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Highly efficient multiplex targeted mutagenesis and genomic structure variation in *Bombyx mori* cells using CRISPR/Cas9

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ABSTRACT

Bombyx mori is an economically important insect and a model organism for studying lepidopteran and arthropod biology. Using a highly efficient CRISPR/Cas9 system, we showed that this system could mediate highly efficient targeted genome editing of a single gene locus, large chromosomal deletion or inversion, and also multiplex genome editing of 6 genes simultaneously in BmNs cell line derived from *B. mori*. The simplicity and high efficiency of our system provide unprecedented possibilities for researchers to implement precise and sophisticated manipulation of a chosen *B. mori* gene in BmNs cells easily in a limited time course, and perhaps new opportunities for functional genomics of *B. mori* and other lepidopteran insects.

1. Introduction

Bombyx mori is a domestic insect with huge economic importance and a model organism for studying lepidopteran and arthropod biology (Goldsmith et al., 2005). As a primary producer of silk, *B. mori* does not only serve as a major contributor to sericulture, which is an important source of income for Japan and some developing countries including India and China, but also has promising potential in the production of recombinant proteins, including drugs and spider silk in place of original cocoon silk. 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 (Goldsmith et al., 2005; Xia et al., 2014). However, genetic manipulation tools are always lacked in this species. In the past, only transposon-based transgenic technology (Tamura et al., 2000; Uchino et al., 2007) and recombinase mediated cassette exchange (Long et al., 2012; Yonemura et al., 2013) were well established. These technologies have received the increasing concerns in the limitations of random insertion, possible instability from the transposons, and potential genomic sequence damage from the site-specific recombinases. To make better understanding and further exploration of this species, more advanced genetic manipulation technologies are urgently required.

The emergence and rapid application of site-specific genome editing using engineered nucleases has greatly reshaped the field of genetic manipulation during the past 10 years. Zinc finger nucleases (ZFNs), chimeric proteins engineered to induce a site-specific double-strand break (DSB), emerged and immediately became the first powerful tool for the genomic manipulation around 2005. At the end of 2010, TAL effector nucleases (TALENs), a tool similar to ZFNs was discovered. The DNA binding motif of a TALEN is an engineered transcription activator-like effector (TAL effector) protein, which recognizes DNA in a modular fashion. TALEN was rapidly recognized to be a better tool, as TALEN is much more predictable and simple to design and assemble. With these innovative tools, genome engineering in model organisms became much easier, and more importantly, genome editing has been achieved in various non-model organisms including rats, rice, tobacco, modeka, yellow cattle fish, wheat, pig, rabbit, cow and monarch butterfly, all of which site specific genetic techniques were not available before. To meet the increasing demand of advanced genetic manipulation technologies in *B. mori*, ZFN was pioneered to achieve site specific mutagenesis and was proved to

Abbreviations: CRISPR/Cas, clustered regularly interspaced short palindromic repeat/CRISPR associated; th, tyrosine hydroxylase gene; re, red egg gene; fl, flugellos locus; kynu, kynureninase gene; T7EI, T7 endonulease I.

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be inefficient (Takasu et al., 2010). TALEN was then introduced in this species and showed considerable efficiency not only for site specific genome editing (Ma et al., 2012), but also for targeted genomic structure variations (Ma et al., 2014b). We also established a high-efficiency system for construction and evaluation of customized TALENs for *B. mori* genome editing (Wang et al., 2013a). However, as the central region of the TALEN is composed of highly conserved tandem arrayed repeat units, the generation of custom TALEN remains a challenge.

Recently, a new approach based on clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 DNA endonucleases to mediated sequence modification appeared and has become one of the most compelling topics in biotechnology. Bacteria and archaea have evolved an RNA-based adaptive immune system that uses CRISPR and Cas proteins to invade viruses and plasmids (Wiedenheft et al., 2012). The Cas9 endonuclease from type II CRISPR/Cas system was programmed to induce double-strand break (DSB) in vitro together with a chimeric gRNA that fused crRNA with tracrRNA (Jinek et al., 2012). Further investigations showed that this system can be used to induce targeted DNA cleavage in cultured human cells (Cho et al., 2013; Cong et al., 2013; Mali et al., 2013) and zebrafish (Chang et al., 2013; Hwang et al., 2013). Different from ZFN and TALEN, the specificity of CRISPR/Cas9 is determined by RNA-DNA pairing. A very important feature of CRISPR/Cas9 system is its simplicity as only short guide RNA needs to be customized to target the genes of interest. Numerous reports demonstrating utility of this technique to delete, add, activate, or suppress targeted genes in various organism including human cells, mice, rats, zebrafish, bacteria, fruit fly, yeast, nematodes, and crops. We suspected that this system could be used to perform large scale genome editing in *B. mori*. And 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 (Wang et al., 2013c).

Here we reported that the CRISPR/Cas9 system could mediate targeted disruption of a single gene locus, genomic structure variation such as large deletion or inversion, and multiplex genome editing of up to six genes simultaneously in BmNs cell line derived from *B. mori*, using a much simpler and more practicable plasmid-based CRISPR/Cas9 method. A transient assay in *B. mori* embryos showing considerable genome editing activity of a whole organism was also performed.

2. Materials and methods

2.1. Design and construction of cas9 and gRNA expression vectors

The construction of Cas9 expression vector, in which the codon-optimized Cas9 was driven by hr3-A4 promoter, was described elsewhere (Ma et al., 2014a). T-U6-gRNA1, in which U6 promoter sequence and the gRNA1 scaffold sequence were placed upstream and downstream of two *BbsI* enzyme sequence was constructed previously (Ma et al., 2014a). Two additional gRNA expression vectors, T-U6-gRNA2 and T-U6-gRNA3, were constructed by insertion of annealed synthetic oligomers into *BbsI* site of T-U6gRNA1. All target sequences were synthesized as oligomers and inserted into the *BbsI* site of T-U6gRNA vectors.

2.2. Transfection of BmNs cell line and DNA extraction

The cell line BmN-SWU1 (BmNs) was established from the ovarian tissues of *B. mori* larvae in our laboratory, and supplemented with Tc-100 medium containing 10% fetal bovine serum (FBS) in 27 °C. Cultures were changed every 2–3 days. BmNs cells were seeded into 24-well plates (Corning) approximately 24 h prior to transfection with 70–90% confluent per well. The cells were transfected with 0.5 μg cas9 plasmid, 0.5 μg gRNA using X-tremeGENE HP DNA Transfection Reagent (Roche) following the manufacturer's recommended protocols. When the cells treated with one more gRNAs, equal amount of gRNAs was used for transfection. The genomic DNA of cells that harvested 3 days after transfection was extracted using E.Z.N.A.® Tissue DNA Kit (Omega) following the manufacturer's protocols.

2.3. T7 endonuclease I assay

The genomic DNA extracted as described above was amplified with primers for target genomic regions. PCR products were purified with 3M NaAC and alcohol, then digested with T7 endonuclease I (T7EI) (NEB) and analyzed on 2% agarose gel. The targeting region of mutant samples were amplified and cloned into pMD19-T Simple Vector (TaKaRa). The sequencing data were analyzed to detect the forms of mutation in target region and estimate the mutation efficiencies.

2.4. RNA and protein extraction

Cells were treated with plasmid DNA as described above, and washed with phosphate buffered saline (PBS). Total RNA from treated cells was extracted using Total RNA kit II (Omega) following manufacturer's protocol. The RNA was quantified by Naonodrop 2000(Thermo Scientific) and normalized to same concentration. The reverse transcription was proceeded using Reverse transcriptase M-MLV (RNase H-)(TaKaRa) with a specific reverse primer 5′ AAAAAGATAACGGACTAGCCTTAT 3′ for gRNA2 scaffold and 5′ TGTGGAACGCTTCACGATT 3′ for U6 gene. The product was amplified in order to detect the expression level of gRNA. Protein of treated cells was extracted using NP-40 lysis buffer (Beyotime) and tested with western-blot using Flag tag antibody (Beyotime).

2.5. Microinjection of B. mori embryos

A diapausing strain, Dazao, which is wildly used as a wild type *B. mori*, was utilized in this study. Embryos were prepared as described by Ma et al. (2013) with minor modifications. The microinjections were performed utilizing TransferMan NK2 micromanipulator and Femto Jet 5247 microinjector (Eppendorf) under an SZX16 microscope (Olympus). The mixture of gRNA plasmid target for kynu gene (kynu-2) and cas9 were microinjected into the embryos within 3 h after oviposition. The treated embryos were incubated at 25 °C for 2 days following by DNA extraction.

3. Results and discussion

3.1. CRISPR/Cas9 system mediated highly efficient site specific mutagenesis

BmBlos2, which involved in the determination of epidermal color in silkworm (Fujii et al., 2010), was widely used as a marker gene to demonstrate the activity of genetic manipulation tools such as ZFN (Takasu et al., 2010), TALEN (Ma et al., 2012) and CRISPR/Cas9 system (Wang et al., 2013c). For estimating whether our CRISPR/Cas9 system could work, we chose *BmBlos2* gene as our first target gene. *BmBlos2* contains four exons and three introns and encodes a 145 amino acids protein (Fig. 2A). Three target regions and sequences (termed B1, B2 and B3, which targeted the 1st, 2nd and 3rd exons, respectively) (Fig. 2A and Table S1) were designed following the rules that U6 promoter transcription only initiated with G and the whole target sequence must end with PAM motif sequence, NGG. As an internal negative control, B2 were designed