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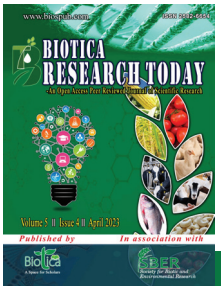

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Recent Advances on CRISPR/Cas9 based Genome Editing in Silkworms *Bombyx mori* L. (Lepidoptera: Bombycidae)

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Abstract

Bombyx mori, the mulberry silkworm, is of the utmost significance in the discipline of sericulture due to extensive production of glossy silk fibres. Apart from playing a crucial role in the textile industry, it is also a model organism for various scientific studies and a bioreactor that facilitates the production of recombinant protein. Therefore, numerous efforts are being made to appropriately alter silkworm genetics. The infamous CRISPR/Cas9 genome editing technique is currently in use for manipulation of vital genes as *BmBlos2, BmKu70, BmFibH, BmCactus, BmJhe, immediate early-1* (*ie-0*) and (*ie-2*) in *B. mori*. Therefore, this review briefly highlights the advancements made to manipulate the genome of silkworms in the current decade using CRISPR/Cas9 as a tool in hand. It is also understood as to how CRISPR-Cas9 systems heightened the basic research on *B. mori* and other organisms, demonstrating the enormous promise of insect biotechnology across a wide range of disciplines.

Keywords: CRISPR/Cas9, Genetics, Silk, Silkworms

Introduction

Due to its high fidelity and reprogrammable feature the CRISPR/Cas9 systems are continuously exploited to understand the complex genomes of various organisms. These repeating CRISPR sequences were first identified in bacteria and archae as a component of their pre-adaptive acquired immune system against bacteriophages. The CRISPR locus comprises of the highly conserved identical repeat sequences (20-40 bp) and the spacer sequences that are generally incorporated into the host genome during the attack by the intruders. Upstream to this locus is *cas* genes, that encodes for vital endonucleases called CRISPR associated proteins or Cas proteins. The whole mechanism is divided into 3 steps *viz*., acquisition, biogenesis and interference (Figure 1) wherein the Cas proteins acting as molecular scissors cleave and degrade the intruder genome. CRISPR/Cas9 is considered as an ideal genome editing tool owing to its high precision, cost effectiveness, simplicity and ability to simultaneously target multiple genes in organisms.

Various components of the CRISPR machinery are Cas nucleases, guide RNA (it guides the Cas endonuclease to cleave the target DNA at a specific site) and PAM (Proto-spacer adjacent motif) sequence bordering the target complementary sequence. Thus, the central concept is Cas proteins aided by gRNA scans the genome for PAM sequence and cleave both the strands of target DNA leading to the formation of Double stranded breaks which are further repaired by cell repair pathways: Homology directed repair and Non-homologous end joining. CRISPR/Cas9 system is currently exploited for the genomic manipulation of various insects as *Drosophila melanogaster*, *B. mori*, *Apis mellifera*, *Tribolium castaneum*, *Aedes aegypti*, *etc*.

Model Organism: *B. mori*

B. mori is an oligophagous insect with four distinct stages (egg, larvae, pupae and adult) in the life cycle. It has a short developmental cycle and also serves as a reliable experimental model for scientific investigations. It is regarded as an ideal organism for human disease evaluation and helps to determine the therapeutic potential and efficacy of various antibiotics for humans. Additionally, it is employed in bioassay studies to assess the toxicity for different insecticides (phoxim and fenvalerate). In addition, it is regarded as a model organism for drug screening, assessment of several virulence factors, and the identification of the genes is crucial for the pathogen's virulence. Furthermore, it evaluates the toxicity of nanomaterials. Thus it is quite evident that the

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model experimental material *B. mori* holds the central stage in several scientific investigations worldwide.

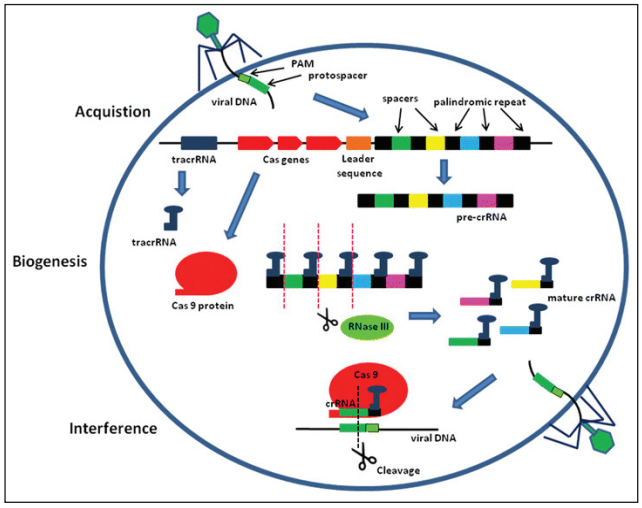

Figure 1: Mechanism of CRISPR action involving acquisition, biogenesis and interference

Utility of CRISPR/Cas9 Systems in *B. mori*

The first successful manipulation of the genome of *B. mori* was demonstrated targeting the *BmBlos2* gene that is integral for the production of opaque integument in the larval instars. Due to CRISPR/Cas9 based editing the larval integument lost pigmentation and appears translucent (Figure 2). Thus, *BmBlos2* is considered as a useful phenotypic marker gene for mutation studies. *BmBlos2* is an orthologous of the *Blos2* gene in humans. This simple example highlights the relevance of using CRISPR/Cas9 not only in *B. mori*, but also various other organisms of interest (Wang *et al.*, 2013). Consequently, six other genes (*ebony, red egg, yellow-e, fugellos, kynureninase* and *tyrosine hydroxylase*) along with the *BmBlos2* gene was targeted to access the multifarious potential of CRISPR/Cas9 and to assess the potential off target activity of the system. Mutations were successfully induced at each gene site leading to altered gene action without much of target activity. Thus, the above experiments validated CRISPR/Cas9 as a flawless and accomplished technology to achieve precise and elaborate target mutagenesis simultaneously of multiple gene loci.

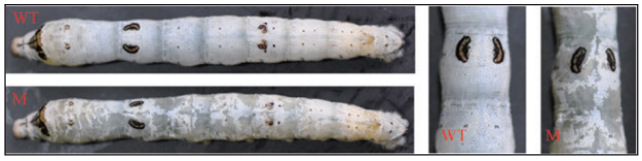

Figure 2: A comparative view of *B. mori* larval cuticle of wild type (WT) and the mutant (M); Mutants can be visualised with translucent skin patches

Similarly, *BmKu70* gene that codes for the highly conserved Ku70 protein was targeted. The Ku70 protein plays a vital role in cell adhesion, apoptosis, *etc*. Previous studies on the gene hypothesised that knocking out of the *BmKu70* gene increases the frequency of homologous repair significantly. Knockout of the gene in the Z chromosome of *B. mori* by genetically manipulating the embryos confirmed the above hypothesis (Ma *et al.*, 2014). These experiments thus open up the way for more basic and fundamental research in model organisms as *B. mori*.

CRISPR/Cas9 systems can also be employed to assess the function of certain genes that are doubtful at present by possibly silencing them and assessing the effect of gene deletion in the target species. Silencing of the IGFLP (Insulin-like growth factor-like peptide) gene in the *B. mori* led to smaller ovaries and reduced fecundity in females. However, the size of laid eggs and subsequent development of the mutant organisms were not significantly affected. It was concluded that IGFLP did not impair fertility of *B. mori*, but is crucial for ovarian development in the insect (Fujinaga *et al.*, 2019).

Genetic manipulations using CRISPR/Cas9 can up or down regulate gene action and subsequently increase or decrease the accumulation of gene product. The *BmFibH* (Fibroin Heavy Chain) gene is usually down regulated in the wild type. Activation of this gene lead to high expression of *BmFibH* in the transformed cells. Further it was also shown that this gene plays a crucial role in silk gland development of the insect. Therefore knocking out *BmFibH* gene resulted in production of pupae with thin shells or naked pupa and subsequent death. Furthermore, knock in of the spider silk genes into *B. mori* genome was appraised. These genes were knocked into the introns of *BmFibL* and *BmFibH* genes (Zhang *et al.*, 2019). Therefore, the silk with improved mechanical and industrial properties can be successfully obtained.

In silkworms infected with bacteria or fungi, there is activation of Toll and Imd pathways and genes relating to production of Anti-microbial peptides and lysozymes. They serve as the innate defence mechanism of the insect. *BmCactus* is an important gene in the Toll pathway that is inactivated due to attack of pathogens. It was witnessed that CRISPR/Cas9 based inactivation of the gene led to a sharp rise in the levels of the anti-microbial peptides and lysozymes in the organism. This holds an immense importance in the field of life science and clinical research.

CRISPR/Cas9 can also be used to target genes involved in hormone regulation within the insect. The duration of the fifth instar larval was found to be 24 hours longer when the *BmEo* (Ecdysteroid oxidase) gene was depleted using the CRISPR-Cas9 method. Similarly knock-down of the *BmJhe* (Juvenile Hormone Esterase) led to increase in the instar duration of the fourth and fifth instar larvae due to reduced JH metabolism in the insect. Increasing the larval duration and subsequent development of larger larva and bigger cocoons ultimately increases the silk productivity. This explains the feasibility of using CRISPR/Cas9 for higher economic gains using silkworms.

Utility of CRISPR/Cas9 in Anti-BmNPV Therapy

The BmNPV infection in the silkworms leads to significant economic losses in the sericulture industry. CRISPR/Cas9 is used to develop an effective anti-viral strategy to combat the infection due to these viruses. Gene *immediate early-1* (*ie-0*) and (*ie-2*) involved in the viral replication and

propagation process were targeted. Disruption of these genes significantly increased the resistance against BmNPV. There was 65% increment in the survival rate of transgenic silkworms. Therefore, genetically improved strains of *B.mori* carrying beneficial mutations can be developed as a strategy to manage BmNPV infection (Dong *et al.*, 2019). Various other genes subjected to CRISPR based manipulations are enlisted in table 1.

Table 1: List of some important *B. mori* genes manipulated

Target gene	Function of the gene	Mutation type	Objective
BmKmo	Eye colouring and Egg formation	Deletion	Phenotypic analysis
BmYki	Organ development and regeneration	Deletion	Functional gene analysis
BmIdgf	Involved in pigmentation	Deletion	Analyse melanisation mechanism

Conclusion

CRISPR/Cas9 is a revolutionary genome editing technology that finds numerous applications in agriculture, industry, medical, biotechnology, clinical research, life science and entomology. Thus it holds tremendous potential to facilitate research in *B. mori*. Gene knock in or knock out leading to developmental of desirable traits and improved resistance to viral infection in silkworms *via* CRISPR/Cas9 is witnessing huge success and popularity worldwide. Further, the same can be used for targeting various processes and pathways involved in sericin and fibroin synthesis and enhancing the properties of silk fibres so obtained. Thus CRISPR/Cas9 and its notable applications in suitably manipulating the silkworm genome can serve as a boon for increasing production and productivity in the sericulture sector.

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