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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 interest[1]. As a result, numerous reports demonstrating utility of this technique to delete, add, activate, or suppress targeted genes in various organism including human cells[2–4], mice[5,6], rats[7], zebrafish[8], bacteria[9], fruit flies[10], yeast[11], nematodes[12], and crops[13]. 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 biology[14]. 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 silk[15]. 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 insects[14]. To make better understanding and further exploration of this species, various biotechnology tools including transgenic *B. mori*[16], RNAi[17], ZFN mediated mutagenesis[18] and TALEN mediated genome editing[19] 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)[20]. 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 cells[21–24]. *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, apoptosis[25], cell adhesion[26], fibronectin binding[27], transposition[28], telomere length maintenance and subtelomeric gene silencing[29]. Here, we reported the multiplex genome editing and heritable

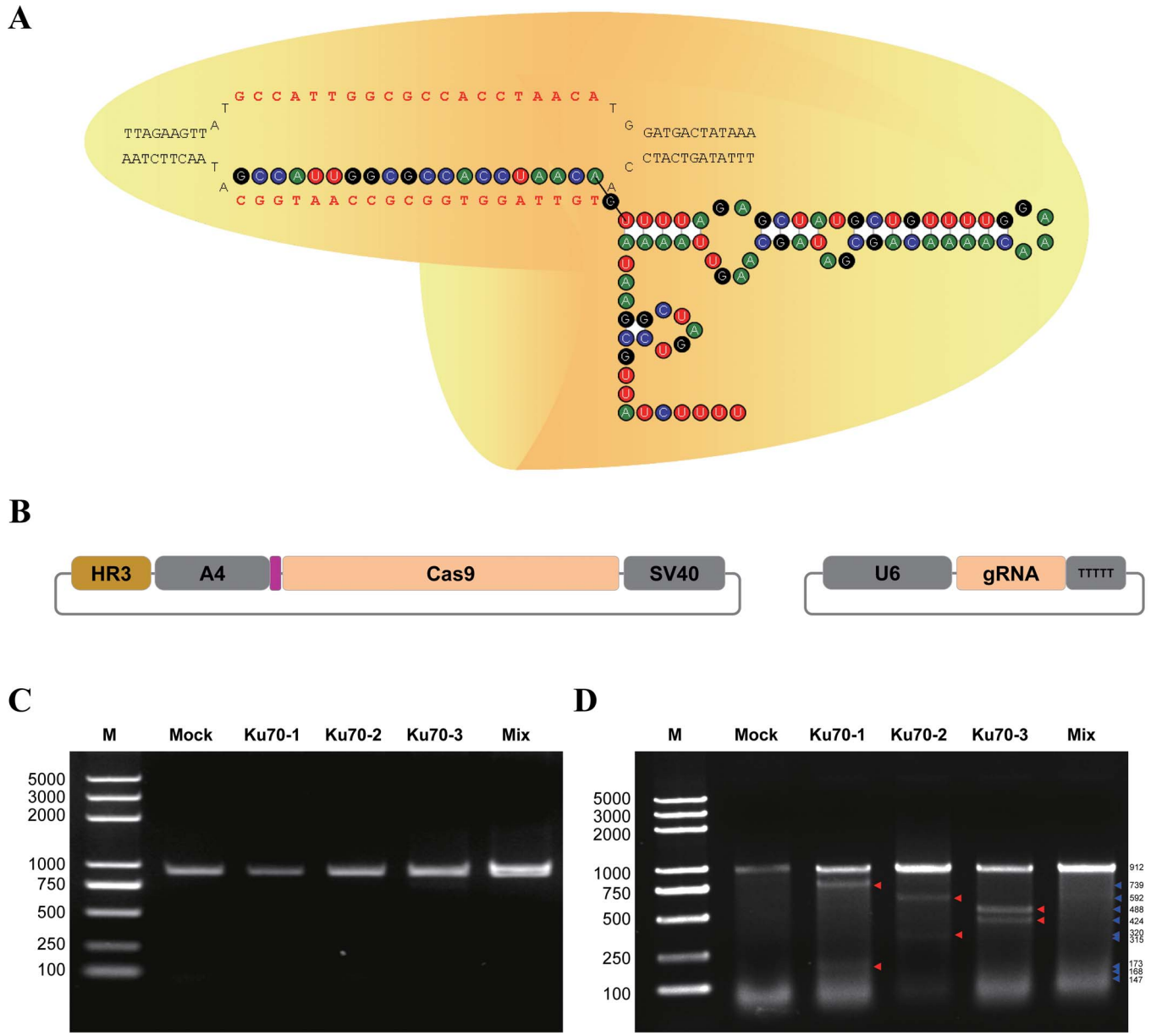

Figure 1 | CRISPR/Cas9 mediated genome editing of *BmKu70* in BmN cell. (A) Schematic representation of Cas9 protein and gRNA (ku70-2) complex binding to target DNA strands. (B) Schematic representation of Cas9 expression vector and gRNA expression vector. A triple flag tag and a nuclear localization signal (NLS) (purple) are fused to the N-terminal of codon-optimized Cas9. (C) PCR detection and T7EI assay (D) of CRISPR/Cas9 activity in BmN cell lines. Mock represents untreated cells. Ku70-1, Ku70-2, Ku70-3 and Mix represent cells treated with different gRNAs together with Cas9. Numbers on the left represents the sizes of the DNA ladder (DL2000 plus).

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 promoter, hr3-A4, that we constructed previously[30]. A nuclear localization sequence (NLS) and a triple flag tag were sequentially fused to the N terminal of Cas9 (Fig. 1A and 1B). The high expression level of gRNA was provided by a polymerase III U6-promoter cloned from *B. mori*[31] (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 *B. mori* 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

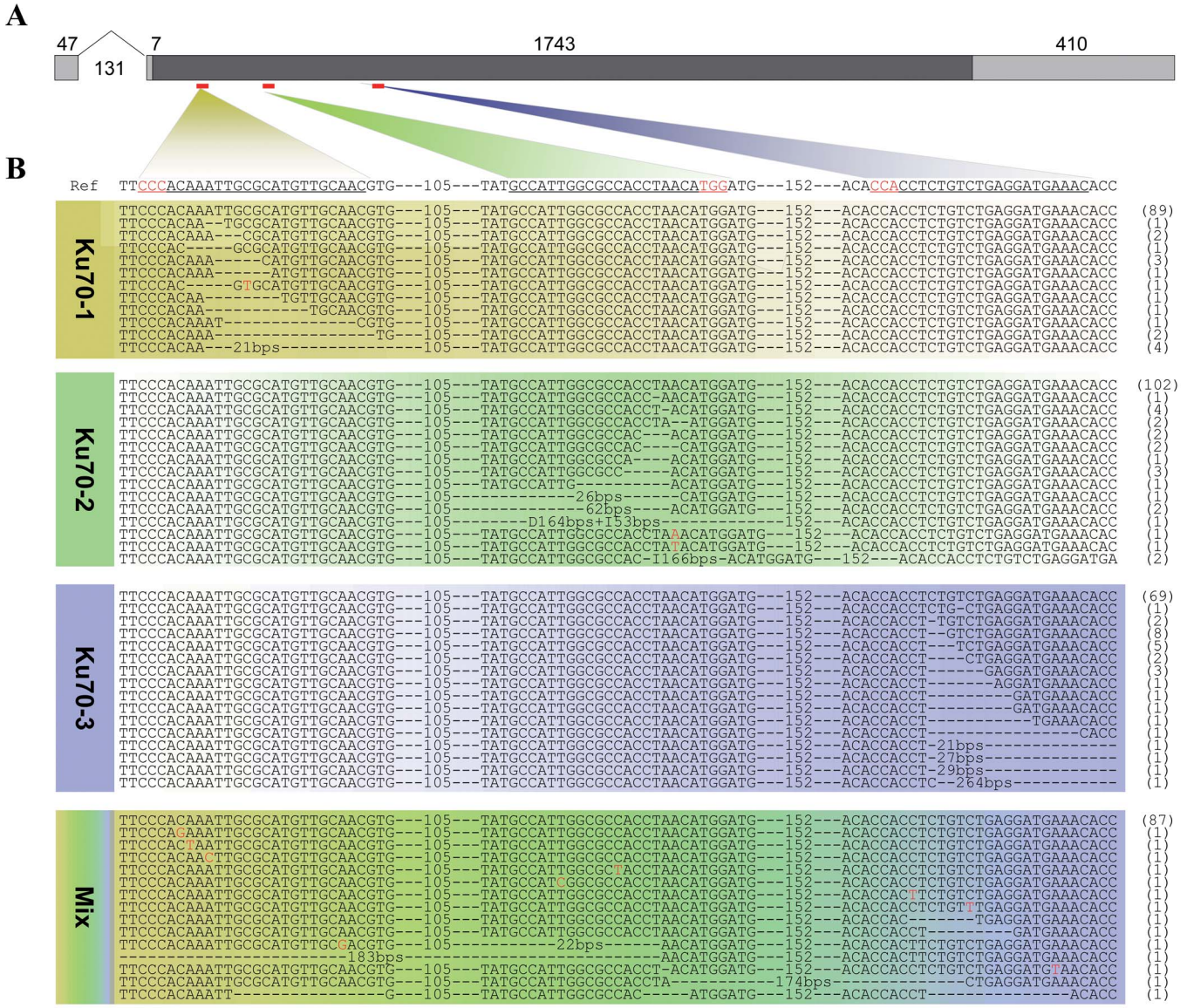

Figure 2 | Sequencing of CRISPR/Cas9 treated cells. (A) Schematic representation of the structure of *Bmku70* gene depicting exons (filled boxes) and gRNA target sequences (sequences at the bottom). The numbers indicate the exact lengths of the introns, exons, coding sequence, or un-translated region. (B) Sequences of mutations. The wild type sequence (ref) is shown at the top. gRNA sites are underlined and the red sequence represents the PAM sequence of each gRNA. The marks on the left indicate treatment of Cas9 together with ku70-1, ku70-2, ku70-3 or a mixture of the three gRNAs. The numbers on the right represents the number of mutations recovered by sequencing. Within the sequences, deletions are indicated by dashed lines, and substitutions/insertions are indicated in red.

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 locus, 1 were mutations at all three locus, 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

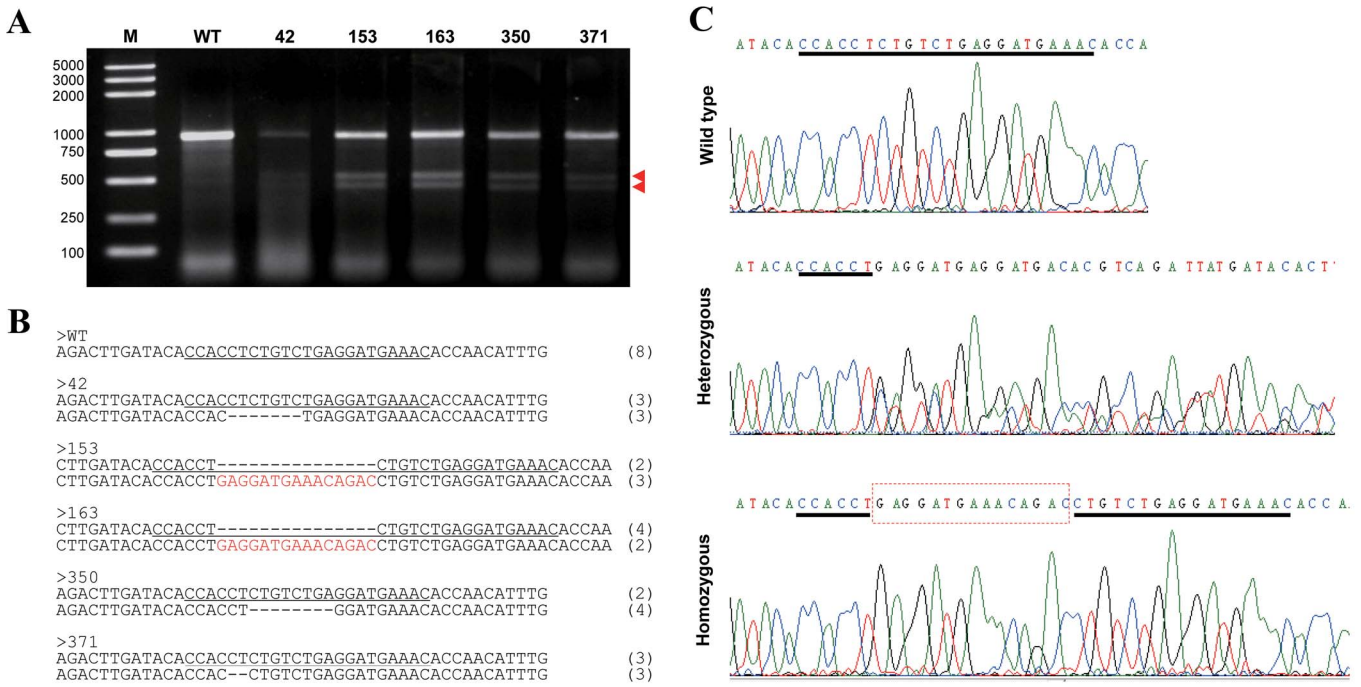

Figure 3 | Genome typing of heritable Bmku70 mutants. (A) T7EI assay of 5 mutants. Numbers on the top of each panel represents serial number of each moth. WT represents wild type *B. mori*. Numbers on the left represent the sizes of the DNA ladder (DL2000 plus). The red arrows on the right indicated the expected sizes of T7E1 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.

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 using T7EI genotyping (Fig. 3A; 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 mutant sequence, we crossed them with each other to generate homozygous mutations. Of 24 sequenced G2 moths, 5 were wild type *Dazao*, 13 were heterozygous and 6 were homozygous mutants (Fig. 3C). The ratio of three genotypes was 1 : 2 : 1, indicating a Mendel inheritance of the mutant. These results indicated that CRISPR/Cas9 system can be used to generate heritable targeted mutations.

To test whether disruption of *Bmku70* could increase the frequency of HR mediated knock-in in *B. mori*, a transient analysis in the embryos of *Bmku70* knocked-out *B. mori* was carried out. *Bm702*, a novel gene located on the Z chromosome that was successfully edited using TALEN[32], was selected as our target gene for knock-in (Fig. 4A). As the frequency of conventional HR in *B. mori* was extremely low, we first introduced a DSB, which was demonstrated to enhance gene targeting in cultured silkworm cells[33], near the TALEN target site using CRISPR/cas9 system. The cleavage activity was measured in cultured cells and the TA-clone sequencing results showed that 14 of 90 sequences carrying deletions ranging from 1 to 31 nucleotides (Fig. 4C). The frequency (15.6%) was equal to ku70-1, which further indicated that the CRISPR/Cas9 system was efficient and stable in *B. mori*. A knock-in donor, in which a 3Xp3-DsRed-sv40[34] expression cassette was flanked by a 1237 bp left homology arm upstream and a 1081 bp right homology arm downstream, respectively, was constructed (Fig. 4B). The donor vector was microinjected with Cas9 and gRNA expression vectors into the embryos of wild type *Dazao* and *Bmku70* knocked-out *B. mori*, respectively. Wild type *Dazao* embryos treated with 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*.

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 of the *BmBLOS2* gene by direct microinjection of Cas9 mRNA and sgRNAs was published[35]. In comparison, our system is much simpler and more practicable because: (1) delivery of plasmid DNA into cells is much easier and the plasmid is more stable than RNA; (2) as both Cas9 and gRNA were expressed by expression vectors directly after transfection, there is no need to acquire Cas9 mRNA and gRNAs through in vitro transcription, which is a time consuming and expensive process; (3) in CRISPR/Cas9 system, only

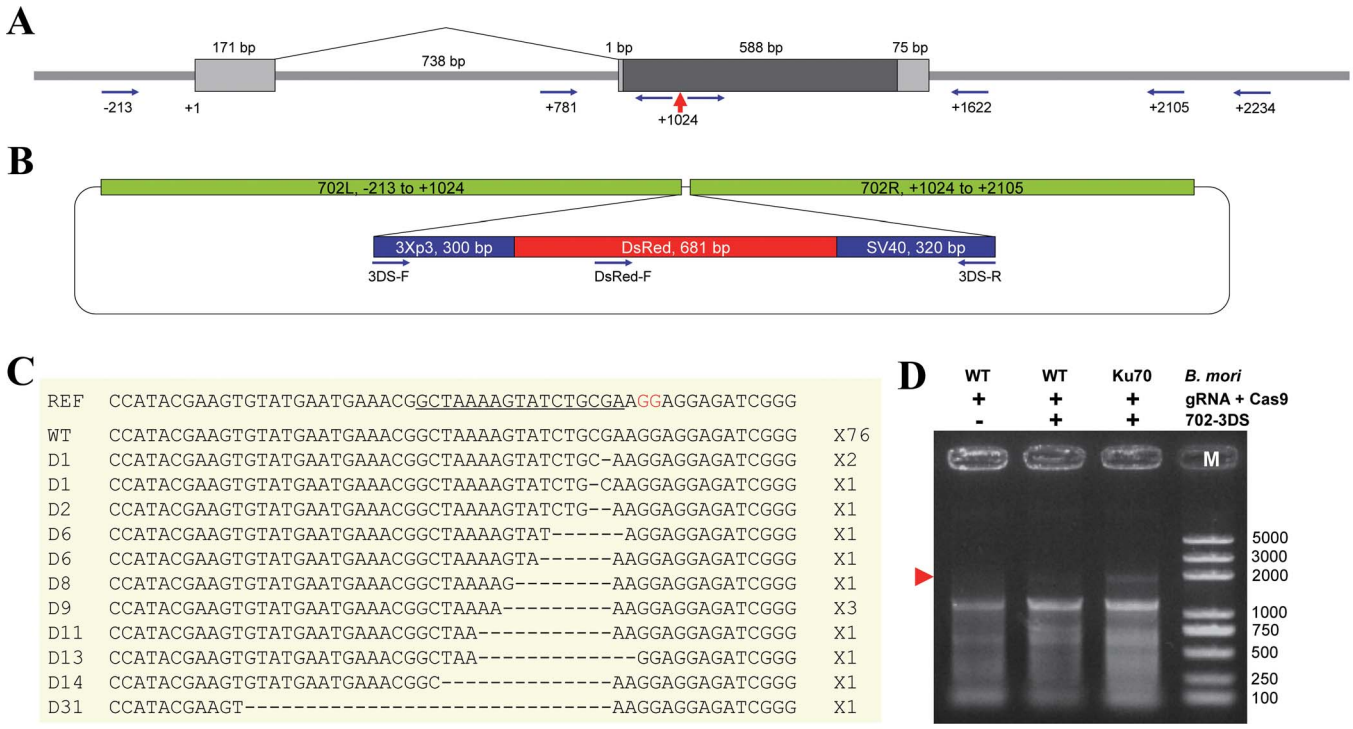

Figure 4 | Transient analysis of Knock-in in embryos. (A) Schematic representation of the structure of *Bm702* gene depicting exons (filled boxes). The numbers on the top indicate the exact lengths of the introns, exons, coding sequence, or un-translated region. The blue arrows and numbers (start from 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 site 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 ZFN[18], TALEN[19] and CRISPR/Cas9[35] 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 good example to perform heritable genome editing in *B. mori* and perhaps other insects. Ku70 is required for NHEJ pathway and also related to antigen diversity, telomere length maintenance and subtelomeric gene silencing. Previous report showed that inactivation of ku70 protein could significantly increase the frequency of homologous recombination mediated targeted gene knock-in in Arabidopsis[24]. 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[hrs1000BmAct4LUCser1PA] vector[30] stored in our laboratory, and subsequently cloned into the vector using *Xho*I and *Spe*I, 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 702(−213)−F/702(+1024)-R and 702(+1024)-F/702(+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 *BamH* I and *Xba* I, forming T-702LR. 3DS was then cloned into T-702LR using *Asc* I, 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/uL. 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 SZX16 microscope (Olympus) as we reported previously[19]. The final concentration of each plasmid was 250 ng/uL. 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

T7EI positive G0 moths were pooled and allowed to develop to the adult stage. 288 male G1 moths and 288 female moths were randomly selected, and sibling crossed with each other to produce G2 eggs (288 broods). Then all 576 moths were subjected to T7EI genome typing. The offspring of T7EI positive moths were heritable mutants.

T7 endonuclease I assay. The PCR product was treated 5 units of T7 endonuclease 1 (NEB) for 15 min at 37°C and then precipitated by addition of 2.5 volumes of ethanol. The precipitated DNA was analyzed by agarose gel electrophoresis.

Sequencing of the mutations. The genomic DNA was extracted using nucleic acid isolation systems PI-1200 (Kurabo), and PCR amplified segments cloned into pMD19-T-Simple and sequenced. All the methods used for PCR sequencing were followed the standard molecular cloning protocols or instructions provided by the manufacturers.

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Author contributions

S.Y.M., J.S.C. and Q.Y.X. conceived and designed the study. S.Y.M., J.S.C., X.G.W., Y.Y.L., J.D.Z., W.L., J.G. and R.S. performed the study. S.Y.M., P.Z., J.S.C. and Q.Y.X. analyzed and wrote the manuscript. All authors reviewed the manuscript.

Additional information

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