CRISPR/Cas-Mediated Genome Editing in Insects

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Insect pests impose a serious threat to agricultural productivity. Initially, for pest management, several breeding approaches were applied which have now been gradually replaced by genome editing (GE) strategies as they are more efficient and less laborious. Due to its specificity and easy handling, CRISPR/Cas9-based genome editing has been applied to a wide range of organisms for various research purposes. For pest control, diverse approaches have been applied utilizing CRISPR/Cas9-like systems, thereby making the pests susceptible to various insecticides, compromising the reproductive fitness of the pest, hindering the metamorphosis of the pest, and there have been many other benefits.

CRISPR/Cas9 insect pest integrated pest management genome editing

1. Introduction

Annually, phytophagous insects damage one fifth of the world's total crop yield. Biotic stress affects the food security of any country by compromising the quality and quantity of the crop productivity. The yield loss due to insect infestation has devastating impacts on the society such as, hunger and poverty. The combined impact of the emergence and/or re-emergence of insect pests and rapidly growing human population calls for immediate inventions and the use of rigorous and integrated agricultural practices. The FAO estimated that plant diseases and pests are responsible for a 20–40% reduction in the global crop yields per year [1]. Researchers predicted a strong decline in the crop yield in response to climate change and weather pattern variations. Climatic changes might increase the risk that is caused by phytophagous pests, thereby turning them into a more harmful threat to the crops [2]. Over the past thousands of years, plant breeding has been exploited for constructing insect resistant crop varieties, however, it is laborious, time-consuming, has a stochastic nature, and the screening process is a very challenging practice [3]. Further, the unavailability of a resistance source in the gene pool has restricted the scope of breeding a resistant cultivar [4][5]. Under such a scenario, the use of toxic and cost-intensive agrochemicals appeared to be the only convenient solution for crop protection. Considering the toll these chemicals take on the ecosystem, there was an urge to develop genetically stable and fixed plant types [6].

Genome editing (GE) can play a pivotal role as it is a more promising and an environmentally friendly answer that can be used to deal with the situation. It all began with the gene-targeting experiments on the protoplast of *Nicotiana tabacum* which were performed in 1988 [7] and the findings in 1993 which supported that DNA double-strand breaks (DSBs) improved the gene-targeting efficacy [8]. Since then, the scientific orientation shifted towards

the development of targeted genome editing techniques. The adoption of GE systems provided remarkable results in the field of the genetic improvement of crops. Genetic engineering rationalized the biological research world with the introduction of methods involving in vivo genome editing. The GE technique results in base substitutions and/or insertions/deletions (indels) in the target DNA. It includes several techniques, for instance, the use of zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and the recently established clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) system. In contrast to TALENs and ZFNs, the CRISPR/Cas system is more direct and easier to handle as it requires a single guide RNA (gRNA) for target determination with the Cas9 nuclease [8][9]. In the recent past, the preference has shifted from breeding insect-resistant cultivars to making CRISPR/Cas9-mediated modifications in the agronomic traits or targeted mutagenesis in the insect genome. The constant modifications to gene knockout strategies, transgene integration, nucleotide substitution, transcription regulation, etc., have made the CRISPR/Cas system an easy-to-apply, cost-effective, and a widely used technique for manipulations at the genetic levels [10][11][12]. Biotic stress resistance is one of the traits that is improved by GE, which makes CRISPR/Cas system highly efficient in enhancing global food security, crop protection, and sustainable agriculture (**Figure 1**). On the subject of insects, many research groups have reviewed the application of various GE techniques, with special attention being paid to the CRISPR/Cas9 system in arthropods; in spite of this, no inclusive report is available that covers all of the insect pests. The recent developments in the field of molecular biology and omics approaches presented that there has been a peak in the usage of CRISPR/Cas9 technology for insect pest management, and the data from these reports are not summarized in any previously published reviews. In this work, researchers emphasize and explain the prospects and applications of CRISPR/Cas technology in different insect groups for pest management. The CRISPR/Cas9 system has the potential of providing promising approaches for the control of insect pests. Therefore, summing up the CRISPR/Cas9-based control strategies against insect pests is significant in achieving global goals such as sustainable development.

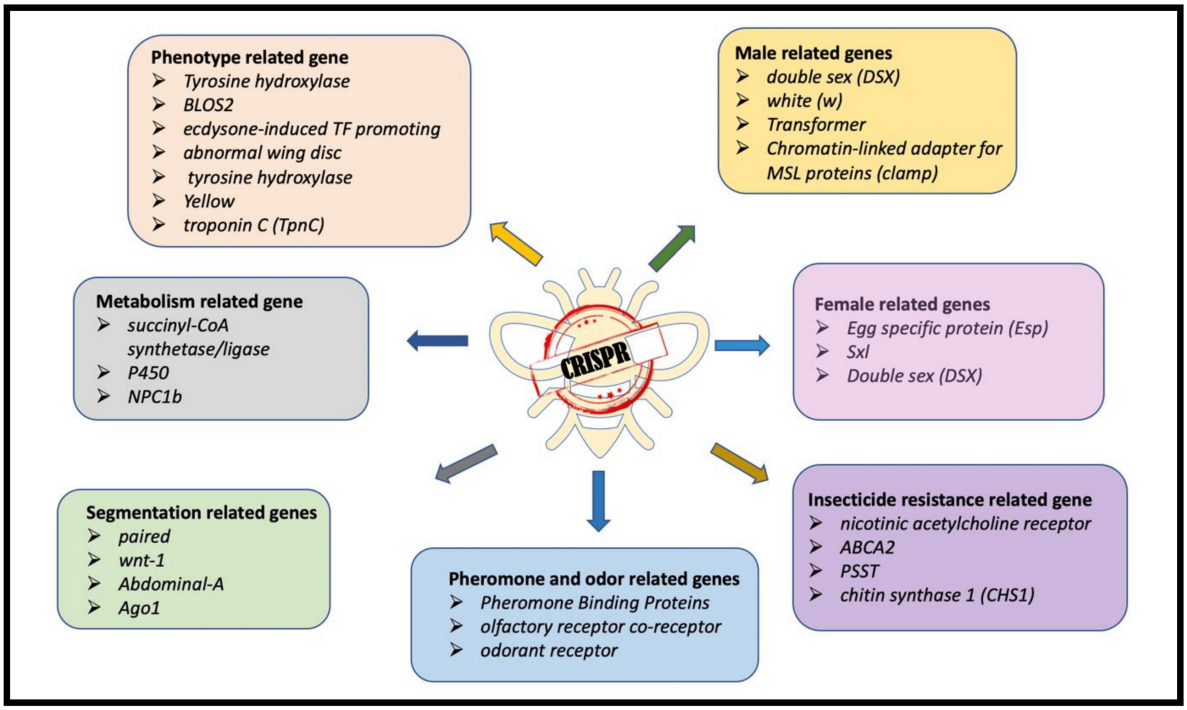

Figure 1. The figure shows various genes targeted by CRISPR/Cas9 for insect pests control.

2. CRISPR/Cas-Mediated Genome Editing in Insects

Biotechnology and molecular biology have experienced a great transformation and advancement since the development of the CRISPR/Cas9 gene-editing system in the mammalian cells in the year 2012 [13]. With the discovery of the homology-dependent cleavage recombination mechanism, the utility of CRISPR/Cas9 was explored in genome editing, and it emerged as an assuring genome editing tool. The growing utility of gene-editing tools as a site-specific genome-editing approach offers infinite opportunities which may have an impact on important agronomic traits such as resistance to biotic stresses [14][15][16][17]. The present text reviews the novel opportunities that CRISPR/Cas9 system offers and how it has attracted all of the attention by offering distinct advantages in the area of insect pest control thus ensuring a good crop yield and food security. A combination of gene editing tools and insect manipulation methods have been already used in *Drosophila melanogaster* Meigen, several tephritids, and mosquitoes which have answered some basic questions about insect biology. Recently, the technology has been utilized for the development of novel pest control strategies [18][19][20][21], and it has been proven to be an efficient approach for pest management [22][23][24]. The advancements in genome editing methods paved the way for inventive pest control methods by the development of genetically modified insects. The CRISPR/Cas technology is evolving as it is very beneficial for efficient tailoring and gene manipulation. The components of the CRISPR/Cas tool (sgRNA and the Cas9 protein) can be delivered in the target organism in form of plasmid DNA, RNA, or a ribonucleo-protein (RNP) complex [25]. Some of the phytophagous insect orders that have been explored for pest management using genome editing by the CRISPR/Cas system are summarized in **Table 1**.

Table 1. CRISPR/Cas9-based genome editing in various insects.

Insect Species	Target Gene	Accession Number	Genetic Trait	Mutation Type	Delivery of CRISPR Components	Findings	References
Drosophila melanogaster	*yellow*, *rosy*	NM_057444.3, NM_079613.3	Pigmentation and Mating	Knockout, Knockin	Plasmid	This was the first report using the CRISPR/Cas9 system to mediate efficient genome engineering in Drosophila.	Gratz et al., 2013
	yellow, *white*	NM_057444.3, NM_079613.3	Pigmentation and Mating	Knockout	Cas9 mRNA and sgRNA	sgRNA concentration-dependant knockout was shown for *yellow* gene, and highly efficient and varied genome editing	Bassett et al., 2013

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						efficiencies were shown by different sgRNAs.	
	yellow	NM_057444.3	Pigmentation and Mating	Knockout	Cas9 mRNA and gRNA	This report used the approach of targeting multiple genes with different sgRNAs, and it attained a remarkably effective targeted mutagenesis.	Yu et al., 2013
	Ast, *Eh*, *capa*, *Ccap*, *Crz*, *npf*, *Mip*, *mir-219*, *mir-315*, *white*	NM_001300582.1, NM_079662.3, NM_079828.3, NM_001275917.2, NM_079626.3, NM_080493.3, NM_140714.4, NR_048289.1, NR_048297.1, X76202.1		Knockout	Plasmid	To obtain a Cas9–sgRNA complex for achieving targeted mutagenesis, two transgene vectors harboring expression cassettes for Cas9 and sgRNA were delivered.	Kondo and Ueda, 2013
	rosy, *DSH3PX1*	NM_079613.3, NM_140091.4	Pigmentation and Mating	Knockout, Knockin	Plasmid	Executed efficient and complex genomic manipulations using CRISPR/Cas9-mediated HDR.	Gratz et al., 2014
	ebony, *yellow*, *wingless*, *wnt*	NM_079707.4, NM_057444.3, NM_078778.5	Segmentation	Knockout, Knockin	Plasmid	Different promoters were used to drive sgRNA expression, and based on promoter properties, different patterns of expression were observed.	Port et al., 2014
	EGFP, *mRFP*		Chromogenic fluorophores	Knockout	Plasmid	Induction of mutations by injection of an sgRNA into Vasa-	Sebo et al., 2014

Insect Species	Target Gene	Accession Number	Genetic Trait	Mutation Type	Delivery of CRISPR Components	Findings	References
						Cas9 transgenic fly embryos.	
	white, *piwi*	NM_057439.2, NM_001298896.1	Pigmentation and Expression of group of small RNA	Knockout, Knockin	Plasmid	Used Cas9 nickase and sgRNA pairs to prevent off-target effects during the generation of indel mutants.	Ren et al., 2014 [26]
	ms(3)k81, *white*, *yellow*	NM_143253.2, NM_057439.2, NM_057444.3	Pigmentation	Knockout, Knockin	Plasmid	CRISPR mediated genome editing was shown in Drosophila.	Xue et al., 2014a
	yellow, *notch*, *bam*, *nos*,*ms(3)k81*, *cid*	NM_057444.3, NM_001258581.2, NM_057452.4, NM_057310.4, NM_143253.2, NM_079006.4	Physiology	Knockout	Plasmid	A CRISPR/Cas9-mediated conditional mutagenesis system combined with tissue-specific expression of Cas9 was used to temporally and spatially inhibit gene expression.	Xue et al., 2014b
	salm	NM_164966.3	Zinc Finger Transcriptional Repressor	Knockin	mRNA, transgene	For flexible modification of fly genome, a two-step method was proposed.	Zhang X. et al., 2014
	ebony, *yellow*, *vermilion*	NM_079707.4, NM_057444.3, NM_078558.3	Pigmentation	Knockout, Knockin	Plasmid, transgene	Donor template and sgRNA plasmids were injected into Cas9 transgenic embryos in Drosophila.	Ren et al., 2014b
	ebony, *yellow*, *white*	NM_079707.4, NM_057444.3, NM_057439.2	Pigmentation	Knockout, Knockin	Plasmid, transgene	A bicistronic Cas9/sgRNA vector was constructed which enhanced the efficiency of gene targeting.	Gokcezade et al., 2014

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	ebony, *yellow*, *wg*, *wls*, *Lis1*, *Se*	NM_079707.4, NM_057444.3, NM_078778.5, NM_140188.4, NM_057812.5, NM_139978.4, NM_057812.5	Pigmentation and Physiology	Knockout, Knockin	Plasmid, transgene	Non-transgenic individuals exhibited lessefficient knockin than transgenic individuals did.	Port et al., 2015 [27]
	yellow	NM_057444.3	Pigmentation	Knockin	Transgene	Heterozygous recessive mutation was converted to homozygous loss of function mutations utilizing mutagenic chain reaction (MCR) technology in *Drosophila*.	Gantz and Bier, 2015
	Dα6	NM_164874.3	Insecticide resistance	Knockin	Plasmid	The G275E mutation of the *nAChR Dα6* subunit is directly related to Spinosad resistance.	Zimmer et al., 2016
	LUBEL	NM_001273232.2	Growth and Development	Knockout	Plasmid	Flies with *LUBEL* mutations exhibited reduced survival and defective climbing in response to heat.	Asaoka et al., 2016
	Scsα	NM_079181.4	Growth and Development	Knockout	Plasmid	Mutant flies could not produce sufficient energy to promote normal growth.	Quan et al., 2017
	clamp	NM_136293.4	Sex Specific	Knockout	Plasmid	The expression of a sex-specific gene was regulated by an essential transcription factor.	Urban et al., 2016

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	chameau, *CG4221*, *CG5961*	NM_135273.5,NM_141949.4		Knockin	mRNA	HDR-mediated genome modifications efficiency was tested, and a problem associated with “ends-in” recombination was resolved.	Yu et al., 2014
	fdl	NM_165908.2		Knockout	Plasmid	Capability of CRISPR/Cas9 system for analysing or manipulating protein glycosylation pathways.	Mabashi-Asazuma et al., 2015 [28]
	mod(mdg4)	NM_163878.2		Knockout	Plasmid	Validation of a functional gene involved in trans-splicing that influenced the development in flies.	Gao et al., 2015 [29]
	act5C, *lig4*, *mus308*	NM_167053.2, NM_132679.3, L76559.1		Knockout, Knockin	Plasmid, transgene	Offered a comprehensive technique for genome editing in Drosophila S2 cells.	Kunzelmann et al., 2016 [30]
	yellow, *white*, *tan*	NM_057444.3, NM_057439.2, NM_132315.1	Pigmentation	Knockin	Plasmid, transgene	Proposed a new process of attaining single or multiple allelic substitutions.	Lamb et al., 2016
	wntless	NM_140188.4	Growth and development	Knockout	Plasmid	A complex of tRNA–sgRNA was proposed to amplify the cleavage efficiency of the Cpf1 and Cas9 nucleases.	Port and Bullock, 2016

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	TpnC	NM_078895.4	Growth and development	Knockout	Plasmid	Confirmed that the myofibril assembly is related to *TpnC* gene.	Chechenova et al., 2017
	Alk	NM_144343.3	Growth and development	Knockout	Plasmid	Revealed that transcription factors can affect *Alk* gene expression by establishing mutations in Alk enhancer regions.	Mendoza-Garcia et al., 2017
Drosophila suzukii	*white (w-)*	NM_057439.2	Pigmentation	Knockout	Plasmid	Absence of mating and copulation failure was reported. The mutation also caused pigmentation deficiency in testis sheath, which could be a probable reason for copulation failure.	Yan et al., 2020
	white, *Sxl*	NM_057439.2, XM_017083263.2	Sex determination	Knockout	Plasmid	*Sxl* gene was proved as excellent gene to suppress the population growth of this destructive pest.	Li and Scott, 2016
	DsRed (red fluorescence protein)			knockin	Plasmid, transgene	The enhancer/promoter of the spermatogenesis-*specific beta-2-tubulin (β2t)* gene was used for expression of fluorescent proteins or effector molecules in testes of pests, and this providing	Ahmed, H. M. et al., 2019

Insect Species	Target Gene	Accession Number	Genetic Trait	Mutation Type	Delivery of CRISPR Components	Findings	References
						basis for reproductive biology studies sexing and monitoring.	
Drosophila subobscura	*yellow*, *white*	XM_034814491.1, XM_034808177.1	Pigmentation	Knockout	mRNA	Gene functions were analyzed in a non-model Drosophila species.	Tanaka et al., 2016 [31]
Anastrepha ludens	*Astra-2*	EU024509.1	Sex determination	Knockout	RNP complex	The mutation caused sterility, thus, the target gene was proposed for helping in pest control.	Li et al., 2019
Bactrocera dorsalis	*White* and *transformer*	AY055817.1, KP342062.1	Sex determination and reproduction	Knockout	RNP complex	CRISPR/Cas9 mediated mutation of *white* and *transformer* genes caused various phenotypic effects.	Zhao et al., 2018
Ceratitis capitata	• eGFP_gRNA2 • eGFP_gRNA2 and 1 mM Scr7 • eGFP_gRNA2b–Cas9 complexes with ssODN_BFP donor template		Homology directed repair	knockin	RNP complex and a single-stranded oligo donor	Conversion of eGFP-to-BFP was demonstrated for establishing an efficient HDR through CRISPR-based genome editing.	Aumann, R. A. et al., 2018
	• *white* eye (we) • *paired* gene (Ccprd)	X89933.1, XM_020858622.2	Segmentation	Knockout	RNP complex	A simple and highly efficient RNP complex-based genome editing approach was reported with the details of	Meccariello, A. et al., 2017

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						designing and preparation.	
Helicoverpa armigera	*NPC1b*	MK555324.1	Growth and dietary uptake of Cholesterol	Knockout	RNP complex	NPC1b is vital for the growth and dietary cholesterol uptake. Thus, a novel pest-management technique can be developed using NPC1b as an insecticidal target.	Zheng, J. C. et al., 2020
	HaCad	JX23382.1	cell–cell adhesion	Knockout	sgRNAs and Cas9 mRNA	sgRNAs and Cas9 mRNA were injected into the fresh eggs, and a high editing efficiency of the HaCad locus was achieved.	Wang, J. et al., 2016
	HaABCA2	KP259911.1	Regulation of enzymes	Knockout	Cas9 mRNA and sgRNA	The knockout of *HaABCA2* confirmed the role of *HaABCA2* in mediating toxicity of both Cry2Aa and Cry2Ab against *H. armigera.*	Wang, J. et al., 2017
	odorant receptor 16 (OR16)	KF768670.1	Olfaction	Knockout	Cas9 mRNA + sgRNA and RNP complex	The results represent the basis for novel olfactory-based strategies of pest population control.	Chang, H. et al., 2017
	white, *ok*, *brown*, and *scarlet*	XM_021344759.2, KU754490.1, KU754480.1, KU754478.1	Pigmentation	Knockout	Cas9 mRNA	The report represented differential distribution of eye pigments in the mutants; this finding may be helpful in	Khan, S. A.,et al., 2017

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						elucidation of biosynthetic pathway.	
	cluster of nine P450 genes	KM016735.1, R095600.1, KM016739.1, KM016740.1, DQ256407.1, KM016743.1, KM016741	Regulation of detoxifying enzymes	Knockout	Cas9 protein and multiple sgRNAs	The report identified the key players in the insecticide metabolism.	Wang, H. et al., 2018
Plutella xylostella	*Pxabd-A*	XM_011570968.3	Body segmentation	Knockout	Cas9 mRNA	CRISPR/Cas9 was used to target genes in *P. xylostella* for the first time which provided new ideas for pest control.	Huang et al., 2016
	Pxdsx	XM_048630440.1	Sex determination	Knockout	Microinjection of RNP complex	The results showed CRISPR/Cas9 system led to altered expression of sex biased genes.	Wang et al., 2019
	PxCHS1	AB271784.1	Development	Knockout	Plasmid	Description of the resistance management strategies for insect pests, it and explained the MoA behind the resistance using CRISPR/Cas9 system.	Douris et al., 2016
	LW		Locomotion	Knockout	RNP complex	The results showed weaker phototaxis and reduced locomotion, thus making it a helpful method for pest control	Chen et al., 2021 [32]
Spodoptera frugiperda	*Sfabd-A*	MH541836.1	Body segmentation	Knockout	RNP complex	The results showed that gene	Wu et al., 2018

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						function validation and the understanding of resistance mechanism can be performed using CRISPR/Cas9 system which can lead to the development of novel pest management approaches.	
	• BLOS2 • E93 • TO	XM_035582273.2 XM_050696092.1 XM_050696079.1	Growth and development	Knockout	Cas9 protein and multiple sgRNAs	The developed mutants were helpful to understand the crucial pathways of *S. frugiperda* and the strategy can also applied for other invasive pests.	Zhu, G. H. et al., 2020 [33]
Spodoptera litura	*Slabd-A*	GCA_002706865.1	Body segmentation	Knockout	Cas9 mRNA and sgRNA	The direct injection of Cas9-coding mRNA and *Slabd-A*-specific sgRNA into the embryos of the *S. litura* led to the induction of the typical *abd-A* deficient phenotypes showing irregular segmentation and unusual pigmentation at the larval stage.	Bi, H. L. et al., 2016
	SlitBLOS2	XM_022977403.1	Molecular marker	Knockout	Cas9 mRNA and sgRNA	The study demonstrated that SlitBLOS2 has a role in the coloration of the integuments, and thus, it provided a marker gene for	Zhu, G. H. et al., 2017

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						functional studies and pest control strategies.	
Spodoptera littoralis	*SlitOrco*		Olfaction	Knockout	mRNA	The *Orco* gene was investigated in the insect *Spodoptera littoralis*. The results were helpful in making a pest control strategy and in gene function analysis.	Koutroumpa et al., 2016
Spodoptera exigua	*Seα6*	MN714701.1		Knockout	RNP complex	The study demonstrated that knocked-out Seα6 was highly resistant to insecticides.	Zuo et al., 2020
Dendrolimus punctatus	*DpWnt-1*	KU640201.1	Development and segmentation	Knockout	mRNA	Proved the necessity of DpWnt-1 signaling in appendage development and anterior segmentation.	Liu H. et al., 2017
Cydia pomonella	*CpomOR1*	FJ385021.1	Olfaction	Knockout	Cas9 mRNA and sgRNA	The report demonstrated mutation in the *CpomOR1* gene via CRISPR/Cas9 affected the egg production and viability in the insect.	Garczynski, S. F. et al., 2017
Ostrinia furnacalis	*OfAgo1*		Growth and development	knockout	sgRNA and Cas9 mRNA	Mutation in *OfAgo1* gene through CRISPR/Cas9 technology caused cuticle disruption.	You et al., 2019

palindromic repeats (CRISPR)-mediated mutagenesis and phenotype rescue by piggyBac transgenesis in a nonmodel Drosophila species. Insect Mol. Biol. 2016, 25, 355–361.

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Agrotis ipsilon	*AiTH*		Growth and development	Knockout	sgRNA and Cas9 mRNA	The *AiTH* gene knockout by CRISPR/Cas9 caused narrowing in the egg shell.	Yang et al., 2018
Hyphantria cunea	Hcdsx		Reproduction	Knockout	sgRNA and Cas9 mRNA	Knocked-out *Hcdsx* gene by CRISPR/Cas9 caused sex-specific sterility, thus making it a pest control method.	Li et al., 2020
Mythimna separata	*NPC1b*	MZ209049.1	Intestinal absorption and sterol trafficking	Knockout	RNP complex	Knockout of *NPC1b* can hamper nutrient absorption.	Tang et al. 2022
Nilaparvata Lugens	*Nl-cn* and *Nl-w*	MH105806.1	Pigmentation	Knockout	Cas9 mRNA and sgRNA	Two genes for eye pigmentation were targeted using CRISPR/Cas9, and the results paved path for gene-function interrogation.	Xue. et al., 2018
Diaphorina citri	*ACP-TRX-2*	XM_026831570.1	Physiology	Knockout	BAPC-assisted delivery of CRISPR components	The method incorporated BAPC-assisted delivery of CRISPR/Cas9 into nymphs and adults, thus resulting an innovative breakthrough in gene editing, it and has shown a significant improvement over efforts using injection of eggs.	Hunter et al., 2018
Diaphorina citriHomalodisca vitripennis,	*Thioredoxin* and *Vermillion*	XM_046819472.1	Physiology andEye color	Knockout	BAPC, plasmid, dsRNA	The BAPC-assisted delivery system developed	Hunteret al., 2019

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Bemisia argentifolii						gene editing methods across the all hemipteran pests by permitting the use of nymphs and adults. BAPC-assisted CRISPR delivery transformed the approaches to protect food crops from different pathogens and insect vectors.	
Bemisia tabaci	*white*	XM_019053144.1	Pigmentation	Knockout	SgRNA + Cas9 protein fused with overy targeting peptide ligand (BtKV)	The method has significantly expanded the capability of CRISPR techniques for whitefly research.	Heu et al., 2020
Euschistus heros	*abnormal wing disc (awd)*, *tyrosine hydroxylase (th) and yellow (yel)*	NP_001119625.1, XP_008182999.1, XP_001948479.1	Body segmentation and pattern	Knockdown and knockout	dsRNA, RNP complex	Use of RNAi and CRISPR/Cas9 techniques for managing insect pests.	Cagliari et al., 2020
Tribolium castaneum	*Tribolium E-cadherin*	XM_961215.3	Dorsal closure defect	Knockout	Plasmid	*Tribolium E-cadherin* gene was targeted for knockout study.	Gilles et al., 2015
Leptinotarsa decemlineata	*vestigial gene (vest)*	XM_023168389.1	Growth and development	Knockout	RNP complex	Functionally characterized *vest* gene and CRISPR/Cas9 protocol was established for mutagenesis.	Gui, S. et al., 2020
Locusta migratoria	*Orco*	JN989549.1	Olfaction	Knockout	mRNA	Functional genetic studies of locusts by generation of loss-of-function mutation for managing insect pests.	Li Y. et al., 2016

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Tetranychus urticae	*PSST*	KX806605.1		Knockout	Plasmid	Substitution in the H92R amino acid of the PSST homolog was related to pyridaben resistance and the mutation into the *Drosophila* PSST homolog using CRISPR/Cas9 genome-editing tools.	Bajda et al., 2017
	phytoene desaturase	MF167355.1		Knockout	RNP complex	Induction of two mutagenetic events using CRISPR/Cas9 providing basis for functional studies.	Dermauw, W. et al., 2020