CRISPR/Cas9 Applications in Functional Genomics of Lepidopteran Pests

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Abstract. CRISPR/Cas9 technology originates from bacteria and represents the third-generation gene-editing tool. It has significantly advanced research on gene functions in Lepidopteran insects. This article studied Spodoptera frugiperda and Plutella xylostella in the order Lepidoptera. And summarized the application of CRISPR/Cas9 technology in studying the body color genes, reproductive genes, insecticide resistance genes, and gene drive systems of the two insects. Studies have found that when knocking out reproductive related genes in insects such as SfPBAN, PxDCr-1, PxNap1, can disrupt their mating habits or lower their ability to reproduce. When editing insect resistance genes such as SfABCB1, PxABCB1, and PxRDL, pests become much more sensitive. When knocking out insect color related genes, such as SfKmo and SfYellow-y, can change the color of their eggs. This article also summarizes the research on the gene drive system in Plutella Xylo stella, which can be combined with CRISPR technology for the dissemination of gene edited products in wild populations. Overall, CRISPR/Cas9 technology is a very useful method for analyzing gene functions in Lepidopteran pests. In practical applications, this technology still faces some challenges, such as gene editing efficiency, ecological safety, and field controllability.

Keywords: CRISPR, Lepidoptera, *Spodoptera frugiperda*, *Plutella Xylo stella*.

1. Introduction

Lepidopteran insects are the second largest order of insects, including moths, beetles, and butterflies. Some insects of this purpose pose a threat to plants, such as Spodoptera frugiperda and Plutella Xylo stella, which are typical pests and cause widespread crop and economic losses, posing a major threat to global agriculture. Due to the high reproduction rate, mobility, and significant pesticide resistance of lepidopteran insects, the effectiveness of traditional strategies such as chemical pesticides, genetically modified crops, and insect viruses has been greatly reduced [1, 2].

The birth of CRISPR/Cas9 technology has brought about a revolutionary change to the research of lepidopteran pests. Most lepidopteran insect species are not genetic model organisms, and many lepidopteran insects, such as cotton bollworms (Helicoverpa armigera), have problems of insensitivity to RNAi or rapid degradation of dsRNA. Therefore, CRISPR/Cas9 technology can replace the traditional gene silencing methods. As the third-generation gene editing technology, CRISPR/Cas9 can achieve targeted editing of single or multiple genes through the precise design of sgRNA. It can identify target genes solely by synthesizing sgRNA. It has core advantages such as high targeting accuracy, simple operation, and controllable editing efficiency. CRISPR/Cas9 technology can be used to study gene functions, conducting research on single or multiple genes through gene knockout, gene knock-in, and multiplex editing. Research on the gene functions of Lepidopteran insects using CRISPR/Cas9 mainly focuses on aspects such as pesticide resistance, pigmentation, and mating and reproduction [3]. For example, OUYANG et al. used CRISPR/Cas9 technology to construct a homozygous mutant population of PxABCB1 gene, demonstrating that this technology can be effectively applied in the study of gene function in Lepidopteran insects.

Although CRISPR/Cas9 technology has accelerated the study of gene function in Lepidopteran insects, there are still many issues that need to be addressed. Off target effects, low knock-in rates,

and imprecise gene editing have always been research challenges for CRISPR/Cas9 technology. The efficiency of germline mutation is still limited, and multi gene editing and fine regulation technologies are not yet mature. For Lepidopteran insects, microinjection technology is highly difficult and has a high mortality rate due to the hard embryonic shell and short injection window.

This study summarizes the latest progress of CRISPR/Cas9 in the research of Lepidopteran pests, with a focus on topics such as insecticide resistance, pigmentation characteristics, reproductive control, and gene drive systems. The aim is to clarify the research status of gene functions of two main Lepidopteran pests, and provide theoretical basis for formulating effective control strategies in agricultural environments.

2. Characteristics of Lepidopteran Insects

Lepidoptera is a large order of insects, including moths and butterflies, which are widely distributed in various climatic and topographical regions [4]. The body of lepidopteran insects is divided into three parts: head, chest, and abdomen. Adult wings are membranous wings with scales on the surface, which is an important basis for identifying species. Adult mouthparts are siphoning type, suitable for sucking nectar and other liquid food; The larvae are chewy and used to feed on plant tissues. Its developmental characteristics are complete metamorphosis, with four life stages of egg, larva, pupa, and adult, and most of them are phytophagous. Because of their feeding habits, many species of Lepidoptera are agricultural pests, which have a huge impact on the global agricultural harvest and economy.

With the development of molecular biology technology, at present, a variety of lepidopteran insects have completed genome sequencing, among which the more representative are silkworm, cotton bollworm, and other groups. According to the results of genome sequencing, there are some differences in the genome size of lepidopteran insects, usually more than 300MB to 1GB, and the content of repetitive sequences is high, which may be closely related to the evolution of lepidopteran insects. At the gene level, the lepidopteran genome contains a large number of gene families related to metabolism, visual perception, and other functions. For example, UGT (UDP glycosyltransferase) family genes related to the production of detoxification metabolic enzymes in lepidopteran pests, or detoxification enzyme genes related to larval feeding on plant tissues, usually expand in phytophagous species to cope with the defense of plant secondary metabolites.

3. CRISPR /Cas9-mediated Gene Function Analysis in *Spodoptera frugiperda*

Spodoptera frugiperda belongs to the Lepidoptera of Noctuidae, which is native to tropical and subtropical regions of the Americas and has been recognized by the Food and Agriculture Organization of the United Nations (FAO) as the world's major migratory pest [5]. The main characteristics of *Spodoptera frugiperda* are a wide host range, strong reproductive ability, and strong adaptability. It can infect 353 species of plants, including more important crops such as corn, rice, and cotton [6]. This report expounds the body color, reproduction, and drug resistance of *Spodoptera frugiperda*.

3.1. Functional analysis of body color-related genes in *Spodoptera frugiperda*

Spodoptera frugiperda, as a typical agricultural pest, its body color visual markers that are particularly important. Salum et al.'s team used CRISPR technology to knock out body color-related genes such as kynurenine 3-monooxygenase (kmo) gene, ebony gene, scarlet gene, and yellow-y gene of *Spodoptera frugiperda*, so as to distinguish its eyes, body, and other colors from the wild type as effective visual markers [7]. After the knockout of the kmo gene, the eggs of the knockout individuals appear emerald green, while the wild type is dark brown, and the body color of the larvae of the knockout individuals will also appear dark brown, different from that of the wild type larvae [8]. The egg coloring degree of yellow-y gene knockout individuals is lower than that of the wild type, and

the knockout larvae have transparent epidermis, the head and epidermis are light yellow, and the melanin of the knockout adults is significantly reduced [9]. Based on this way to study pigment genes, researchers can understand the mechanism of pigment production at various stages. This method is expected to be applied in pest management species based on gene editing.

3.2. Functional Analysis of Reproduction-Related Genes in *Spodoptera frugiperda*

In order to solve the problem of *Spodoptera frugiperda*, the mating of *Spodoptera frugiperda* was interfered with. Sex pheromone synthesis activating neuropeptide (PBAN) regulates the synthesis of sex pheromones in female moths, which is a key regulator of their reproductive behavior. Ashok et al.'s team used CRISPR/Cas9 to design targeted sgRNA, and achieved gene editing by injecting ribonucleoprotein (RNP) complex (Cas9+sgRNA) through an embryo microscope. Knockout of the PBAN gene led to female moths being unable to attract male moths after knockout, and the number of eggs laid after mating was 0, or the hatching rate was 0. The number of eggs laid by the knockout male moths and wild female moths decreased after mating, and the hatching rate decreased to 73% [10]. The mutation of the PBAN gene can significantly disrupt mating behavior and reduce the number of eggs laid and hatching rate, especially in the attraction of female moths to male moths, which provides a specific scheme for pest control based on reproductive interference.

3.3. Functional Analysis of Resistance-Related Genes in *Spodoptera frugiperda*

The widespread use of chemical pesticides has led to genetic resistance and reduced the efficiency of common chemical pesticides. To solve the problem of drug resistance, Li et al.'s team used CRISPR/Cas9 gene editing technology to edit the ATP binding cassette (ABC) transporter of *Spodoptera frugiperda*, and constructed a sfABCB1 knockout strain of *Spodoptera frugiperda* (sfABCB1-KO), and detected the sensitivity of common chemical pesticides such as beta cypermethrin and compared it with wild type (WT) [11]. Through biological monitoring, the sensitivity of the third instar larvae of the ABCB1-KO strain to Emamectin, beta-cypermethrin, and chlorantraniliprole was increased by 4.18 times, 2.94 times, and 2.89 times, respectively, according to the estimation of the median lethal concentration (LC50) [11]. The Shi et al. team used CRISPR/Cas9 for gene editing of UDP glycosyltransferases (UGTs) - related gene family, developed sfUGT50A15 knockout line (sfUGT50A15-KO), and detected its sensitivity to chlorantraniliprole and Emamectin benzoic acid, and compared it with wild type (WT) [6]. Through biological monitoring, based on the LC50 value, the sensitivity of the third instar larvae of the sfUGT50A15-KO strain to chlorantraniliprole increased by 5.67 times, and the sensitivity to Emamectin benzoic acid increased by 5.00 times, and the mortality was significantly higher than that of the wild type under high concentration of Emamectin benzoic acid [6]. Based on the study of the resistance related genes of *Spodoptera frugiperda*, this paper provides new ideas for the pest control of *Spodoptera frugiperda*.

4. CRISPR /Cas9-mediated Gene Function Analysis in *Plutella xylo stella*

The *Plutella Xylo stella* is an arthropod species belonging to the genus *Plutella* within the family Plutellidae of the order Lepidoptera. It is distributed throughout China. The larvae of the *Plutella Xylo stella* feed on plants of the Cruciferae plants, and the adult insects mostly appear at night and have phototaxis. The *Plutella Xylo stella* has high reproductive capacity, strong ecological adaptability, and robust insecticide resistance, causing serious harm to agricultural production. Chinese scientists You Minsheng and others have for the first time published the whole-genome sequencing results of the diamondback moth, indicating that *Plutella Xylo stella* contains 18,071 protein-coding genes and 1,412 unique genes [12]. The results of whole-genome sequencing provide a basic framework, candidate gene pool, and technical targets for subsequent research on the gene functions of *Plutella Xylo stella*.

4.1. Functional Analysis of Insecticide Resistance-Related Genes in *Plutella xylo stella*

At present, Bt toxin and fipronil are used as insecticides to eliminate *Plutella Xylo stella*. The PxABCB1 (Pluttela Xylo stella ATP-binding cassette transporter B1) gene is a key gene of the Bt toxin receptor in biological insecticides. The PxRDL1 (Plutella Xylo stella Resistant to Dieldrin-like 1) gene and the PxRDL2 (Plutella Xylo stella Resistant to Dieldrin-like 2) Genes are the key genes for the receptor of the chemical insecticide fipronil. Ouyang et al. used CRISPR/Cas9 to construct a homozygous mutant population with a 5-base deletion in exon 3 of the PxABCB1 gene, resulting in resistance to Bt Cry1Ac toxin that was 63-fold higher than the susceptible strain, confirming PxABCB1 as a key functional receptor for Bt Cry1Ac [13]. Ben-Jie et al. obtained homozygous mutant strains with a 4-nt deletion on exon 4 of PxRdl1 and a 52-nt deletion on exon 1 of PxRdl2 through CRISPR/Cas9 technology by knocking out the PxRdl1 and PxRdl2 genes [14]. Bioassay showed that the resistance of the PxRdl1 knockout line to fipronil increased by 10.4 times, and the sensitivity of the PxRdl2 knockout line increased by 4.4 times, indicating that PxRDL1 is the main target of fipronil and that PxRDL2 has an antagonistic effect [14]. The research on these three genes is of great importance for comprehending the resistance mechanism of the *Plutella Xylo stella* to two insecticides, and it will help to control *Plutella Xylo stella* in agriculture in the future through methods such as early warning by detecting resistance, optimizing insecticide formulations, and genetic engineering intervention.

4.2. Functional Analysis of Reproduction Regulation Genes in *Plutella xylo stella*

Studies have shown that the PxDcr gene, PxNap1 (Plutella Xylo stella Nucleosome Assembly Protein 1) gene, PxOsp (Plutella xylostella Ovary-serine protease) gene, PxTAR1 (Plutella Xylo stella Tyramine Receptor 1) gene, and Pxfl (2)d (Plutella Xylo stella female-lethal (2) -duplicate) gene, etc., are involved in the regulation of their reproductive capacity. Pengrong et al. knocked out the PxDcr-1 and PxDcr-2 genes of *Plutella Xylo stella* through the CRISPR/Cas9 system and obtained a homozygous mutant strain after six generations of self-pollination [15]. The results showed that the PxDcr-1 deletion strain exhibited high mortality, low emergence rate, egg-laying rate, and hatching rate, and the ovaries of female cabbage moths were poorly developed [15]. Chenxu et al. knocked out the PxNap1 gene (targeting exon 3) of *Plutella Xylo stella* through the CRISPR/Cas9 system and obtained mutants [16]. The results indicated that PxNap1 is crucial for the formation and migration of eupyrene sperm, and its absence leads to significant male infertility; apyrene sperm is normal, and female reproduction is not affected; dynamic metabolism supports the sperm process of nucleated sperm, and Nap1 regulates the related metabolic network [16]. Muhammad et al. knocked out the PxOsp gene of *Plutella Xylo stella* through the CRISPR/Cas9 system and obtained homozygous mutants [17]. The results showed that PxOsp is a femfemale specific essential gene, and its absence causes complete recessive female infertility; males are not affected and are suitable as targets for biological control strategies such as gSIT [17]. Wei et al. knocked out the PxTAR1 gene of *Plutella Xylo stella* (targeting the TM5 region) through the CRISPR/Cas9 system and obtained a 7 bp deletion homozygous mutant (Mut7) [18]. The results showed that the sex pheromone content of the mutant female moths decreased significantly; the decline in the attractiveness of a spouse leads to a decrease in the mating rate; at the same time, the number of eggs laid and the hatching rate decreased simultaneously [18].

Based on the above results, the deletion of the PxDcr-1 gene will lead to a decrease in the reproductive and hatching rates of *Plutella Xylo stella*, the deletion of the PxNap1 gene will cause a decline in the reproductive ability of male *Plutella Xylo stella*, and the deletion of the PxOsp and PxTAR1 genes will lead to a decrease in the fertility of female *Plutella Xylo stella*. The research on the reproductive genes related to *Plutella Xylo stella* will help to control *Plutella Xylo stella* through genetic intervention or gene regulation in agriculture in the future.

4.3. Research on Gene Drive Systems in *Plutella xylo stella*

Gene drive is a natural phenomenon in which a specific gene is preferentially passed on to the next generation during the genetic process. The artificial gene drive system is based on CRISPR/Cas9 technology, integrating promoters, Cas9 coding sequences, sgRNA expression boxes, and target functional elements into specific genomic loci. When Cas9 cleaves another allele in a germ cell species, the cell replicates the driver element to the cleaved site through a homologous recombination mechanism, transforming the individual from heterozygous to homozygous. This drive system can spread efficiently within the population, thereby rapidly altering the genetic composition of the population. There are many difficulties in developing gene drive systems for Lepidopteran insects, such as selecting regulatory elements that can effectively control the expression of Cas9 and sgRNA, and ensuring the controllability and safety of the system.

Xue Jiao and her team conducted the first study on the gene drive system of the diamondback moth. They utilized the endogenous regulatory elements of *Plutella Xylo stella* to construct an expression vector, and integrated it into the target gene site through homologous directed repair by combining it with an sgRNA expression cassette, forming a split gene drive system [19]. Their results indicate that the CRISPR system has high splicing efficiency in somatic cells and low splicing activity in germ line cells [19]. Xue Jiao et al.'s experiment constructed a gene drive system that can achieve efficient somatic splicing and partial species splicing, providing experience for genetic control of *Plutella xylostella* and other Lepidopteran insects. In the future, more effective reproductive cell specific regulatory elements can be studied to improve the efficiency of reproductive cell cutting and transformation.

5. Conclusion

This study describes the application of CRISPR/Cas9 in the study of gene function of lepidopteran pests, mainly focusing on *Spodoptera frugiperda* and *Plutella xylostella*, and the progress and achievements of research on the related genes in body color, reproduction, and drug resistance. This technology can accurately change the phenotype and function of pests, such as producing different body colors from the wild type, reducing the reproductive ability, or improving the sensitivity of chemical insecticides, showing great potential in pest control and management. The embryo operation system can be optimized in the future; Develop an efficient delivery system for Lepidoptera insects. The standardized population and genetic resource database were established to improve the stability of the model. Promote systematic research on more Lepidoptera insect species, and establish representative model species. However, the current research is mostly limited to laboratory gene function verification, and the field application is still in a theoretical state. The transformation still faces the challenges of ecological security and population regulation efficiency. Future research needs to further evaluate the adaptability of gene knockout individuals to the wild and their impact on the whole ecosystem, and carry out rigorous field release tests. At the same time, the efficiency of gene editing was optimized to screen for more suitable target genes. In a word, CRISPR/Cas9 has opened up a new path for pest control and brought new hope for agricultural pest control.

Authors Contribution

All the authors contributed equally and their names were listed in alphabetical order.

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