworm strains

A non-diapausing strain, N4w, which is wild-type (WT) for the *H-chain* gene, was used in all experiments. This strain is maintained

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at the Nakagaki Laboratory (Shinshu University, Nagano, Japan). The larvae were reared on fresh mulberry leaves at 25 °C. Ethical approval was not required for the study of *B. mori*.

2.2. TALEN design and expression vector construction

To avoid the repetitive portion of the H-chain, the 1st exon of this gene was examined for TALEN design. A 54-bp target sequence was selected, and the corresponding TALEN sequences (TALEN–HL and TALEN–HR) were generated using the DNA backbone according to Ma et al. [9]. The TAL effector domain was assembled using a commercial service (ViewSolid Biotech, Beijing, China). The complete TALEN coding sequences were then inserted into expression vectors containing the T7 promoter and the FokI coding sequence (pSW-peas-T) by NheI + BamHI digestion. Vector construction followed standard molecular cloning protocols and kit instructions.

2.3. Luciferase activity measurement

A luciferase SSA (single-strand annealing) reporter was used in this experiment to evaluate TALEN activity according to [11]. A pGL4-SSA-H vector was constructed to include two truncated luciferase fragments separated by a stop codon and a 54-bp *H-chain* recognition site. The target sequence was inserted into the XmaI + SmaI sites between the two truncated luciferase fragments of the pGL4-SSA reporter vector. The vector was transfected into Human embryonic kidney (HEK) 293T cells with or without the TALEN-H vector. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagles medium (DMEM) (D5796) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). Two hundred nanograms of pGL4-SSA-H reporter plasmid with or without TALEN-H expression plasmids was transfected into 4 thousands of cells by using Lipofectamine LTX (Invitrogen) in a 96-well plate. The cells were collected after 24 h of incubation to measure their luciferase activity was assayed using a dual-luciferase reporter assay system (Promega).

2.4. In vitro mRNA synthesis

TALEN constructs in the pSW expression vector were prepared using a HiSpeed plasmid midi kit (Qiagene), and the vectors were linearized using NotI. HL and HR mRNA were transcribed in vitro using an mMESSAGE mMACHINE kit (Ambion) using a T7 promoter. The mRNA was precipitated with LiCl, washed with 70% ethanol, and dried in a vacuum centrifuge.

2.5. Silkworm embryo microinjection

The N4w embryos were prepared for microinjection as previously described [1] (Tamura et al., 2000). HL and HR mRNA were mixed in 0.5 mM phosphate buffer (pH 7.0) at a molar ratio of 1:1. The concentration of each mRNA was 200 ng/μl. The mRNA samples were microinjected into silkworm embryos (G0 generation) 4–8 h after oviposition. The microinjections were performed using a Transfer-Man NK2 micromanipulator and a Femto Jet 5247 microinjector (Eppendorf) under an SZX16 microscope (Olympus). The injection opening was sealed with instant glue (Topvalu), and the injected embryos were incubated at 25 °C in a humidified atmosphere.

2.6. Crossing scheme and screening strategy

The silk produced by an H-KO mutant primarily comprises sericins because the *sericin* genes are intact. Therefore, we can screen for the mutation by evaluating the cocoon. However, individual silkworms inherit two alleles of the *H-chain* gene, and only silkworms in which both alleles are knocked out can produce visible sericin cocoons. Theoretically, silkworms could generate biallelic mutations from the microinjected G0 generation and perhaps exhibit a sericin cocoon-making capacity. However, hereditary mutations should be selected from the following (G1) silkworm generation. Based on this strategy, G0 moths were sibling crossed to generate G1 silkworms. The hatched G1 silkworms were allowed to develop at 25 °C and were reared separately. Sericin cocoon-making mutations were then selected from the G1 broods. The mutations were further confirmed by genome sequencing. Mutants in the same brood were crossed to generate G2 silkworms.

2.7. Mutation sequencing

Among the 5 screened G1 mutations, 3 were randomly subjected to DNA extraction and sequencing. Genomic DNA was extracted using a Dneasy Blood & Tissue Kit according to the manufacturer's instructions. Approximately 25 mg of moth tissue was collected for each DNA sample, and DNA fragments containing the targeted site were amplified using PCR. The PCR products were inserted into a pMD-20T vector and sequenced using a commercial service (Operon Co., Tokyo, Japan).

Two mutated *H-chains* should have been detected in each DNA sample because all H-KO silkworms were considered biallelic mutations. Thirty-six clones from each mutation were selected for sequencing to improve the discovery frequency of both mutated alleles in each mutated brood.

2.8. Mutant silk gland sectioning

The silk glands from fifth instar larvae of the G2-mutated silkworms and the wild-type N4w strain were dissected in 0.02 M phosphate-buffered saline (PBS). The samples were fixed in 4% paraformaldehyde and dehydrated in 30% sucrose for 2 h. The treated specimens were then positioned on a cryostat object disk on which an appropriate amount of tissue freezing medium had been placed. The specimens were sectioned at 8–10 μm thickness at −25 °C using a freezing microtome. The sections were visualized using an Olympus BX51TR after staining.

2.9. Silk protein analysis

Cocoon silks or luminal silk-gland proteins were dissolved in sample buffer containing 2% SDS and 5% β-mercaptoethanol, heated for 5 min in a boiling water bath, and centrifuged for 3 min at 10,000 rpm. The prepared samples were loaded onto polyacrylamide gels (4% stacking gel, 8% separating gel), and electrophoresis was performed under denaturing conditions. The gels were stained using Coomassie brilliant blue R-250 and destained using 25% methanol in 10% acetic acid.

3. Results

3.1. Target site selection and TALEN activity measured using an HEK293-based SSA assay

The *H-chain* gene, which encodes the primary protein of silk, was selected as the target objective. Silkworm *H-chain* is characterized by a highly repetitive core flanked by two non-repetitive 5′-and 3′-terminal regions [12]. We designed one pair of TALENs to target the 1st exon, which encodes the 5′-terminal region of *H-chain*, to avoid the repetitive portion of this gene (Fig. 1). The luciferase SSA reporter gene was used to test TALEN-H activity in HEK 293T cells. The cells transfected with TALEN-H and the associated SSA reporter exhibited a 900% dose-dependent increase in TALEN-H

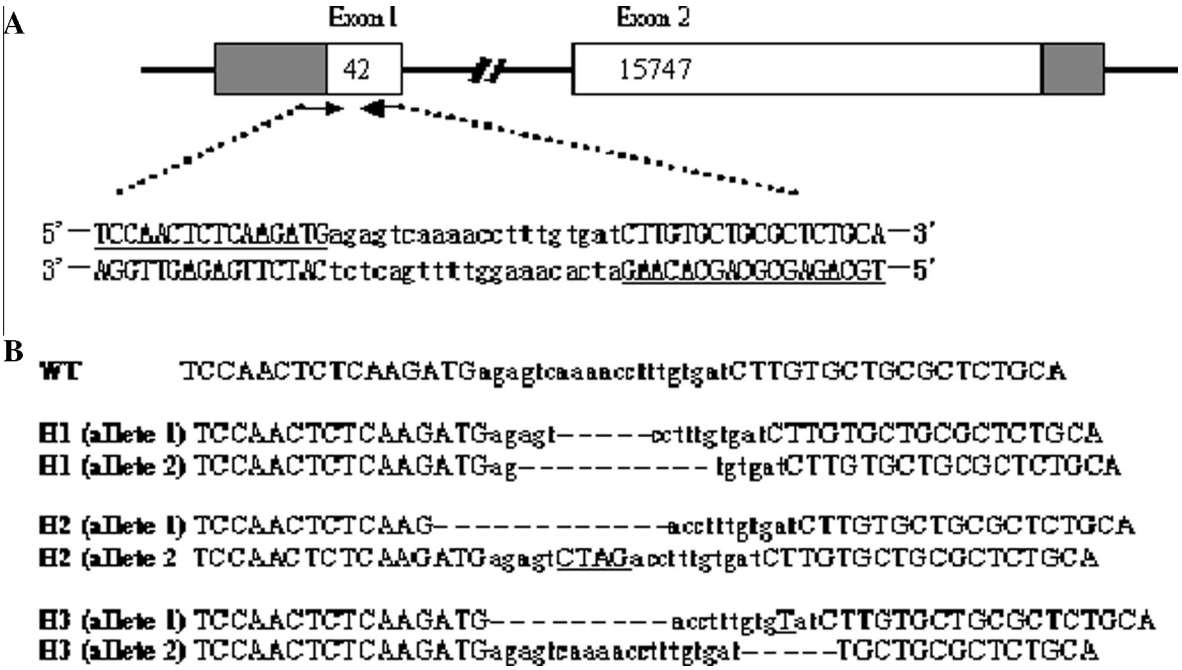

Fig. 1. The structure of the *H-chain* gene and the sequences of the mutations introduced using TALEN-H. (A) A schematic representation of the gene structure of the *H-chain* gene, depicting an intron (broken line), exons (blank boxes), and the TALEN target sequences (sequences written at the bottom). The numbers indicate the exact lengths of the exons. The grey boxes represent the 5′UTR (left) and the 3′UTR (right). The underlined sequence represents the recognition site of the corresponding TALEN monomer. (B) Sequences of mutations recovered from different broods. The wild-type (WT) sequence is shown at the top, together with the TALEN targeting sequence (capital letters). Deletions are indicated by dashes, and insertions are indicated by capital letters within the boxes.

activity compared with transfection of the SSA reporter vector alone (Fig. S1).

3.2. Selective sericin cocoon

In vitro-synthesized mRNA was microinjected into silkworm embryos at the syncytial preblastoderm stage. Five hundred and twenty-eight preblastoderm stage embryos were injected with TALEN-H, and 166 individual G0 silkworms hatched. Sixty-two G1 broods were obtained from the sibling cross of the G0 moths. The silkworms of the late hatched 28 G1 broods, and approximately 6000 individuals were selected for breeding until the cocoon stage. Eighteen sericin cocoons from 5 broods were detected from all of the G1 cocoons (Fig. 2).

3.3. Mutant sequence analysis

We amplified genomic DNA fragments flanking the target region using PCR and analyzed the mutant sequences to determine whether the mutation occurred at the specified locus and to identify what types of mutations might have been introduced by the TALENs. An alignment of the DNA target sequences from 3 G1 sericin cocoon-making silkworms is presented in Fig. 1. Three mutant silkworms were randomly chosen and named TH1, TH2, and TH3. Sequencing analysis revealed the presence of 2 types of mutations: small deletions and insertions.

3.4. Internal silk gland structure and cocoon silk protein analysis

In normal silkworms, the posterior silk gland, which is responsible for fibroin synthesis, is very long and convoluted. Morphological observations showed that the posterior silk glands of the H-KO silkworms were strongly degraded (Fig. 2), and negligible amounts of fibroin was observed in the posterior silk gland of the mutants compared with that found in normal glands (Fig. 3). Also, no fibroin was detected in the middle silk gland of the mutant. SDS–PAGE analysis suggested that the sericin cocoon contained no H-chain,

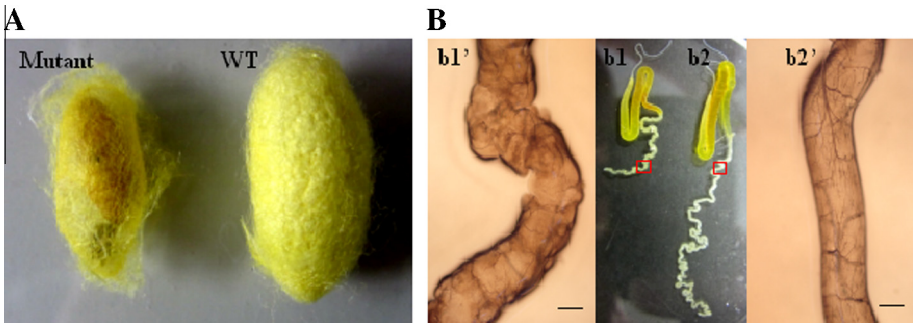

Fig. 2. Comparison of the cocoon and silk gland of the mutant and WT (N4w) silkworms. (A) Comparison of a mutant cocoon and a WT one. Because no fibroin was synthesized, the cocoon of the mutant silkworm exhibited sericin products that were more filmy than those of the WT cocoon. (B) Comparison of the silk gland of the mutant and WT. The posterior silk gland of the mutant was strongly degraded and its cells were abnormal (b1). b1′ is a magnified view of the frame present in b1.b2 displays the silk gland of the control, and b2′ is a magnified view of the frame present in B2. Scale bars = 200 μm.

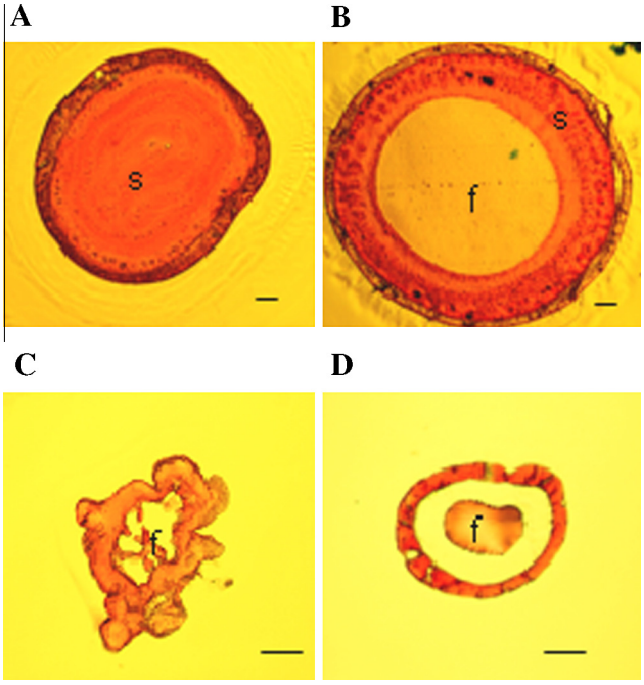

Fig. 3. Cross-sections of the silk glands of 5th instar mutant and WT (N4w) silkworms. The sections were stained using a mixture of 0.04% (w/v) methyl green, 0.30% (w/v) acid fuchsin, and 0.80% (w/v) picric acid. Sericin is stained red by acid fuchsin, and fibroin is stained yellow by picric acid. (A) Middle silk gland of mutant. (B) Middle silk gland of WT. (C) Posterior silk gland of mutant. (D) Posterior silk gland of WT. Of the middle silk gland, Sericin and fibroin were clearly observed in the control silkworm; however, no fibroin layer was observed in the mutant. The posterior silk gland of the WT was regular and contained a mass of fibroin materials, unlike the mutant. Notably, the fibroin in the posterior silk gland had not yet formed a compact fibrous structure; the fibroin is stained red, similar to sericin. 'S' represents sericin, and 'f' represents fibroin. Scale bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which is consistent with the results observed in the silk gland (Fig. 4).

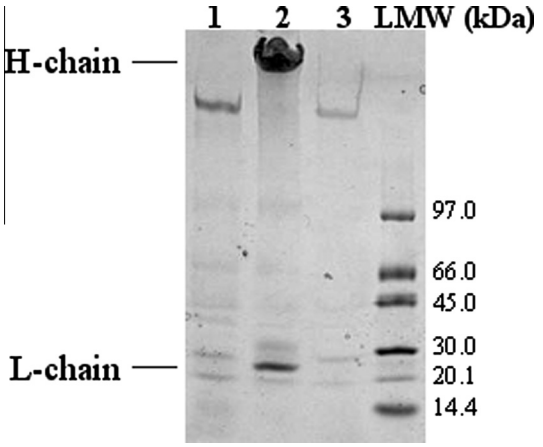

Fig. 4. SDS–PAGE of luminal silk-gland proteins and cocoon silk under reducing conditions. LMW, low molecular weight standard (BIO-RAD); the numbers to the right represent molecular masses (kDa). Lane 1, the middle lumen contents of the middle silk gland of the mutant; Lane 2, the cocoon silk of a WT (N4w) silkworm; Lane 3, the cocoon silk of the mutant silkworm. The positions of the H-chain and L-chain in Lane 2 are indicated. Neither the H-chain nor the L-chain was detected in the mutant.

4. Discussion

TALENs are a recently developed genome-editing technology, and the applicability of this technique in silkworms has been established [9]. To date, *BmBlos* is the only gene to have been successfully edited using TALENs in silkworm [9,10]. *BmBlos* is responsible for the transparent skin phenotype and is located on the Z chromosome; therefore, this gene is present in only one allele. These characteristics simplify the screening for *BmBlos* mutant individuals. In this study, we report the first mutation of a biallelic gene located on the euchromosome using TALENs, In the interest of establishing a potential highly efficient system for producing recombinant proteins in silkworm.

Delayed hatching has been shown to generate more mutations in transgenic silkworms [9]. Therefore, only G1 individuals from the late-hatched G0 silkworms were selected for breeding to reduce the scope of the screening. However, an undesirable consequence occurs when using this screening strategy: unless two mutated silkworms are selected for crossing, we would fail to detect mutated sericin cocoons. In this case, the use of other screening strategies, such as Surveyor Nuclease technology, could detect single-allele mutations. In this study, mutated sericin cocoons were found in 5/28 (18%) G1 broods. Considering the random choice of G0 silkworms for sibling crossing, the ratio of mutants containing at least one *H-chain* allele should be much higher than 18%. This indicates the high efficiency of the TALEN method and supports our screening strategy.

Negligible quantities of fibroin were observed in the posterior silk gland. Because the H-chain had been knocked out, the L-chain was expected to predominate in the fibroin. However, no distinct L-chain was detected in the middle silk gland as well as the sericin cocoon. Perhaps the quantity of the L-chain was too low to create sufficient pressure to pour into the medial silk gland, resulting in the inability to detect this protein in the cocoon. The H–L subunit is believed to play a key role in the solubility of fibroin during intracellular transport, secretion, and luminal transport through the silk gland before spinning [13]. Although the wild-type L-chain gene was present in the H-KO silkworms, this chain was not normally secreted, further confirming that the combination of the H and L subunits of silk fibroin is important for the efficient secretion of fibroin.

A silk fibroin secretion-deficient naked pupa silkworm (Nd-s^d) has been used to express recombinant proteins [13]. The H–L subunit cannot be formed and secreted normally in the Nd-s^d silkworm due to the presence of the mutant L-chain in this silkworm strain. A recombinant protein was fused to a normal L fibroin and was expressed using the Nd-s^d system. The resulting recombinant proteins were secreted in a molar ratio equal to that of the H-chain, and the final product of the fusion gene constituted approximately as high as 10% of the cocoon silk [14]. The H-KO silkworm has at least two advantages in expressing recombinant proteins in contrast to Nd-s^d system. First, compared with the diapause Nd-s^d, the non-diapause characteristic of the H-KO mutation would aid in generating transgenic silkworms for future experiments. Second, and most importantly, the H-KO system is more efficient at producing recombinant proteins. An obvious deficiency of the Nd-s^d system is that the recombinant protein must be fused with the L-chain and co-expressed with the very large H-chain protein in the form of an H–L subunit pair. Thus, this system would markedly limit the expression of recombinant proteins. The H-KO system would allow us to use the strongest *H-chain* promoter when producing recombinant proteins, and the recombinant protein would be produced in a molar ratio of 1:1 with the small L-chain in the fibroin core. Nevertheless, because the large H-chain protein would no longer be synthesized, more amino acids would be available to produce the recombinant protein. If the system was used to modify the silk, for example, to produce spider silk,

the generation of a predominantly spider silk-containing-silk would be possible because the molecular weight of spidorin is much higher than that of L-chain. In addition, because of the lack of insoluble fibroin, if a drug were to be expressed using the H-KO system, it would be easily purified from the fibroin core.

Furthermore, some strategies can be used to facilitate the producing the recombinant protein in H-KO system. For example, it has been reported that overexpression of the silkworm *Ras1* oncogene can prevent silk gland destruction, thus improving fibroin synthesis [15]. Co-expression of proteins such as *Ras1* and our target gene might be needed to produce more recombinant protein using the H-KO system.

As described above, although the non-diapause characteristics of the H-KO mutation favor the generation of transgenic silkworms, sustaining these varieties is time-consuming. To solve this problem, we first hybridized a transgenic silkworm with a diapause strain to generate the G1 diapaused generation (G1); then, the G1 siblings were crossed to generate G2 silkworms. Finally, diapause homozygotes were selected from the G2 generation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.092.

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