Frontiers of Genetic Engineering: Cutting-Edge Genome Editing for Silkworms and Honeybees

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Abstract

Nowadays, entomology is one of the most studied domains due to the pivotal role of insects in ecology, agriculture, the pharmaceutical industry, and medicine. Insects are the most diverse and numerous group of species, and their impact represents a high interest for the scientific community. Due to their well-documented high applicability in various areas, two of the most studied insects are silkworms and honeybees. One of the most important roles of silkworms is their role in medicine and the pharmaceutic industry as bioreactors and model organisms. Honeybees represent the main pollinator for numerous crops and wild plants and present a major contribution to the food chain. Despite their beneficial role in nature and the numerous products obtained from the hive, the bees face several stressors, both biotic and abiotic. The most important progress in this direction has been made by applying genome editing tools to enhance their productivity and agricultural sustainability. Until now, researchers have obtained disease-resistant individuals, limiting the high need for chemical treatments and promoting environmental health. These advancements exhibit progress in biotechnological innovations, including the production of innovative biomaterials for medical applications, underscoring the broad impact of these techniques on the economy, ecology, and medicine.

Keywords: biotechnological innovations, honeybees, medicine, silkworms

1. Introduction

The studies in the entomology area have seen remarkable advancements in recent years [1]. One reason for this direction can be attributed to the more recent advancements in the techniques used for genome editing, which can be coupled with the advancements in genome sequencing and genome resequencing for species of interest [2-4]. Another reason for their study can be attributed to the significance of the insect of their products from the perspective of their economic value, which is the case for species of insects such as *Bombyx mori* and *Apis mellifera* [3].

2. Bombyx mori

The silkworm, *B. mori*, is one of the most important insects for the textile industry, producing silk, one of the most appreciated textiles. In the last decade, its role has been extensively enlarged, for instance, it is one of the most feasible model organisms to be used in research. Also, it exhibits a key role in the pharmaceutical industry by being used as a bioreactor to produce recombinant proteins. Besides these two great applications, the silkworm receives a great level of attention from the scientific community due to the role of silk as a biomaterial [5-7]. When it comes to the biomaterials area, fibroin, the main protein found in the silk thread, exhibits extraordinary properties, like great mechanical resistance, biocompatibility or biodegradability. Up to this moment, there have

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been obtained numerous recombinant proteins and a wide range of biomaterials, from nanoparticles to biofilms [8-11].

2. Apis mellifera

Honey bees are well known for the wide variety of benefits they bring to humanity, starting with a wide variety of products we can obtain from the hive and ending with the pollination services, which have a significant positive effect on crop production and also ensure the maintenance of biodiversity and environmental protection [12, 13]. Compared to silkworms, the honey bee genome seems to be edited to answer specific research questions and not for industrial applications [1]. The more recent advancements in genome editing techniques disrupt specific genes, which, in turn, can help to understand better the biological processes that influence their eusocial functions [2;1]. The honey bees are placed at the pinnacle of eusociality and could serve as an excellent model to study as many of the eusocial concepts are rotted at the intersection between sociology, genetics and evolution [14- 16].

3. Genome editing technologies

Up to this day, there have been described three major players when it comes to genome editing technologies, namely the Zinc finger nucleases (ZFNs), Transcription activator-like effectors (TALENs), and Clustered regularly interspaced short palindromic repeats (CRISPR) systems [17-22]. Each listed technology implies unique mechanisms that exhibit specific advantages and drawbacks. These powerful tools lead to living organisms' genome edits, contributing to progress in genetic research, improving disease treatment, recombinant protein production, and enhancing agricultural practices. The choice between the three systems depends on the specific requirements of the research, including specificity, type of genetic alteration targeted, cost, and complexity, respectively [23, 24] (Figure 1).

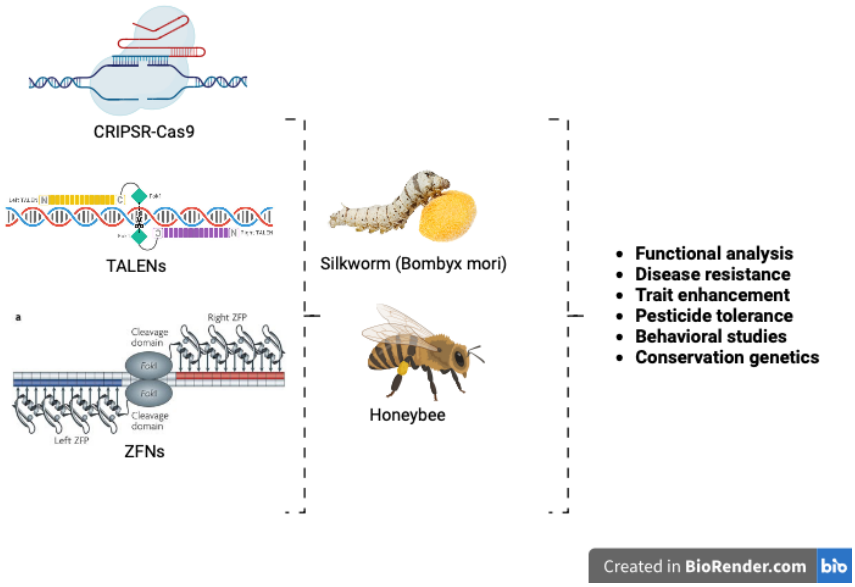

Figure 1. Graphical representation of genome editing technologies applied in sericulture and apiculture (Created with BioRender).

The first editing system that has been used is the ZFNs technology, that involves specific restriction enzymes. These elements are designed to target specific sequences in order to induce double-strand breaks. Even if it is a highly specific technique, designing and constructing the proteins involves high costs that limit large-scale usage. The system has two major players that are merged, more specifically, DNA-binding proteins, and a FokI nuclease domain. The zinc finger domains can be engineered to target specific DNA sequences, directing the FokI nuclease to induce a double-strand break at a specific location in the genome. When a disruption in the DNA sequence occurs, the

cell assures certain repair mechanisms, which can be harnessed to introduce mutations through non-homologous end joining, or to insert a new DNA sequence by the process of homology-directed repair. ZFNs have been particularly useful in looking into the genetic basis of insect phenotypic traits or behaviors. By creating targeted knockouts or modifications in specific genes and observing their impact on the phenotypic changes, the gene function can be elucidated. This is crucial for understanding complex biological processes such as development, reproduction, and sensory perception in insects [23, 25, 26].

In terms of sericulture, the ZFNs system has been employed for inducing targeted mutations. For instance, one of the first studies of this genome editing tool describes its successful use to knock down the gene encoding the pivotal protein found in the silk tread, namely the *Bmfih-H* gene. The research focus was to remove the endogenous protein in order to increase the expression level of target exogenous recombinant proteins, thus highlighting the great role of silkworms as bioreactors. Their results revealed that by knocking down the *Bmfih-H* gene the larvae exhibited a smaller silk gland and a thin cocoon that contained sericin, however, this process significantly increased the production of exogenous proteins [25-28]. Moreover, the ZFNs technology has been used to facilitate homologous recombination in silkworms. This application is particularly valuable for inserting exogenous genes at specific loci. This assures progress in the sericultural sector by enhancing silk production and developing silk threads that exhibit certain properties.

By inducing specific gene knockouts, ZFNs lead to a better understanding of gene functions, including studies on genes responsible for coloration, growth, and resistance to disease, contributing to better understanding and potentially improving sericulture practices. ZFN technology has been extensively explored to improve certain characteristics of silkworms like disease resistance, better silk quality, and increased production. These modifications play a crucial role in the text industry, as well as in the pharmaceutical and medical fields [26,28].

The second system, TALENs, respectively, involves a principle that is similar to the one of the ZFNs, however, it is distinct when it comes to the DNA-binding mechanism. This construct implies bacterial proteins that bind DNA sequences with high specificity, and transcription activator-like effectors (TALEs), respectively. Similar to the previously described technology, these are fused to a nuclease that performs DNA cleavage. Comparing them, it is more feasible to design TALENs in order to target specific genomic locations than ZFNs, moreover, it involves lower costs [24].

In terms of TALENs applicability in the sericulture industry, numerous studies successfully reported its utilization to perform specific genome edits.

Table 1 lists the main studies that involved the TALENs technology as a genome editing tool in silkworms.

Table 1. The applicability of TALENs technology on *Bombyx mori*

Target gene	Study scientific relevance	Gene function	Reference
ku80	Evaluating the feasibility of knocking-in target genes	Non-homologous end joining	[29]
blos2	Functional gene analysis	Larval epidermis	[28]
ptpmt1	Functional gene analysis	Egg formation	[30]
let-7	Functional gene analysis	Pupal metamorphosis	
fibl	Mass silk production	Silk production	[31]
fibh	Recombinant proteins production	Silk production	[32]
fhx	Functional gene analysis	Silk production	[33]
dsx	Functional gene analysis	Sex determination	[34]
abcb1	Evaluation of susceptibility determination	ATP-powered translocation	[25]

One of the most groundbreaking tools in terms of genetic engineering that has revolutionized the fields of life science-related fields, is represented by the CRISPR-Cas system. It was originally discovered as a part of the immune system in bacteria, more specifically, for the first time, it has

been observed in *E. coli*. Nowadays, it has been adapted to edit genes in a wide range of organisms, including insects, animals, plants, and humans [35-39]. In terms of using the CRISPR-Cas system in the medical field, Wang et al. (2014) successfully used the CRISPR-Cas9 technology to perform deletions in the ccr5 gene and inactivate it. This specific gene is correlated with one incurable disease, that affects people all over the globe caused by a retrovirus named the human immunodeficiency virus or HIV. When the infection occurs, the CD4+T cells are the first target of the virus. The first step of the infection mechanism is membrane fusion, more specifically the retrovirus' envelope glycoprotein (gp120) binds to the primary receptor CD4 localized on the membrane of the CD4+t cells. To successfully infect the host cell, the virus requires the presence of two specific chemokine co-receptors: CCR5 and CXCR4. Thus, mutations on both alleles of the genes responsible for the synthesis of the co-receptors, lead to an individual's resistance against HIV infection.

Regarding entomology, the CRISPR-Cas system has become a significant tool, providing new ways to explore and manipulate insect genetics for research, pest control, and other applications. CRISPR-Cas enables researchers to perform targeted gene editing in insects, allowing them to investigate the function of specific genes. For example, by knocking out genes, their roles in insect development, reproduction, and behavior, can be elucidated. This is crucial for understanding basic biological processes and can also offer insights into the development of targeted pest control strategies that are less harmful to non-target species and the environment [40].

When it comes to the sericultural applicability of the CRISPR-Cas system, major progress has been made.

Table 2 lists the most recent studies in this direction.

Table 2. The most recent studies of CRISPR-Cas applicability in sericulture

Target gene	Gene function	Delivery method	Reference
let-7	Silk gland development	Microinjection	[41]
mamo	Melanin pigmentation		[42]
fhx-L1	Silk production		[33]
hh	Adult morphogenesis		[43]
fru	Sexual behavior		[44]
period, timeless, clock and cycle	Circadian clock		[45]
dome	Wing development		[46]
foxo	Insulin-like signaling pathway		[47]
toll10-3	Cellular immunity		[48]
eckl1	Silk Gland Development		
yki	Ovary maturation		[49]
sob	Wing morphology formation		[50]
phyhd1	Controls egg side		[51]

In a novel study, Mei et al., (2024) used the CRISPR-Cas9 system to knock down the *BmC/EBPZ* gene to evaluate its function. In numerous studies, one of the most used mutants is represented by a recessive male sterile lineage (GMS), that exhibits small larvae as a specific characteristic. By knocking down the *BmC/EBPZ* gene, the authors observed a similar phenotype in the individuals as the characteristics identified in the GMS mutants. They concluded that the targeted gene plays a key role in the GMS mutation [52].

In another recent study, the CRISPR-Cas9 technology was applied in order to knock out the gene in order to perform functional analysis. The authors inactivate the target gene in two parts specific loci of the silk gland, namely middle and posterior silk glands. The *BmEcKL1* gene encodes a member of the wide group of ecdysteroid kinase-like family. They are known to play a key role in the detoxification process. Later studies led to a hypothesis that this target gene is involved in silk gland development. By successfully inactivating the *BmEcKL1* gene, the authors confirmed the hypothesis of the gene's implication in silk protein production. moreover, their results revealed that by knocking out this gene in the two parts of the silk

gland, the silk production yield was significantly increased. These findings are incredibly important not only for the textile industry but for the medical field [53].

In the same direction, in order to enhance the silk yield, one of the most important approaches is to manipulate the regulatory mechanism of the silk proteins. Keeping this in mind, it is essential to continually gain knowledge in this subject. Cao et al. (2022) used the CRISPR-Cas9 system to knock out the *Bmdimm* gene in order to elucidate its role in the regulatory mechanism of the main protein of the silk thread, fibroin. By targeting this specific gene, they observed a negative impact on the silk yield and shorter larval stages. Moreover, great weight loss was identified in both larvae and adults [54].

On another topic, Feng et al. (2024) used the same technology in order to target a specific intramembrane protease, namely *SPP*, a highly conservated element, that exhibits a great role in immune surveillance when it comes to viral proteins. It displays a pivotal impact on numerous organisms in the viral infection resistance. By knocking down the target gene through the CRISPR-Cas9 system, the authors aimed to obtain silkworms that exhibit great resistance against the Bombyx mori Nuclear Polyhedrosis Virus (BmNPV). This study is of great importance due to the significant losses that this pathogen causes. The results of this research revealed that this approach has great potential to obtain B. mori breeds that exhibit significant resistance to BmNPV [55].

4. Advancements in gene editing for honey bees

In 2016, Kohno et al. successfully applied a CRISPR-Cas9 technique to *A. mellifera* and obtained bees with knock-out genes. The targeted gene, in this case, was mrjp1 (Major royal jelly protein) [56]. In this study, the application of this technique on the embryo germline cells resulted in two queens, one of which was able to produce genome-edited drones [56]. A later study using this technique targeted the mKast gene (middle-type Kenyon cell-preferential arrestin-related protein) which is tough to regulate worker bee behaviour and it was successfully applied to obtaining drones in which the respective gene was not expressed. To validate results the semen of the knock-out drones was harvested and used to artificially inseminate queens thus obtaining homozygous mutant workers [57]. The results suggest that *mKast* gene does not seems crucial for the development and maturation of drones. And further studies of this gene for *A. mellifera* could reveal important information regarding its implications in social behaviour [57].

The reported success rate for successfully editing the mrjp1 and mKast gene using CRISPR/CAS9 was relatively low [56; 57]. However a More recent CRISPR-Cas9 methods have allowed for a higher success rate than previously reported methods. Three major improvements were reported as important for the higher success rate are the improvement of injection site along with its timing and delivery for the embryo; the embryos were injected using a sgRNA (single guide RNA) and CAS9 protein complex and managed to obtain a success rate for the candidate genes above 70% while previous studies obtained a success rate slightly above 10 % [59].

The high success rate and the one-step balletic knockout will allow this improved technique to perform gene functional studies in honey bees. Moreover, it allows the possibility of analysing genes critical for the development of the embryo, larva, or pupa [59].

Another study that uses CRISPR-Cas9 in honey bees focused on the study of taste receptors in honey bees, by editing the *AmGr3* gene in worker bees the team was able to inactivate the gene, the resulting mutants presented a loss of responsiveness to fructose with no significant difference in the response to sucrose confirming that this conserved receptor is highly specific for fructose [60]. Compared with other insect species the number of gustatory receptor genes seems relatively low for *Apis mellifera* with a total of only 10 gustatory receptor genes and only three of them seem to have a higher expression level in the worker bees brain during feeding [60-62]. Further studies that used CRISPR-Cas9 on the gustatory receptor genes *AmGr1* and *AmGr2* revealed a reduced reaction for worker bees that did not have *AmGr1* active proving that this gene is responsible for a response to sucrose and glucose. At the same time, it seems that *AmGr2* acts as a co-receptor for *AmGr1*[63]. Moreover, it was noticed that *AmGr2* does not modulate the fructose-specific receptor AmGr3 [63].

Another study on honey bees used CRISPR-Cas9 to knock out the *Amyellow-y* gene to understand its functional role better [64]. This gene is involved in the pigmentation process and individuals for whom

this gene does not work correctly present defects in pigmentation. By targeting the exon 2 of the *Amyellow-y* gene, they were able to obtain both worker and mutant drones that presented a yellowish appearance [64]. By analyzing other genes involved in the melanin synthesis pathway, it was noticed that the *Amyellow*-y plays a crucial role, and it was observed a significant upregulation for the *laccase2* gene for mutant drones compared with the wild type. *Lacase2* seems to influence cuticular pigmentation and hardening, and an experiment that involved the knockdown of this gene resulted in an impairment in exoskeleton differentiation [65]. Nie et al. even proposed a practical application as the adults for the knock-out *Amyellow-y* gene as mutant drones can be easily identified by phenotype and this technique could be applied as a potential marker for screening genomic editing in *A. mellifera* [64].

In order to gain more insights into the development of honey bees and caste differentiation in 2019, a team of scientists used the CRISPR-Cas9 approach on specific genes related to sex differentiation [66]. Using the newer methods developed for honey bees, the team developed a high percentage of double mutant larvae with inactivated genes [66], 2019; McAfee et al., 2019). Using these mutants with the inactivated feminizer (*fem*) gene, it was possible to prove that this gene has a direct input in sex determination, and as a result, it became clearer that the differentiation between future workers and future queens is not only given by nutrition [66]. Moreover, by, mutating the *dsx* (double sex) gene, which operates downstream of the fem gene, resulted in more valuable insights. The mutant workers with the *dsx* gene presented intersex reproductive organs, proving its implication in the control of the female differentiation of the reproductive organs [66].

Later studies using CRISPR-Cas9 were able to confirm that the glubschauge (*glu*) gene regulates eye differentiation in honey bees and acts as a sex-specific developmental regulator [67]. These new findings brought a significant contribution from the molecular point of view in understanding how sexually dimorphic structures are formed between castes. Moreover, it suggests the existence of a specific mechanism in which sexual dimorphism in some body parts is regulated by specific regulator genes [67].

Table 3 lists the most recent studies in this direction.

Table 3 . The most recent studies of CRISPR-Cas applicability in apidology

Target gene	Gene function	Delivery method	Reference
mrjp1	main factor for differentiation of the queen larva from worker larvae.	Microinjection	[56]
mKast	regulate the behaviors of worker bees		[57]
AmGr3	a specific fructose receptor		[60]
AmGr1	sugar-induced response to (glucose, sucrose, maltose, trehalose and melezitoze)		[63]
AmGr2	co-receptor for sucrose and glucose perception		[63]
Amyellow-y	gene involved in the pigmentation process		[64]
fem	instructs female development and maintains the female signal during development,		[66]
dsx	regulating sexual reproductive organs development		[66]
glu	regulator of sex-specific eye morphology		[67]

4. Conclusions

The silkworm, *B. mori*, holds a prominent position when it comes to life sciences-related domains, due to its great roles as a model organism and bioreactor. Tremendous progress has been made in applying genome editing tools in order to obtain transgenic larvae for recombinant production. Also, these systems are used to edit specific genes to reveal gene function or enhance silk properties.

Limitations to elucidate the specific functions of a targeted gene: it seems that the respective gene cannot have an essential function during the larval or pupa stage [56].

On the other hand, there are many ethical and legal restrictions regarding the rise of genetically modified honey bees, and it is recommended that these experiments bee kept indoors [1; 58].

Honeybees, as a superorganism, offer a fascinating example of social organization and collective intelligence in nature [14]. Their study provides

valuable insights into the principles of biological organization and the evolution of complex social systems [68]. However, to fully understand the evolution of sociality in this superorganism and the role of different genes in influencing the hive's individuals, further comprehensive research is warranted.

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