CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in Bombyx mori

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CRISPR/Cas9, a bacterial adaptive immune system derived genome-editing technique, has become to be one of the most compelling topics in biotechnology. Bombyx mori is an economically important insect and a model organism for studying lepidopteran and arthropod biology. Here we reported highly efficient and multiplex genome editing in B. mori cell line and heritable site-directed mutagenesis of BmKu70, which is required for NHEJ pathway and also related to antigen diversity, telomere length maintenance and subtelomeric gene silencing, using CRISPR/Cas9 system. We established a simple and practicable method and obtained several BmKu70 knockout B. mori lines, and showed that the frequency of HR was increased in embryos of the BmKu70 knockout B. mori. The mutant lines obtained in this study could be a candidate genetic resource for efficient knock-in and fundamental research of DNA repair in B. mori. We also provided a strategy and procedure to perform heritable genome editing of target genes with no significant phenotype effect.

CRISPR/Cas9, a bacterial adaptive immune system derived genome-editing technique, has become to be one of the most compelling topics in biotechnology. Different from other genome editing tools such as zinc finger nucleases (ZFN) and transcription activator like effector nucleases (TALEN), CRISPR/Cas9 uses a protein-RNA complex to target and cleavage the target sequence. The specificity and activity is determined by RNA-DNA pairing. Therefore, CRISPR/Cas9 system is more efficient as RNA-DNA paring is usually more stable than protein-DNA interaction and much easier to implement as only short guide RNA needs to be customized to target the genes of interest1. As a result, numerous reports demonstrating utility of this technique to delete, add, activate, or suppress targeted genes in various organism including human cells2–4, mice5, rats6, zebrafish7, bacteria8, fruit flies9, yeast10, nematodes11, and crops12. With CRISPR/Cas9, scientists can create research models much more quickly, study genes much faster, and even perform gene therapy of human disease and molecular farming of crops and animals.

Bombyx mori is an economically important insect and a model organism for studying lepidopteran and arthropod biology13. As a primary producer of silk, B. mori not only serve as a major contributor to sericulture, which is an important part of economic income of some developing countries, but also has promising potential in the production of recombinant proteins, including drugs and spider silk in place of original cocoon silk14. As a research model, with complete genome sequences and numerous natural genetic resources, genetic and physiologic studies on B. mori have greatly accelerated fundamental findings on pheromones, hormones, brain structures, physiology, and genetics of insects14. To make better understanding and further exploration of this species, various biotechnology tools including transgenic B. mori15, RNAi16, ZFN mediated mutagenesis17 and TALEN mediated genome editing18 have been established. However, site-directed insertion of a recombinant DNA fragment into desired locus of B. mori genome remains a great challenge, even despite of numerous attempts using ZFN and TALEN mediated homologous recombination (HR)19. We suspect that this is because of the preferred choice of nonhomologous end joining (NHEJ) pathway during repairing of double strand breaks (DSBs). It was reported that disruption of ku70 could increase the frequency of HR mediated knock-in in various organisms from fungus, plant to human cells20–23. Ku70, an evolutionarily conserved protein from bacteria to human, is part of the ku heterodimer, which binds to DNA DSB and is required for NHEJ pathway. It is also related to antigen diversity, apoptosis24, cell adhesion25, fibronectin binding26, transposition27, telomere length maintenance and subtelomeric gene silencing28. Here, we reported the multiplex genome editing and heritable
mutagenesis of BmKu70 using CRISPR/Cas9 system, and showed that the frequency of HR mediated knock-in was increased in BmKu70 knocked-out B. mori.

Results

With the aim to demonstrate the potential of CRISPR/Cas9 system in B. mori and obtain a modified B. mori line that enable efficient targeted knock-in, we select BmKu70 as the target locus. For efficient genome editing, sufficient amount of Cas9 protein and gRNAs should be co-existed in a cell. To ensure high expression level of Cas9 protein in B. mori, we optimized the codon of Cas9 according to the codon usage in B. mori, and fused it to a very powerful pro-moter, hr3-A4, that we constructed previously30. A nuclear localization sequence (NLS) and a triple flag tag were sequentially fused to the N terminal of codon-optimized Cas9 (Fig. 1A and 1B). The high expression level of gRNA was provided by a polymerase III U6-promoter cloned from B. mori31 (Fig. 1B). Three gRNAs (ku70-1, ku70-2 and ku70-3) were designed to target exon 2 of BmKu70 gene (Fig. 2A; supplementary information, Table S1). To validate the activity of these gRNAs and Cas9 protein, ku70-1, ku70-2, and ku70-3 expression vector were co-transfected with Cas9 expression vector, respectively, into a cultured BmN cell line (BmN). Genotyping using T7 endonuclease I (T7EI) showed that PCR fragments (Fig. 1C) containing 3 gRNA target sites could be cut into expected bands in all 3 samples transfected with Cas9 and gRNAs, but not Cas9 only (Fig. 1D), suggesting that CRISPR/Cas9 system can mediate efficient genome editing in BmN cell. To confirm the T7EI genotyping, we determined the sequence of the PCR fragments using TA-clone sequencing. As the 3 gRNA target sites are very close to each other on the genomic region, we used a pair of primers flanking 3 gRNA target sites to perform PCR amplification and TA-cloning. A total of 232 TA-clone sequencing results showed that 18 out of 107, 24 out of 126, and 30
out of 99 sequences from samples treated with ku70-1, ku70-2 and ku70-3 gRNAs were mutated exactly at the ku70-1, ku70-2 and ku70-3 sites, respectively (Fig. 2B). Different gRNAs showed substantially different efficiencies of mutation rates, ranging from 16.8% (ku70-1) to 30.3% (ku70-3). We suspected that this is due to the gRNA sequence, probably by forming different secondary structures. The type of mutation was diverse with small deletions (87.5%), large deletions (8.3%) and a combination of two or more aforementioned mutations (4.2%).

Given that the specificity of CRISPR/Cas9 genome editing is solely dependent on a single gRNA, which was demonstrated above and results reported by others, we sought to determine whether multiple genomic loci could be targeted simultaneously, and whether CRISPR/Cas9 can mediate targeted genomic structure variation such as large deletion, inversion or duplication. We co-transfected the Cas9 expression vector with a mix of ku70-1, ku70-2 and ku70-3 gRNAs expression vectors. Genotyping was performed by PCR amplification, T7EI assay and TA-cloning (Fig. 1C and 1D; Fig. 2B). Unlike transfected with single gRNA, no obvious cutting band was observed in T7EI assay result (Fig. 1D). We suspected that this was due to the double or triple mutations occurred simultaneously at one DNA molecular. A total of 101 TA-clones were generated and sequenced. To our surprise, 13.9% (14 out of 101) of the sequences were mutated, of which 9 were mutations at a single locus, 2 were mutations at two loci, 1 were mutations at all three loci, indicating that the CRISPR/Cas9 can be applied to generate multiplex gene disruptions in a single *B. mori* genome. More importantly, we also observed large chromosomal deletions (1 between ku70-1 and ku70-2, 1 between ku70-2 and ku70-3) within the targeted region (Fig. 2B), suggesting that this system can be applied to induce targeted chromosomal deletions and to manipulate non-coding RNAs, regulatory regions or transgene markers.

To further test whether this system can induced heritable mutations, we co-injected the Cas9 expression vector with ku70-3 gRNA...
expression vector into the preblastama B. mori embryos. A total of 399 embryos were injected and 301 (75.4%) were hatched, indicating no significant toxic effect of Cas9 and gRNA expression. All the hatched larvae were reared on fresh mulberry leaves to adult moths (G0) and 88 of them were subjected to mating and oviposition and then T7EI assay. Most of the samples showed T7EI positive bands and 21 of them were observed to be chimeric mutations with higher efficiency (supplementary information, Fig. S1). Offspring from the 21 moths were pooled and reared to moths to screen putative mutants. Finally, 5 mutants were obtained from 576 screened G1 moths and 21 of them were observed to be chimeric mutations with higher efficiency (supplementary information, Fig. S2). TA-clone sequencing was also performed to reveal the exact mutant sequences. All the mutants were heterozygous with one wild type allele and one allele harboring 7 bp deletions (NO.42), 15 bp insertions (NO.153 and 163), 8 bp deletions (NO.350) or 2 bp deletions (NO.371) (Fig. 3B). As NO.153 and 163 mutants have the same heterozygous mutations.

Discussion

In conclusion, we successfully performed highly efficient and multiplex genome editing, including highly efficient targeted mutagenesis, multiplex genome editing of two or three mutations simultaneously and targeted chromosomal deletions, in B. mori cell line and heritable site-directed mutagenesis using CRISPR/Cas9 system. During the preparation of this manuscript, a paper demonstrating targeted mutagenesis using CRISPR/Cas9 system was published. This system is much simpler and more practicable because: (1) delivery of plasmid DNA into cells is much easier and the plasmid is more stable and stable in the cell line; (2) in CRISPR/Cas9 system, only mRNA and gRNAs through in vitro transcription, which is a time consuming and expensive process; (3) in CRISPR/Cas9 system, only the Cas9 and gRNA expression vectors were used as control. Junction PCR using primers on the inserted DsRed and genomic DNA downstream the right homology arm was performed to detect the knock-in events. The results showed that positive bands with expected size could be detected in embryos treated with donor vector plus Cas9 and gRNA expression vectors, but not Cas9 and gRNA expression vectors only. Interestingly, the band from Bmku70 knocked-out embryos was brighter than wild type Dazao, which indicated that the frequency of HR was enhanced in Bmku70 knockout B. mori.

Figure 3 | Genome typing of heritable Bmku70 mutants. (A) T7EI assay of 5 mutants. Numbers on the top of each panel represents serial number of each moth. WT represents wild type B. mori. Numbers on the left represent the sizes of the DNA ladder (DL2000 plus). The red arrows on the right indicated the expected sizes of T7EI digestion. (B) Sequences of mutations. The wild type sequence (ref) is shown at the top. Within the sequences, deletions are indicated by dashed lines, and insertions are indicated in red. The numbers on the right represents the number of mutations recovered by sequencing. (C) Chromatograms of DNA sequences of wild type Dazao, heterozygous and homozygous mutation of ku70 (number 153, in which 15 bp sequence was inserted into the target site). The gRNA target site was underlined, and the inserted sequence was indicated by a red outlined rectangle.

Figure 3C | Genotyping of heritable Bmku70 mutants. (A) T7EI assay of 5 mutants. Numbers on the top of each panel represents serial number of each moth. WT represents wild type B. mori. Numbers on the left represent the sizes of the DNA ladder (DL2000 plus). The red arrows on the right indicated the expected sizes of T7EI digestion. (B) Sequences of mutations. The wild type sequence (ref) is shown at the top. Within the sequences, deletions are indicated by dashed lines, and insertions are indicated in red. The numbers on the right represents the number of mutations recovered by sequencing. (C) Chromatograms of DNA sequences of wild type Dazao, heterozygous and homozygous mutation of ku70 (number 153, in which 15 bp sequence was inserted into the target site). The gRNA target site was underlined, and the inserted sequence was indicated by a red outlined rectangle.
Figure 4 | Transient analysis of Knock-in in embryos. (A) Schematic representation of the structure of Bm702 gene depicting exons (filled boxes). The numbers on the top indicate the exact lengths of the introns, exons, coding sequence, or un-translated region. The blue arrows and numbers (start from the transcription start site) bellow indicate the position of primers. The position of 702 gRNA target site is indicated by a red arrow. (B) Schematic representation of the structure of knock-in donor vector. The homology arms are indicated by green rectangles. (C) Sequences of mutations. The wild type sequence (ref) is shown at the top. gRNA is underlined and the red sequence represents the PAM sequence. The numbers on the right represents the number of mutations recovered by sequencing. Within the sequences, deletions are indicated by dashed lines. (D) Junction PCR results using primers DsRed-F and 702(+2234)-R. Numbers on the right represent the sizes of the DNA ladder (DL2000 plus). The red arrows on the left indicated the expected sizes of junction PCR.

gRNA needs to be customized to target the genes of interest, we also constructed a simple strategy developed by Cong et al.3 that utilized two adjacent BbsI sites. To target a gene of interest in B. mori, the only thing that required is just to insert the 20 bp-long guide sequence between the BbsI sites using annealed oligonucleotides. More importantly, unlike BmBlos2 gene disrupted by ZFN18, TALEN19 and CRISPR/Cas920 in previous reports, in this study, we choose a target gene with no significant phenotype and successfully screened heritable mutants. The strategy and procedure used in this study set up a simple and easy-to-use system of CRISPR/Cas9, which can be used in other species like Drosophila21, Zebrafish22, and perhaps other insects. Ku70 is required for NHEJ pathway and also telomeric gene silencing. Previous report showed that inactivation of Ku70 protein could significantly increase the frequency of homologous recombination mediated targeted gene knock-in in Arabidopsis23. We also showed that the HR frequency was increased in Bmku70 knock-out B. mori. These results indicated that the Bmku70 knock-out B. mori lines obtained in this study could be a candidate genetic resource for efficient knock-in and fundamental research of DNA repair in B. mori.

Methods

Design and construction of cas9 and gRNA expression vectors. Coding sequence of Cas9 was codon optimized, fused to a triple flag tag and a nuclear localization signal. The whole sequence (supplementary information) was synthesized and inserted into pUC57-T-simple using Genscript service, forming pUC57-Cas9. To generate the cas9 expression vector, an enhanced promoter in silkworm cell lines Hr3-A4 promoter was amplified with primers (hA4-F and hA4-R) from 1180[hisI1008RmAct4lUcArrPA] vector24 stored in our laboratory, and subsequently cloned into the vector using Xhol and SpeI, forming pUC57-hA4-Cas9. U6 promoter sequence and the gRNA sequence were placed upstream and downstream of two BbsI enzyme sequences, and the whole sequence (supplementary information) was also synthesized and inserted into pUC57-T-simple using genscript service, forming pUC57-gRNA. Guiding sequence of ku70-1, ku70-2, ku70-3 and 702 gRNAs were synthesized as two reversely complemented oligos, annealed and inserted to BbsI treated pUC57-gRNA, forming pUC57-ku70-1, pUC57-ku70-2, pUC57-ku70-3 and pUC57-702, respectively.

Construction of knock-in donor. The left homology arm (702L) and right homology arm (702R) were amplified from the genomic DNA of wild type Dazao using primers 702L(-213)-F/702L(+1024)-R and 702R(+1024)-F/702R(+2105)-R, respectively. The 3Xp3-DsRed-sv40 (3DS) was amplified from plasmid pBac[3Xp3-DsRed, fa]. All the PCR fragments were inserted into a TA-cloning vector, pMD19-T-Simple Vector (Takara), forming T-702L, T-702R and T-3DS. 702L was cloned into T-702R using AscI, forming the final knock-in donor vector, 702-3DS. All the vectors were sequenced to confirm the sequences and directions of inserted fragments.

Transfection of BmN cell lines. Plasmids for transfection were purified with a Plasmid Mini Kit (Qiagen). Plasmids were mixed with Cellfectin II reagent (Invitrogen) at a ratio of 1 : 5 (w/v) and then transfected into the BmN cell lines. The final concentration of each plasmid was 100 ng/µl. The cells were collected 72 hours post transfection. All transfections were done three times independently and in triplicate.

Microinjection of B. mori embryos. A diapausing strain, Dazao, which is wildly used as a wild type silkworm, was utilized in this study. The larvae were reared with fresh mulberry leaves at 25°C, 75% RH. Parental embryos (P) were incubated at 15°C and 75% RH to produce nondiapusing eggs (G0), which are suitable for microinjection. The microinjections were performed utilizing TransferMan NK2 micromanipulator and Femto Jet 5247 microinjector (Eppendorf) under an SXZ16 microscope (Olympus) as we reported previously24. The final concentration of each plasmid was 250 ng/µl. Embryos were injected within 2 hours after oviposition. The injection opening was sealed with instant glue (Konishi co.) and injected embryos were incubated at 25°C and 90%RH. Larvae hatched from the injected embryos were collected and reared on fresh mulberry leaves.

Crossing strategy. The G0 moths were sibling crossed with each other, and the 88 moths were subjected to genome typing using T7EI. Only G1 offspring from 21 most
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