Genome editing of BmFib-H gene provides an empty Bombyx mori silk gland for a highly efficient bioreactor

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Evolution has produced some remarkable creatures, of which silk gland is a fascinating organ that exists in a variety of insects and almost half of the 34,000 spider species. The impressive ability to secrete huge amount of pure silk protein, and to store proteins at an extremely high concentration (up to 25%) make the silk gland of Bombyx mori hold great promise to be a cost-effective platform for production of recombinant proteins. However, the extremely low production yields of the numerous reported expression systems greatly hindered the exploration and application of silk gland bioreactors. Using customized zinc finger nucleases (ZFN), we successfully performed genome editing of BmFib-H gene, which encodes the largest and most abundant silk protein, in B. mori with efficiency higher than any previously reported. The resulted BmFib-H knocked-out B. mori showed a smaller and empty silk gland, abnormally developed posterior silk gland cells, an extremely thin cocoon that contain only sericin proteins, and a slightly heavier pupae. We also showed that removal of endogenous BmFib-H protein could significantly increase the expression level of exogenous protein. Furthermore, we demonstrated that the bioreactor is suitable for large scale production of protein-based materials.

With the developing understanding of disease pathogens and the identification of new molecular targets, biopharmaceutical proteins such as vaccines, hormones and protein-based biomaterials are in increasing demand for both analytical and clinical applications. Bioreactors using genetic modified organisms, which enable production of recombinant proteins in a commercial scale, emerged and underwent decades of development to meet this demand. However, few proteins produced in such bioreactors are now in clinical trials and only one has been approved for marketing, mainly due to low production yield and costly protein purification process using genetic modified plants or livestock. As the silk producing organ of many insect and spider species, silk gland has been extraordinarily conspicuous and well-studied, owing to the impressive ability to secrete huge amount of silk protein and to store proteins at high concentration without aggregation or denaturation. This ability was greatly enhanced in silkworm, Bombyx mori, by thousands of years' domestication. Nowadays, approximately 1,000,000 tons of cocoon and 200,000 tons of silk were produced each year worldwide. Eating about 20 g mulberry leaves, one commercial B. mori larvae can produce 0.5 g pure silk protein (dry weight, 25% of total worms). Therefore, the silk gland of B. mori holds great promise to be a cost-effective platform for commercial scale production of recombinant proteins.

During the past decade, several transgenic silk gland based expression systems were established and various recombinant proteins including collagen, globular protein, human basic fibroblast growth factor, human serum albumin, feline interferon, human mu-opoid receptor, mouse monoclonal antibody and spider silk were successfully produced in these systems. Meanwhile, many strenuous attempts utilizing different kinds of regulatory elements including promoters, enhancers, UTRs, mutant strains and insulators were performed to increase the expression level of exogenous proteins. However, the actual yields of these proteins were much lower than expected, and they can not be dissolved and purified without some harsh reagents, in which the proteins may lose their biological activities.

As a giant organ whose main, if not only, mission was synthesis and secretion of silk protein, the extremely extraordinary protein production ability may mainly serve the silk protein production and thus repress the expression of exogenous proteins. Thus we proposed that reducing or removal of the endogenous silk proteins might increase the expression level of exogenous recombinant proteins. The successive emerging of ZFN, transcription activator-like effector nucleases (TALENs) and clustered regularly interspersed short palindromic...
repeats (CRISPR)/Cas9 technologies enable genome editing in various organisms. And more encouragingly, these systems have been established in *B. mori*, in which targeted mutagenesis had never been achieved before. In the current study, we showed that genetic removal of fibroin heavy chain protein by ZFN re-programmed the silk gland to produce exogenous recombinant proteins at an explosive level.

**Results**

We first performed targeted mutagenesis of fibroin heavy chain (*BmFib-H*) gene, which encodes the largest and most abundant silk protein, using custom ZFN. *BmFib-H* consists of a highly repetitive core flanked by non-repetitive 5’ and 3’ ends, and encodes a considerably large protein comprising N-terminal and C-terminal hydrophilic domains and 12 highly repetitive Gly-Ala-rich regions. A pair of ZFN designed to target the 5’-non-repetitive region of *BmFib-H* (Fig. 1a, supplementary Figs. 1 and 2) was microinjected into 318 embryos of a non-diapausing *B. mori* strain in mRNA form. Twenty-five broods (65.79%) with mutant worms were obtained from 38 total broods. To confirm the considerable high efficiency, which seemed to be much higher than previously reported ZFN or TALEN induced mutations in *B. mori*, the same mRNA was also microinjected into Dazao, a diapausing *B. mori* strain whose genomic sequence was revealed and used as a reference strain. 189 mutant worms with similar efficiency (73.47%) were obtained from 271 injected Dazao embryos (Fig 1d, supplementary Tables 1 and 2). Three mutant phenotypes were observed in both knock-out experiments. About half of 609 total mutant silkworms could produce extremely thin-

![Figure 1](https://www.nature.com/scientificreports)

**Figure 1 | Generation and characterization of BmFib-H knock-out B. mori line.** (a) Schematic representation of the structure of *BmFib-H* gene depicting the non-repetitive regions (green boxes), repetitive regions (brown boxes), and ZFN target site (red eclair icon). (b) Part of the mutant sequences (deletion) generated by ZFN mediated genome editing. The wild-type sequence is shown at the top. The letter “D” represents deletions and the following numbers represent the size of deletion. The number within the “( )” represents the number of mutations recovered by sequence. The ZFN recognition sites are underlined. Deletions are indicated by dashed lines. (c) Ratios of different mutation types. (d) Microinjections of *B. mori* embryos and genome editing efficiency. (e-g) Cocoons (e), dissected silk gland (f), and pupae (g) of wild type Dazao and Fib-H-11. Silk gland were dissected at the wandering stage and imaged under light macro-scope and confocal fluorescent microscope. ASG, MSG, PSG represent the anterior, middle and posterior sections of the silk gland, respectively. Red box represents one silk gland cell. Pupae were imaged and weighted at 4th day post pupation. The number of investigated pupae was 50 for each group. The scale bars represent 1 cm unless specifically labeled within this figure.
layered cocoons and developed normally, while the rest were naked pupae or arrested during the larvae-pupae transition stages (supplementary Fig. 3). A total of 2250 T-clones were generated using PCR fragments amplified from genomic DNA of 450 mutant individuals to indentify the mutant sequences. Similar to ZFN-induced mutations reported previously, various mutation types including deletions, insertions and nucleotide substitutions were found, most of which were micro deletions or deletions companied with small insertions (Fig. 1b,c, supplementary Figs. 5 and 6). No significant differences were found among different phenotypes. However, to our surprise, about one-third of the mutations were in-frame mutations with single nucleotide substitution, deletion of 3n nucleotides, or insertion of 3n nucleotides, resulting point changes of fibroin heavy chain protein (Fig. 1c). And genotypes of all the mutant individuals harboring in-frame mutations were heterozygous with one wild-type allele (data not shown), indicating that the in-frame mutations were dominant. To confirm the dominance/recessive of the mutations, two typical mutations (Fib-H-D3, in which 3 nucleotides were deleted, and Fib-H-I1, in which 1 nucleotide was inserted) were subjected to genetic analysis and the results showed that Fib-H-D3 was a dominant mutation and Fib-H-I1 was a recessive mutation (supplementary Tables 4 and 5). Fib-H-I1, in which one nucleotide was inserted at the cleavage site and only a truncated N terminal hydrophilic domain of Fib-H was produced (supplementary Fig. 7), was used in the following studies.

Compared with wild-type Dazao, the posterior silk glands (PSG) of Fib-H-I1 were smaller, while the anterior and middle silk glands (ASG and MSG) were normal in size (Fig. 1f and supplementary Fig. 4). We counted the number of cells in MSG and PSG in the 1st and 5th larval stages, and no differences were observed. However, the PSG cells of Fib-H-I1 were smaller and abnormal in shape (Fig. 1f). This phenotype was in accordance with the fact that silk gland is an endoreplicating tissue and its growth solely depends on cell size increase. It is unclear how the cell size increase in PSG is regulated. The results here may provide some important information to reveal this mystery, because we shorted the PSG just by removing its product. More importantly, despite of the size of PSG and ASG cell, the lumen of Fib-H-I1 PSG was hollow inside, and MSG was almost empty with a very small amount of sericin proteins secreted by MSG cells (Fig. 1f and supplementary Fig. 4). To confirm this observation, the cocoons of Fib-H-I1 were firstly analyzed using scanning electron microscopy (SEM) and protein electrophoresis. Compared with wild-type Dazao, the cocoons of Fib-H-I1 were much lighter and thinner, while the pupae were little heavier (Fig. 1e, g), indicating the in-frame mutations were heterozygous with one wild-type allele (data not shown), indicating that the in-frame mutations were dominant. To confirm the dominance/recessive of the mutations, two typical mutations (Fib-H-D3, in which 3 nucleotides were deleted, and Fib-H-I1, in which 1 nucleotide was inserted) were subjected to genetic analysis and the results showed that Fib-H-D3 was a dominant mutation and Fib-H-I1 was a recessive mutation (supplementary Tables 4 and 5). Fib-H-I1, in which one nucleotide was inserted at the cleavage site and only a truncated N terminal hydrophilic domain of Fib-H was produced (supplementary Fig. 7), was used in the following studies.

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The most attractive feature of silk gland is its ability to storage protein in an extremely high concentration without aggregation, which is recognized to hold great potential to produce protein-based materials. However, previous attempts using any existing B. mori strains only generate a mixture of a very small amount of target proteins (such as collagen and spider silk) and abundant endogenous silk proteins. Given its “empty” feature and “full” protein production ability, we suspected that Fib-H-I1 silk gland could be an ideal system for expression of protein based materials. For this demonstration, we designed an artificial silk protein (art-Fib-H) by assembling N terminal (contains the 1st repetitive region and 1st amorphous domain) and C terminal (contains the 11th amorphous domain and 12th repetitive region) regions of BmFib-H. For easy detection, an EGFP protein was fused between 1st and 11th amorphous domains (Fig. 3a). One transgenic B. mori line, Fib-H-I1-TR, in which art-Fib-H was expressed under the control of a BmFib-H promoter, was obtained. Fib-H-I1-TR was also crossed to wild type strain (Dazao) to generate WT-TR. Fluorescence detection showed that art-Fib-H could be expressed in PSG cells and secreted into silk gland lumen in both Fib-H-I1-TR and WT-TR. Interestingly, the EGFP fluorescence exhibit distinguished pattern in the lumen of different subdivision: homogeneous in PSG, punctuate distribution in PMSG and ASG, and streaming in MMSG and AMSG (Fig. 3a). This might indicate a conformational change of art-Fib-H proteins when moving from PSG to ASG, during which the protein concentration, metal ions content, pH and shear stress were gradually changed to facilitate fiber formation. Compared with Fib-H-I1, the cocoons of Fib-H-I1-TR were thicker and 1.7 fold heavier, and the cocoon shell rate was also higher (Fig. 3d, e, f). This result was consistent with the comparisons between Fib-H-I1 and WT(Fig. 1g), and suggested that art-Fib-H was successfully expressed in Fib-H-I1-TR and could form cocoons together with sericin proteins produced in MSG. SEM analysis of the cocoons revealed that silk fibers of Fib-H-I1-TR were thicker than Fib-H-I1 and formed typical bifilar fibers in the inner layer of cocoon, which is the typical phenotype of fibroin containing fibers in the inner layer (Fig. 3c, d). The expression of art-Fib-H was also confirmed by gel analysis and western blotting using antibodies...
against N-terminal of Fib-H, full length Fib-L and P25 (Fig. 4). The mechanical properties of cocoons were also analyzed and the result showed that the cocoons of Fib-H-I1-TR were much stronger than Fib-H-I1 (Fig. 3b), indicating art-Fib-H proteins were correctly assembled into silk fibers. BmFib-H protein and spider dragline silk proteins, which are very similar to each other, were the most famous and typical proteins that used as biomaterials. The successful production of artificial silk designed from BmFib-H protein has showed the possibility to utilize Fib-H-I1 silk gland as a unique high efficient bioreactor for the production of protein-based materials and will surely pave the way for production of other biomaterials.

**Discussion**

In summary, we generated several novel B. mori mutants by targeted editing of BmFib-H gene using customized ZFN. The genome editing efficiency was higher than any previously reported. In one of the mutant lines, knock-out of BmFib-H gene resulted in a smaller and empty silk gland, abnormally developed posterior silk gland cells, an extremely thin cocoon that contain only sericin proteins, and a slightly heavier pupae. Using an EGFP fusion protein as a reporter, we showed that the genome edited silk gland could be used as a highly efficient bioreactor for recombinant protein production, whose productivity is superior to any of the known expression systems. Furthermore, by designing and successful production of an artificial B. mori silk protein, we demonstrated that the bioreactor is suitable for large scale production of protein-based materials.

Genetically modified organisms have been used as bioreactors since 1980s, during which increasing the expression yield and developing strategies for large scale production has been long-lasting challenge. Given the highly protein synthesis activity, the high protein content (more than 95%), and the simplicity of the silk protein components (only 11 known major proteins), the silk gland of B.
mori has held promise as a low-cost, high-yield bioreactor. And this promise was proved by successful production of numerous recombinant proteins. The present silk gland bioreactor generated by genome editing of BmFib-H gene further increase the content of recombinant proteins to about 50% of total cocoons, placing this bioreactor superior to any of the known bioreactors. It is reasonable to assume that other proteins such as collagen, human serum albumin and monoclonal antibodies could be expressed in this superior bioreactor with a similar yield. Furthermore, B. mori has been used for production of silk for thousand of years and are now large-scaled producing in China, Indian, Brazil and some other developing countries every year. It is very easy to achieve industrial scale production of biopharmaceutical proteins, which are in increasing demand for both analytical and clinical applications.

Figure 3 | Production of artificial silk protein in BmFib-H knock-out B. mori line. (a) Schematic representation of the structure of artificial silk protein and fluorescent imaging of different subdivision of transgenic silk gland. (b) Mechanical properties of cocoon sheet of Fib-H-I1 and Fib-H-I1-R3. For all histogram, the test number was 10. (c) SEM observation of WT, Fib-H-I1 and Fib-H-I1-R3 cocoons. (d) Diameters of silk fibers from cocoons of Fib-H-I1 and Fib-H-I1-R3. Diameters were measured from the SEM photograph, and 50 samples were used for each test. (e, f) Cocoon weight and cocoon shell rate of Fib-H-I1 and Fib-H-I1-R3. The scale bars represent 1 cm unless specifically labeled within this figure.

Figure 4 | The detection of protein in the cocoons. The proteins were analyzed by SDS-PAGE and western blotting. M represents protein markers. The numbers on the left represent the sizes of each band of the protein marker. The red arrows on the right represent the expected size of each protein. Anti Fib-H, Anti Fib-L and Anti P25 represent antibodies against the N terminal Fib-H, the full length Fib-L and P25 respectively. Proteins were run at a 4-20% gel.
Designing and construction of art-Fib-H. The sequence of N terminal (contains the 1st repetitive region and 1st amorphous domain) and C terminal (contains the 11th amorphous domain and 12th repetitive region) regions of BmFib-H and EGFP (supplementary sequence) was synthesized and inserted into pUC57-T simple using GenScript service, forming pUC57-T-art-Fib-H. The promoter sequence of BmFib-H was amplified from the genomic DNA of Dazao strains and inserted into pUC57-T- art-Fib-H using HindIII and SpeI. The resulting plasmid was digested with AscI and the fragment containing the BmFib-H promoter and art-Fib-H was inserted into a piggyBac transposon derived transformation vector pBac(3xp3-DsRed, af)

Microinjection of B. mori embryos. Two B. mori strains, which were reared on fresh mulberry leaves, were used in our study. Nistiari (N4) is a non-diapassing strain and the microinjection of embryo as in Tamura et al. described

Analysis of the cocoon proteins. 25 mg cocoon shell from 10 randomly chosen cocoons was dissolved in 1 ml of 60% lithium bisulfate (LSCN). Protein concentration was measured by the Bradford protein assay and then subjected to SDS–PAGE (6% or 4–20%, Bio-Rad). The sample preparation and treatment protocol was as described by Zhao et al. The gel was visualized by Coomasie brilliant blue staining or silver staining. Western blotting was performed using polyclonal antibodies against the N terminal sequences of Fib-H (provided by Prof. Cong Zhong Zhou from University of Science and Technology of China), the fibroin light chain and fix/25 (provided by Prof. Chun Liu from our Lab).

Microscopy and mechanical testing. The morphology of cocoons was observed using an SEM (ISS-5000, NeoScope). A piece of silk gland was placed on a glass slide and imaged under macro-scope (MVX10, Olympus). To avoid the autofluorescence of silk glands, all B. mori larva used for fluorescent imaging were reared on artificial diet. For imaging of silk glands cells or subdivisions, silk glands were fixed in PBT (PBS with 1% Triton X-100) containing 4% paraformaldehyde, for 60 min. The nuclei were counterstained with DAPI and then visualized under fluorescence microscope (IX51, Olympus) or confocal microscope (FV1000, Olympus).

to Tomita et al.’s calculation, production of 5 kg of recombinant proteins using our bioreactor only need about 10 kg of cocoon material, which can be achieved in a facility with a floor surface of about 5 m² and 1 worker. Besides the amazing recombinant production ability, we also showed that the superior bioreactor could be used to produce protein-based materials, which are also in great need for biomedical applications but lack of efficient production system. As silk gland is the only organ that can store proteins at an extremely high concentration without aggregation or denaturalization, we believe that the genome edited silk gland generated here could be a unique high efficient bioreactor for the production of protein-based materials.

The mechanism of silk proteins secretion and transport has long been an interesting but unresolved matter. The H-L subunit was thought to be important for the solubility of fibroin during intracellular transport, secretion, and luminal transport through the silk gland. Based on this knowledge, Fib-L and P25 proteins should be absent in the cocoons of the mutant line Fib-H-I1 and recovered in the line Fib-H-II-TR as the small version of Fib-H (art-Fib-H) would form a disulfide bond and interact with P25. However, our results (Fig. 4) showed that both Fib-L and P25 proteins existed in the cocoons of Fib-H deficient mutant line, Fib-H-I1 and other lines including WT, WT/Fib-H-I1 and Fib-H-II-TR. This result indicated that the H-L subunit is not necessary for the secretion of Fib-L. Given the phenotypic effect of Fib-L mutation observed in the Nd-sd mutant, we suspected that the H-L subunit is only necessary for the intracellular transport, secretion, and luminal transport of large fibroin proteins such as Fib-H. The small proteins such as Fib-L, P25, and some other silk proteins are able to secrete and transport in an independent manner. However, to completely reveal the mechanism, more investigations such as knock-out or down/up regulating Fib-L and P25 are required.

The fast-changing development of genome editing technologies in recent years and their rapid applications in increasing amount of organisms have greatly reshaped the field of genetic manipulation, especially in those non-model organisms such as B. mori that targeted mutagenesis was not achievable. ZFNs, TALENs CRISPR/Cas9 system emerged subsequently and served as the troika for genome editing tools. Recently, we showed that TALEN and CRISPR/Cas9 could introduce highly efficient targeted mutagenesis, large chromosomal deletion and even sophisticated genomic structure variations in B. mori

Methods

ZFNs. ZFNs designed to cleave the N-terminal non-repetitive region of the BmFib-H gene were purchased from Sigma-Aldrich. These ZFNs had a five-six-zinc finger protein recognizing 15/18 bases (supplementary Fig. 2). The activities of two pairs of ZFNs for BmFib-H locus were measured by the yeast MEI-1 assay as described

mRNA synthesis. Plasmid DNA was prepared using a plasmid midi kit (Qiagen), digested with XbaI (Promega) and phenol/chloroform purified. miRNAs were in vitro transcribed using polyadenylated using the MessageMaxTM T7 ARCA Capped Message Transcription Kit and Poly(A) Polymerase Tailing Kit (Epicentre Biotechnologies). The resulting mRNA was purified using the MegaClear Kit (Ambion) before transcription. The resulting mRNA was purified using the MegaClear Kit (Ambion) before transcription. The resulting mRNA was purified using the MegaClear Kit (Ambion) before transcription. The resulting mRNA was purified using the MegaClear Kit (Ambion) before transcription.

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Sequencing of the mutations. The genomic DNA was extracted using nucleic acid isolation systems PICO-1200 (Kurabo), and PCR amplified segments were inserted to a T-vector (TransGen) and sequenced using commercial service (Beijing Genomics Institute). All the methods used for PCR and T-cloning were followed the standard molecular cloning protocols or instructions provided by the manufacturers.

Analysis of the cocoon proteins. 25 mg cocoon shell from 10 randomly chosen cocoons was dissolved in 1 ml of 60% lithium bisulfate (LSCN). Protein concentration was measured by the Bradford protein assay and then subjected to SDS–PAGE (6% or 4–20%, Bio-Rad). The sample preparation and treatment protocol was as described by Zhao et al. The gel was visualized by Coomasie brilliant blue staining or silver staining. Western blotting was performed using polyclonal antibodies against the N terminal sequences of Fib-H (provided by Prof. Cong Zhong Zhou from University of Science and Technology of China), the fibroin light chain and fix/25 (provided by Prof. Chun Liu from our Lab).

Staining and imaging of silk gland. Silk glands of wandering larvae were dissected out in PBS. For whole silk gland imaging, the silk glands were placed on a glass slide and imaged under macro-scope (MVX10, Olympus). To avoid the autofluorescence of silk glands, all B. mori larva used for fluorescent imaging were reared on artificial diet. For imaging of silk glands cells or subdivisions, silk glands were fixed in PBT (PBS with 1% Triton X-100) containing 4% paraformaldehyde, for 60 min. The nuclei were counterstained with DAPI and then visualized under fluorescence microscope (IX51, Olympus) or confocal microscope (FV1000, Olympus).
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Author contributions
S.Y.M. and Q.Y.X. conceived and designed the study. S.Y.M., R.S., X.G.W., Y.Y.L., J.S.C., J.G., W.L., J.D.Z. and P.Z. performed and analyzed all the experiments. S.Y.M. and Q.Y.X. wrote the manuscript with support from all authors.

Additional information
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