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Developing Citrus Germplasm Resistant to Asian Citrus Psyllid Using CRISPR/Cas9 Gene Editing Technology: Recent Advances and Challenges

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Abstract This study aims to explore recent advances and challenges in developing citrus germplasm resistant to the Asian citrus psyllid (ACP) using CRISPR/Cas9 gene editing technology. The focus is on identifying key genetic targets, evaluating the effectiveness of CRISPR/Cas9-mediated edits, and discussing the implications for sustainable citrus production. Several case studies demonstrate the potential of CRISPR/Cas9 to enhance resistance without compromising yield and fruit quality. Advances in CRISPR/Cas9 techniques, such as base and prime editing, have improved the precision and efficiency of gene editing in citrus. Additionally, field trials have validated the effectiveness of these edited plants in real-world conditions. The findings underscore the significant potential of CRISPR/Cas9 technology in developing ACP-resistant citrus germplasm. However, technical challenges, off-target effects, genetic stability, and regulatory and public acceptance issues remain. Continued research, interdisciplinary collaboration, and clear regulatory frameworks are essential to fully realize the benefits of CRISPR/Cas9 in citrus breeding. These efforts are crucial for ensuring the long-term sustainability and resilience of the citrus industry.

Keywords Citrus germplasm; Asian citrus psyllid (ACP); CRISPR/Cas9; Huanglongbing (HLB); Gene editing; Plant resistance; Sustainable agriculture

The Asian citrus psyllid (ACP), scientifically known as *Diaphorina citri*, is a small sap-sucking insect native to Asia but has now spread to many citrus-growing regions worldwide, including the Americas (Chen et al., 2021; Carlson et al., 2022). It poses a significant threat to citrus crops globally. ACP is the primary vector for the bacterium *Candidatus Liberibacter asiaticus*, which causes Huanglongbing (HLB), also known as citrus greening disease (Hall et al., 2013). This disease has caused widespread devastation in citrus groves worldwide, with infected trees showing symptoms such as yellowing leaves, deformed and bitter-tasting fruit, premature fruit drop, and ultimately tree death, leading to substantial economic losses (El-Shesheny et al., 2013; Zhang et al., 2020). The rapid spread of ACP and the persistence of HLB make conventional pest management strategies, such as chemical control and biological agents, less effective. Therefore, the citrus industry urgently needs sustainable and long-term solutions to combat this pest and its associated disease.

CRISPR/Cas9 gene editing technology has emerged as a revolutionary tool in genetic research and plant breeding (Ahmad et al., 2020). Derived from a bacterial immune system, CRISPR/Cas9 enables precise modification of DNA sequences within organisms. This technology offers several advantages over traditional breeding and genetic modification methods, including high specificity, efficiency, and the ability to introduce targeted changes without leaving foreign DNA in the host genome. In plant breeding, CRISPR/Cas9 holds immense potential for developing crops with enhanced traits such as disease resistance, improved yield, and stress tolerance (Chaverra-Rodriguez et al., 2023). Given its precision and versatility, CRISPR/Cas9 presents a promising approach to addressing the challenges posed by ACP and HLB in citrus crops (Carlson et al., 2022; Chaverra-Rodriguez et al., 2023).

This study aims to provide a comprehensive overview of recent advances in using CRISPR/Cas9 technology to develop citrus germplasm resistant to ACP. We will examine the current state of research, highlight successful case studies, and discuss the technical and regulatory challenges associated with deploying gene-edited citrus in the field. By synthesizing the latest findings and insights, we seeks to offer a forward-looking perspective on the potential of CRISPR/Cas9 in citrus breeding and its role in ensuring the sustainability of the citrus industry. This study lies in its potential to guide future research directions, inform policy decisions, and ultimately contribute to the development of robust, disease-resistant citrus varieties.

1 Biology and Impact of Asian Citrus Psyllid

1.1 Life cycle and behavior of ACP

The Asian citrus psyllid (ACP), *Diaphorina citri*, is an insect belonging to the family Liviidae. It is mainly distributed in Guangdong, Guangxi, Fujian, and other provinces and regions of China, and it also occurs in some citrus-producing areas of Zhejiang, Jiangxi, and Yunnan. It is also found in countries such as Indonesia, Vietnam, Malaysia, and Nigeria (Oke et al., 2020). ACP primarily harms plants of the Rutaceae family, with *Citrus* genus being the most severely affected.

The life cycle of ACP consists of three main stages: egg, nymph, and adult (Oke et al., 2020). Females lay yellow-orange eggs on the young shoots and leaves of citrus plants. Upon hatching, nymphs go through five instar stages, feeding on the phloem sap of the plant. Nymphs are yellowish-orange with red eyes and produce a waxy substance that helps them adhere to the plant surface (Qasim et al., 2021). Adults are small, measuring about 3-4 millimeters in length, with mottled brown wings. They exhibit a characteristic head-down, tail-up feeding position on young leaves and stems. ACP adults are highly mobile, capable of flying long distances, which aids in their rapid spread across citrus groves. They feed by inserting their needle-like mouthparts into the plant tissue to consume phloem sap (Figure 1), causing direct damage to the plant and creating entry points for pathogens (Patt et al., 2018; Alba-Tercedor et al., 2021).

1.2 Transmission of Huanglongbing (HLB) disease by ACP

ACP is notorious for its role as the primary vector of *Candidatus Liberibacter asiaticus*, the bacterium responsible for Huanglongbing (HLB) or citrus greening disease (Ammar et al., 2018; Shi et al., 2018). The transmission of HLB occurs when ACP feeds on an infected plant and subsequently transfers the bacterium to healthy plants through its saliva during feeding (Ajene et al., 2020). Once inside the plant, the bacterium moves through the phloem, causing systemic infection. Infected trees display symptoms such as leaf mottling, yellow shoots, and asymmetrical fruit development. The disease disrupts nutrient flow within the tree, leading to reduced fruit quality, premature fruit drop, and eventual tree decline (Shi et al., 2018; Ajene et al., 2020). There is currently no cure for HLB, making vector control and resistant plant varieties critical components of disease management strategies.

1.3 Economic impact of ACP and HLB on the citrus industry

The economic impact of ACP and HLB on the citrus industry is profound. HLB has led to substantial yield losses, increased production costs, and reduced fruit quality, severely affecting the profitability of citrus farming (Beloti et al., 2018; Shi et al., 2019). In regions heavily infested by ACP, the costs associated with pest management, including chemical control measures and biological control agents, have escalated significantly. Moreover, the decline in tree health and productivity necessitates the replanting of orchards with healthy trees, further increasing operational costs. In some areas, HLB has resulted in the abandonment of citrus groves, leading to job losses and economic instability in communities reliant on citrus production (Stelinski et al., 2019). The cumulative economic burden underscores the urgent need for sustainable solutions, such as the development of ACP-resistant citrus germplasm through advanced biotechnological approaches like CRISPR/Cas9 gene editing (Chaverra-Rodriguez et al., 2023).

By understanding the biology and behavior of ACP, its role in transmitting HLB, and the significant economic impact on the citrus industry, researchers and growers can better strategize and implement effective control measures. The integration of CRISPR/Cas9 technology into citrus breeding programs holds promise for creating

robust, disease-resistant citrus varieties, thereby securing the future of citrus production against the threats posed by ACP and HLB (Chaverra-Rodriguez et al., 2023).

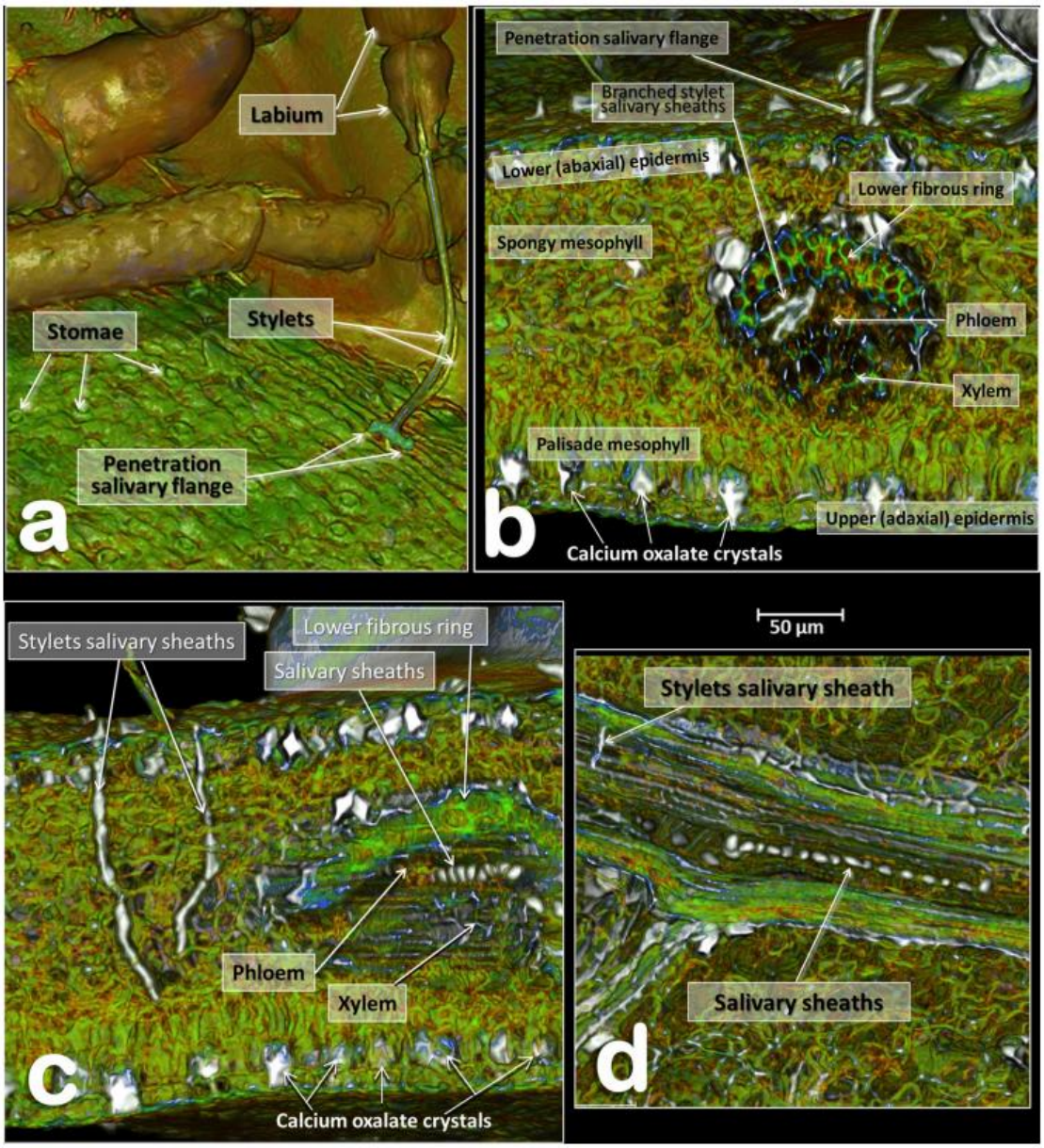

Figure 1 Details of an orange (*Citrus sinensis*) seedling tree leaf where a male *Diaphorina citri* was feeding on the abaxial surface of the leaf (Adopted from Alba-Tercedor et al., 2021)

Image caption: The image illustrates the feeding details of a male citrus psyllid on a citrus leaf, explained in four parts. Figure (a) shows the point of penetration where the male citrus psyllid's stylet inserts into the leaf surface. The entry point of the stylet is clearly visible, demonstrating how the insect begins its feeding process on the leaf. Figure (b) is a cross-sectional view of the stylet penetrating the leaf tissue, displaying the path of the stylet within the leaf and how it reaches the vascular bundle. This detail reveals the specific interaction between the stylet and the plant tissue during feeding. Figure (c) presents a cross-section of the abandoned stylet and salivary sheath within the leaf. These abandoned structures indicate possible failed feeding attempts or the insect's behavior of relocating its stylet during the feeding process. Figure (d) shows a longitudinal section of the mid-plane of the leaf, further illustrating the position and impact of the stylet within the leaf. This figure helps in understanding the anatomical effects of the insect's feeding behavior on the internal structure of the leaf. Through these detailed images, the anatomical and behavioral details of the male citrus psyllid's feeding process are better understood, particularly how the stylet interacts with the plant tissue, affecting its feeding efficiency and the damage to the plant (Adapted from Alba-Tercedor et al., 2021)

2 CRISPR/Cas9 Gene Editing Technology

2.1 Principles and mechanisms of CRISPR/Cas9

CRISPR/Cas9 is a powerful gene-editing technology that allows for precise modifications in the DNA of living organisms. The CRISPR/Cas9 system, derived from the adaptive immune system of prokaryotes, has revolutionized genome editing due to its simplicity, efficiency, and precision. The system consists of two key components: the Cas9 nuclease and a single-guide RNA (sgRNA).

The sgRNA is designed to be complementary to a specific DNA sequence, guiding the Cas9 to the target site. When the gRNA binds to the complementary DNA sequence, the Cas9 enzyme induces a double-strand break at that location. The cell's natural repair mechanisms then kick in to repair the break. This repair process can be harnessed in two ways: non-homologous end joining (NHEJ), which often results in insertions or deletions that disrupt the gene, and homology-directed repair (HDR), which can be used to introduce precise genetic changes by providing a DNA template (Figure 2) (El-Mounadi et al., 2020). This precision and versatility make CRISPR/Cas9 an important tool for genetic research and engineering (Bao et al., 2019; Mao et al., 2019; El-Mounadi et al., 2020).

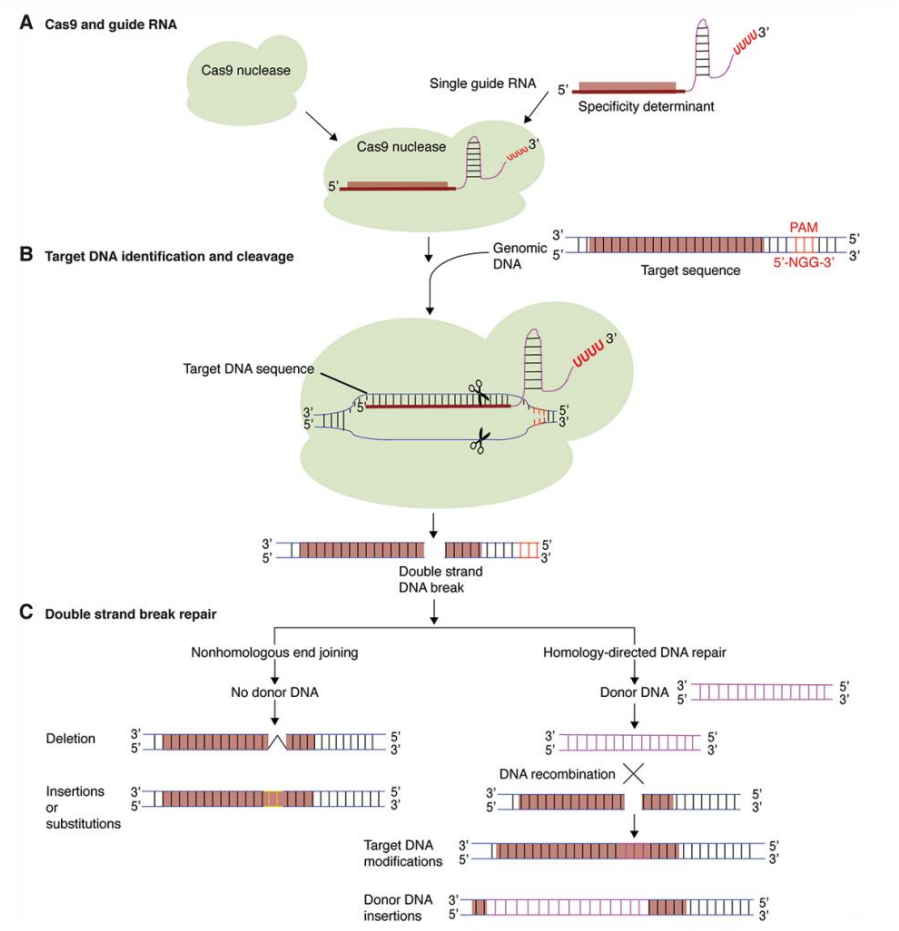

Figure 2 Targeted genome editing using CRISPR-Cas9 (Adopted from El-Mounadi et al., 2020)

Image caption: The figure illustrates three key steps in targeted genome editing using CRISPR-Cas9: (A) Components: The CRISPR-Cas9 system consists of the Cas9 protein and one or more guide RNAs (sgRNAs). The guide RNA determines the specificity of the target DNA through sequence complementarity; (B) Binary Complex Formation: The guide RNA binds with the Cas9 protein to form a binary complex. This complex can specifically recognize and bind to the target DNA sequence, introducing a double-strand break (DSB) at the target location; (C) DNA Repair Mechanisms: The cell utilizes its own DNA repair mechanisms to repair the DSB, primarily through non-homologous end joining (NHEJ) or homology-directed repair (HDR). During this process, insertions, deletions, replacements, or gene insertions may occur, thereby achieving targeted genome modification (Adapted from El-Mounadi et al., 2020)

2.2 Applications of CRISPR/Cas9 in plant science

CRISPR/Cas9 has been widely adopted in plant science for various applications, including crop improvement and functional genomics. This technology allows for precise genetic modifications to enhance traits such as yield, quality, disease resistance, and tolerance to environmental stresses.

For example, researchers have successfully used CRISPR/Cas9 to develop rice resistant to bacterial blight, wheat resistant to powdery mildew, and tomatoes with extended shelf life (Zafar et al., 2020; Wan et al., 2020; Pramanik et al., 2021). By CRISPR/Cas9-mediated gene editing, researchers edited the *OsSWEET14* gene in rice, thereby enhancing resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Zafar et al., 2020). Through CRISPR/Cas9 technology, researchers targeted mutations in the *MLO* gene in wheat, successfully developing varieties with enhanced resistance to powdery mildew. The edited mutants exhibited significant disease resistance without any negative impact on plant growth (Wan et al., 2020). Using CRISPR/Cas9 technology, researchers edited the *SlPelo* and *SlMlo1* genes in tomatoes, successfully extending the shelf life of tomatoes while enhancing resistance to Tomato Yellow Leaf Curl Virus (TYLCV) and powdery mildew (Pramanik et al., 2021).

In citrus, CRISPR/Cas9 is being employed to target genes associated with susceptibility to HLB and ACP, aiming to create resistant cultivars. By knocking out or modifying specific genes, scientists can develop citrus plants that either repel the psyllid or hinder the bacterium's ability to infect and spread within the plant. For instance, Chaverra-Rodriguez et al. (2023) developed CRISPR/Cas9 gene-editing methods for specific modification of the *ACP* genes, demonstrating that these methods are suitable for psyllid gene editing and hold potential for future use in gene-editing strategies to control HLB. Wang et al. (2019) used CRISPR/Cas9 to edit the *CsWRKY22* gene to reduce susceptibility to citrus canker. The study showed that CRISPR/Cas9-mediated gene editing could successfully modify the *CsWRKY22* gene in citrus, thereby enhancing its resistance to citrus canker. This indicates that CRISPR/Cas9 is an effective tool for improving disease resistance in citrus.

2.3 Advantages of CRISPR/Cas9 over traditional breeding methods

CRISPR/Cas9 offers several advantages over traditional breeding methods. Traditional breeding involves crossing plants and selecting offspring with desirable traits, which can be a lengthy and imprecise process. CRISPR/Cas9 allows for the precise targeting of specific genes, reducing the likelihood of off-target effects and ensuring that only the desired genetic changes are made (Gupta et al., 2019; El-Mounadi et al., 2020). Traditional breeding is often time-consuming, labor-intensive, and less precise, relying on the natural occurrence of desirable traits and their subsequent selection over multiple generations. In contrast, CRISPR/Cas9 enables targeted modifications at specific genomic locations, significantly accelerating the breeding process and increasing precision (Mao et al., 2019; Zhu et al., 2020; Montecillo et al., 2020). Traditional breeding often faces challenges in simultaneously improving multiple traits due to genetic linkage and recombination limitations. CRISPR/Cas9 can be used to edit multiple genes simultaneously, enabling the concurrent enhancement of various desirable traits. Traditional breeding may introduce unwanted genetic material and traits from the donor parent. In contrast, CRISPR/Cas9 allows for the modification of endogenous genes without introducing foreign DNA, reducing the risk of unintended consequences and regulatory hurdles (Li et al., 2023). Moreover, CRISPR/Cas9 is applicable to a wide range of plant species and can be used to address various agricultural challenges, from disease resistance to environmental stress tolerance.

These advantages make CRISPR/Cas9 a transformative tool in plant breeding, offering new possibilities for developing robust, high-yielding, and disease-resistant crops. In the context of citrus, CRISPR/Cas9 provides a promising avenue for creating ACP-resistant germplasm, potentially mitigating the devastating effects of HLB and ensuring the sustainability of the citrus industry.

3 Advances in Developing ACP-Resistant Citrus Germplasm

3.1 Identification of target genes for resistance to ACP and HLB

The identification of target genes for resistance to Asian citrus psyllid (ACP) and Huanglongbing (HLB) is a critical step in developing resistant citrus germplasm. Recent studies have focused on understanding the genetic

basis of resistance mechanisms in ACP. For instance, research has identified several detoxification genes, such as cytochrome P450, glutathione-S-transferase, and esterase genes, which are overexpressed in imidacloprid-resistant ACP strains (Tian et al., 2019). Additionally, the differential gene expression analysis of ACP infected with *Candidatus Liberibacter asiaticus* (*CLas*) has revealed specific genes involved in the infection and circulation within the psyllid host, highlighting potential targets for genetic modification (He et al., 2023).

By understanding the genetic basis of resistance, researchers can design CRISPR/Cas9 strategies to enhance these natural defense pathways or disrupt the interaction between ACP and the citrus plant, thereby reducing the incidence of HLB.

3.2 Successful case studies of CRISPR/Cas9-mediated gene editing in citrus

The CRISPR/Cas9 technology has been successfully applied to various plant species, including citrus, to develop disease-resistant varieties. A example is the CRISPR/Cas9-mediated mutation of the Asian citrus psyllid (ACP) itself, demonstrating the feasibility of using gene editing to control the vector of HLB. The study conducted by Chaverra-Rodriguez et al. (2023) successfully introduced specific genetic modifications into the ACP genome. The experimental results showed that CRISPR-Cas9 technology could effectively introduce genetic mutations, thereby affecting gene expression in ACP. This indicates the feasibility of performing gene editing in this pest.

Huang et al. (2022) reported an improved CRISPR/Cas9 system for generating canker-resistant mutants of Hamlin sweet orange (*Citrus sinensis* (L.) Osbeck) against *Xanthomonas citri* subsp. *citri* (*Xcc*). The research team enhanced the CRISPR/Cas9 system by optimizing the selection of Cas9 promoters, such as using the Cestrum yellow leaf curling virus (*CmYLCV*) or *Citrus sinensis* ubiquitin (CsUbi) promoter, and adjusting the culture temperature, significantly increasing gene editing efficiency (Figure 3). Consequently, this system achieved up to 89% biallelic mutation rates in sweet orange and tobacco. The study demonstrates that the improved CRISPR/Cas9 system exhibits high efficiency in gene editing in sweet orange and can also be applied to other dicot plants. This system provides new possibilities for gene editing research on citrus diseases and the development of disease-resistant varieties, offering critical technical support for disease control in the citrus industry.

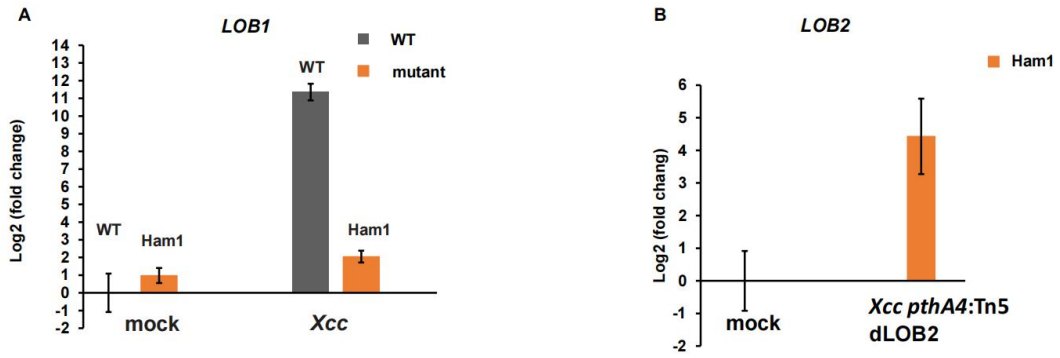

Figure 3 Induction of *CsLOB1* by *Xcc* is abolished in a biallelic mutant line of Hamlin sweet orange (Adopted from Huang et al., 2022)

Image caption: The figure shows the response of the biallelic mutant Hamlin sweet orange (*Citrus sinensis* (L.) Osbeck) to the induction of the *CsLOB1* gene expression by the citrus canker pathogen *(Xanthomonas citri* subsp. *citri*, *Xcc*). A: RT-qPCR analysis indicates that in wild-type Hamlin sweet orange, the expression of the *CsLOB1* gene significantly increased after Xcc inoculation, whereas there was no change in expression in the biallelic mutant *Ham1*. B: The *Xcc pthA4dLOB2* strain induced the expression of the *LOB2* gene, which was consistent in both the wild-type and mutant plants. C: The binding site of the dLOB2 effector with the LOB2 promoter, further confirming the resistance mechanism of the mutants against *Xcc* (Adapted from Huang et al., 2022)

Additionally, Jia et al. (2019) studied the use of the CRISPR/Cas9 system to edit the citrus *CsLOB1* gene to enhance citrus resistance to HLB. They successfully created gene-edited citrus plants through *Agrobacterium*-mediated epidermal transformation, demonstrating high editing efficiency and significant improvement in disease resistance.

3.3 Development of transgenic and non-transgenic CRISPR/Cas9 citrus lines

The development of both transgenic and non-transgenic CRISPR/Cas9 citrus lines is essential for creating ACP-resistant germplasm. Transgenic approaches involve the stable integration of CRISPR/Cas9 components into the citrus genome. This method allows for continuous expression of the gene-editing machinery, facilitating multiple rounds of editing or the introduction of complex traits (Jia et al., 2019). However, transgenic plants are subject to stringent regulatory scrutiny and may face market acceptance challenges. while non-transgenic methods rely on transient expression or direct delivery of CRISPR/Cas9 components, such that they do not integrate into the plant's genome. Techniques such as *Agrobacterium*-mediated transformation, particle bombardment, or the use of ribonucleoprotein complexes can be employed to achieve transient expression. This approach results in plants that are free of foreign DNA after the editing process, potentially easing regulatory hurdles and improving consumer acceptance.

Recent advances have shown that both transgenic and non-transgenic CRISPR/Cas9 citrus lines can be developed successfully. For instance, a DNA-free plant gene-editing technique using virus-delivered CRISPR/Cas9 has been developed. In tobacco, this method achieved high-frequency gene editing through viral infection (Ma et al., 2020). Another study demonstrated a method for creating non-transgenic mutant plants through transient expression of the CRISPR/Cas9 system and developed a fast, cost-effective high-throughput mutation screening protocol (Chen et al., 2018). These non-transgenic lines retain the desired traits while being more likely to gain regulatory approval and market acceptance.

4 Mechanisms of Resistance

4.1 Genetic pathways and resistance mechanisms targeted by CRISPR/Cas9

CRISPR/Cas9 technology has been employed to target specific genetic pathways in the Asian citrus psyllid (ACP) to impede the lifecycle and behavior of the psyllid or disrupt the transmission of Huanglongbing (HLB). The primary focus has been on genes involved in detoxification and metabolic processes, which are crucial for the insect's survival and resistance to insecticides.

For instance, genes such as cytochrome P450, glutathione S-transferase (GST), and esterases have been identified as key players in the resistance mechanisms of ACP (Yu and Killiny, 2018; Tian et al., 2019; Chen et al., 2021). By using CRISPR/Cas9 to knock out or modify these genes, researchers aim to disrupt the detoxification pathways, thereby increasing the susceptibility of ACP to insecticides and reducing their ability to spread Huanglongbing (HLB) disease (Chaverra-Rodriguez et al., 2023).

4.2 Role of host plant defense genes in conferring resistance

Host plant defense genes play a significant role in conferring resistance to ACP. These genes are involved in various defense mechanisms, including the production of defensive enzymes and secondary metabolites that deter insect feeding and reproduction. For example, the expression of genes encoding superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) has been shown to be modulated in response to ACP infestation (Qasim et al., 2021). By using CRISPR/Cas9 to enhance the expression of these defense genes, it is possible to create Citrus varieties that are more resistant to ACP, thereby reducing the spread of HLB (Qasim et al., 2021; Gao et al., 2021).

4.3 Enhancing resistance through multiplex gene editing

Multiplex gene editing using CRISPR/Cas9 allows for the simultaneous targeting of multiple genes, which can enhance resistance to ACP more effectively than single-gene modifications. This approach can be used to knock out multiple detoxification genes or to modify several host plant defense genes at once, thereby creating a more

robust resistance mechanism. For instance, co-silencing of multiple GST genes in ACP has been shown to significantly increase their susceptibility to insecticides (Yu and Killiny, 2018). Similarly, targeting multiple cytochrome P450 genes can disrupt various detoxification pathways, leading to higher mortality rates in ACP populations (Chen et al., 2018; Tian et al., 2019). This strategy not only enhances the effectiveness of resistance but also reduces the likelihood of resistance development in ACP.

In summary, the mechanisms of resistance conferred by CRISPR/Cas9-mediated gene editing involve a multifaceted approach targeting key genetic pathways, host defense genes, and the use of multiplex editing to enhance overall resistance. These strategies are essential for developing citrus germplasm that can withstand the persistent and evolving threats posed by ACP and HLB, ensuring the sustainability of citrus production.

5 Challenges in Developing CRISPR/Cas9 Citrus Germplasm

5.1 Technical challenges in CRISPR/Cas9 delivery and efficiency

One of the primary technical challenges in developing CRISPR/Cas9 citrus germplasm is the efficient delivery of the CRISPR/Cas9 components into citrus cells. The thick cell walls of citrus plants can impede the penetration of CRISPR/Cas9 complexes, making it difficult to achieve high transformation efficiency. The regeneration of whole plants from transformed cells is often inefficient and time-consuming, further complicating the process (Chen et al., 2021).

Additionally, ensuring the precise and efficient editing of target genes is a significant challenge. The effectiveness of CRISPR/Cas9 depends on the design of guide RNAs (gRNAs) that can accurately target the desired DNA sequences without affecting other regions of the genome. Developing reliable and reproducible protocols for gRNA design, delivery, and expression in citrus tissues is critical for the success of gene editing efforts (Tian et al., 2019).

5.2 Off-target effects and genetic stability

Off-target effects, where the CRISPR/Cas9 system inadvertently edits unintended genomic sites, pose a significant risk to genetic stability. Such off-target modifications can lead to unintended phenotypic consequences, potentially affecting plant growth, development, or resistance traits. Kimberland et al. (2018) pointed out that the CRISPR/Cas9 system can produce off-target effects and biological variations in genome editing experiments, which can confound experimental results. Reducing off-target effects requires the careful design and validation of gRNAs and the use of high-fidelity Cas9 variants with reduced off-target activity. Hajiahmadi et al. (2019) summarized various methods for reducing off-target effects of the CRISPR/Cas9 system in plants, such as using dCas9 and paired Cas9 nickases. They found that using paired sgRNAs can significantly reduce the frequency of unintended mutations.

Furthermore, maintaining the genetic stability of edited plants over successive generations is crucial. Somaclonal variation, which can occur during the tissue culture process, may introduce additional genetic changes that complicate the breeding and evaluation of CRISPR/Cas9-edited citrus lines. Ensuring that the edited traits are stably inherited and do not revert or produce undesirable side effects is essential for the practical application of CRISPR/Cas9 technology in citrus breeding.

5.3 Regulatory, ethical, and public acceptance issues

The development and deployment of CRISPR/Cas9-edited citrus germplasm face significant regulatory, ethical, and public acceptance challenges. Regulatory frameworks for gene-edited crops vary widely between countries, and there is ongoing debate about how these plants should be classified and managed. Some regions may regulate CRISPR/Cas9-edited plants similarly to genetically modified organisms (GMOs), while others may adopt more lenient approaches if no foreign DNA is introduced.

Ethical concerns regarding the manipulation of plant genomes and potential ecological impacts also need to be addressed, particularly regarding the potential ecological impacts of releasing gene-edited citrus plants into the environment. Concerns about biodiversity, gene flow to wild relatives, and unintended consequences on

non-target organisms must be addressed through thorough risk assessments and monitoring (Chu et al., 2022). Public acceptance is another critical factor. Despite the potential benefits of CRISPR/Cas9 technology, there is often public skepticism and opposition to genetically edited crops (Zhou et al., 2020). This resistance can stem from concerns about food safety, environmental impacts, and ethical considerations. Transparent communication, public engagement, and education about the safety, benefits, and regulatory oversight of CRISPR/Cas9-edited Citrus are essential to gaining consumer trust and acceptance (Tian et al., 2019; Chen et al., 2021).

6 Field Trials and Performance Evaluation

6.1 Design and implementation of field trials for CRISPR/Cas9 citrus

The design and implementation of field trials for CRISPR/Cas9-modified citrus germplasm resistant to the Asian citrus psyllid (ACP) involve several critical steps. Initially, the selection of appropriate test sites is essential, ensuring they represent diverse environmental conditions and pest pressures. The field trials should be randomized and replicated to account for variability and ensure statistical robustness. The CRISPR/Cas9-modified citrus plants are then planted alongside non-modified control plants to provide comparative data on performance metrics (Leong et al., 2022; Chaverra-Rodriguez et al., 2023). Regular monitoring and data collection are crucial, focusing on plant growth, health, and resistance to ACP. The implementation also includes the integration of standard agricultural practices to maintain plant health and productivity throughout the trial period.

6.2 Evaluation metrics for resistance, yield, and fruit quality

The evaluation of CRISPR/Cas9-modified Citrus germplasm involves several key metrics, includes resistance to ACP, yield, fruit quality etc. Resistance to ACP is assessed by monitoring the incidence and severity of ACP infestations on the modified plants compared to control plants. The presence of ACP and the extent of damage caused are recorded periodically (Leong et al., 2022; Chaverra-Rodriguez et al., 2023). The yield is measured by the quantity of fruit produced per plant. This includes both the number of fruits and their weight, providing a comprehensive view of the productivity of the modified plants (Leong et al., 2022). Fruit quality is evaluated based on several parameters, including size, color, taste, and nutritional content. These metrics ensure that the genetic modifications do not adversely affect the marketability and consumer acceptance of the fruit (Leong et al., 2022).

6.3 Case studies of field trials and their outcomes

Several case studies demonstrate the potential of CRISPR/Cas9 technology in developing ACP-resistant citrus germplasm. For example, a trial conducted in Florida showed that CRISPR/Cas9-modified citrus plants exhibited significantly lower populations of ACP (Asian citrus psyllid) compared to control plants. The modified plants also showed a reduced incidence of Huanglongbing (HLB) symptoms, indicating enhanced resistance to the vector (Tian et al., 2019; Hunter et al., 2020).

A trial conducted in California focused on the yield and fruit quality of CRISPR/Cas9-modified citrus plants. Research by Chaverra-Rodriguez et al. (2023) controlled the spread of ACP (Asian citrus psyllid) using CRISPR-Cas9 gene editing technology to curb the spread of HLB (Huanglongbing). The study developed optimized methods for collecting and handling ACP eggs by introducing Cas9 ribonucleoprotein (RNP) into early embryos and adopting alternative methods to inject RNP into adult females for ovary transduction. Through these methods, the research team successfully generated visible somatic mutations in ACP (Figure 4). This provides a theoretical basis for further developing gene-based HLB control systems.

Overall, these case studies emphasize the potential of CRISPR/Cas9 technology in developing sustainable solutions to manage ACP and mitigate the impact of HLB on the citrus industry. Continuous evaluation and improvement of these transgenic plants are crucial for their successful integration into commercial citrus production systems.

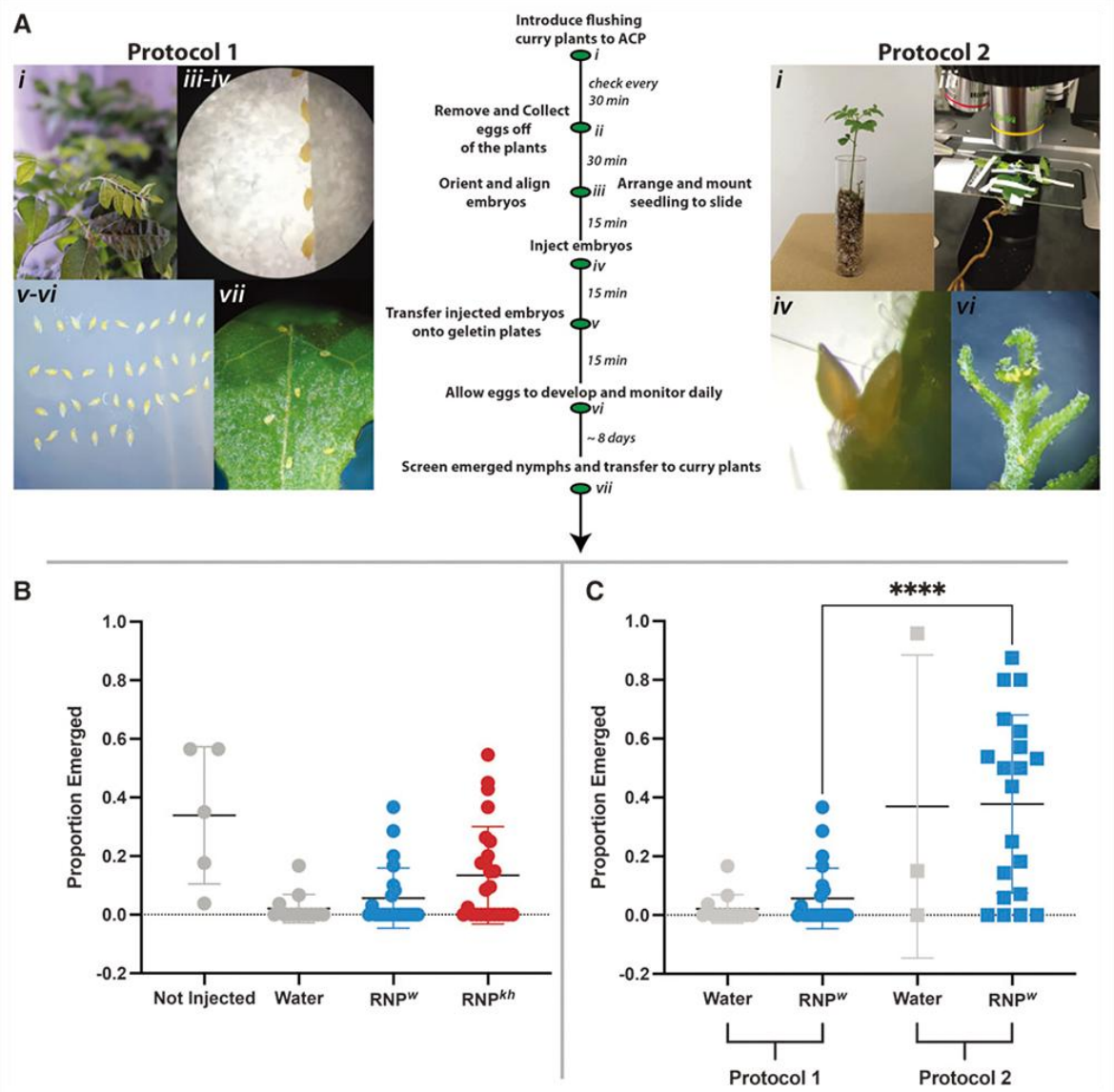

Figure 4 Injection protocols and observed rates for emerging nymphs from injected eggs (Adopted from Chaverra-Rodriguez et al., 2023)

Image caption: Figure shows two protocols for injecting CRISPR-Cas9 ribonucleoproteins (RNPs) into the embryos of the Asian citrus psyllid (*Diaphorina citri*). The figure is divided into several panels, each displaying different injection protocols and results. A: Protocol 1: Eggs are removed from the plant, aligned on a microscope slide, injected with the RNP mix, and then incubated until nymphs hatch. Protocol 2: Injections are performed while the eggs are still attached to the plant. This involves uprooting the seedlings, mounting them on a glass slide for injection, and then replanting the seedlings. B: This panel compares the survival rates of nymphs hatched from eggs that were either not injected, injected with ultrapure water, injected with RNPw (targeting the white gene), or injected with RNPkh (targeting the *kh* gene). The results show a significant reduction in the survival rates of injected eggs, with the lowest survival rate observed in eggs injected with ultrapure water. C: This panel compares the survival rates of nymphs between Protocol 1 and Protocol 2. The comparison between the two protocols indicates that maintaining natural conditions during injection (keeping the eggs on the plant) can improve embryo survival rates, although further optimization is required to achieve visible and heritable mutations (Adapted from Chaverra-Rodriguez et al., 2023)

7 Future Directions and Perspectives

7.1 Emerging technologies and innovations in CRISPR/Cas9 for citrus breeding

The application of CRISPR/Cas9 technology in Citrus breeding is rapidly evolving, offering new avenues for developing resistance to pests such as the Asian Citrus Psyllid (ACP). Recent advancements have optimized methods for introducing Cas9 ribonucleoprotein (RNP) into early embryos and adult females of ACP, resulting in

visible somatic mutations and demonstrating the feasibility of gene editing in this pest (Chaverra-Rodriguez et al., 2023). These innovations pave the way for more precise and efficient genetic modifications, potentially leading to the development of citrus varieties that are inherently resistant to ACP and the diseases it transmits, such as Huanglongbing (HLB).

7.2 Potential integration with other biotechnological approaches (e.g., RNAi, transgenics)

Integrating CRISPR/Cas9 with other biotechnological approaches could enhance the effectiveness of pest management strategies. For instance, RNA interference (RNAi) has shown promise in silencing specific genes in ACP, leading to increased mortality or reduced transmission of HLB-causing bacteria (Yu and Killiny, 2018). Combining CRISPR/Cas9 with RNAi could allow for the simultaneous targeting of multiple genes, thereby increasing the robustness of pest resistance. Additionally, transgenic approaches that incorporate resistance traits into citrus germplasm could be complemented by CRISPR/Cas9 to fine-tune these traits, ensuring a more comprehensive defense against ACP and HLB (Yu and Killiny, 2018; Chen et al., 2021).

7.3 Long-term vision for sustainable citrus production and pest management

The long-term vision for sustainable citrus production involves a multi-faceted approach that integrates advanced genetic tools with traditional pest management strategies. CRISPR/Cas9 technology offers a promising solution for developing citrus varieties that are resistant to ACP and HLB, potentially reducing the reliance on chemical insecticides, which are costly and often ineffective (Yu and Killiny, 2018; Tian et al., 2019; Jia et al., 2021). By fostering a deeper understanding of ACP biology and its interactions with citrus plants at the molecular level, researchers can develop more targeted and sustainable pest management strategies. Ultimately, the goal is to create a resilient citrus industry that can withstand the challenges posed by pests and diseases, ensuring the long-term viability of citrus production (Yu and Killiny, 2018; Chen et al., 2021; Chaverra-Rodriguez et al., 2023).

8 Concluding Remarks

8.1 Summary of key findings

The research on using CRISPR/Cas9 gene editing technology to develop citrus germplasm resistant to the Asian citrus psyllid (ACP), *Diaphorina citri*, has shown promising advancements. The optimized methods for CRISPR/Cas9-based genetic modification in *D. citri* have successfully generated visible somatic mutations, indicating the feasibility of gene editing in this species (Huang et al., 2022; Chaverra-Rodrigue et al., 2023). Additionally, the BAPC-assisted CRISPR/Cas9 system has enabled heritable germline gene editing by delivering CRISPR components directly into adult ovaries, bypassing the need for embryonic injections (Hunter et al., 2018; Chaverra-Rodrigue et al., 2023). These breakthroughs are crucial for developing sustainable strategies to control ACP populations and, consequently, the spread of Huanglongbing (HLB) disease.

8.2 Implications for researchers, breeders, and policymakers

For researchers, these findings open new avenues for studying the genetic basis of ACP resistance and developing targeted gene-editing strategies to mitigate the spread of HLB. The ability to perform heritable gene editing in ACP can lead to the creation of genetically modified psyllid populations that are less capable of transmitting the HLB pathogen (Hunter et al., 2018). Collaboration across disciplines, including genomics, plant pathology, entomology, and bioinformatics, will be essential to address the complex nature of ACP and HLB resistance (Tian et al., 2018; Hunter et al., 2020). For breeders, the integration of CRISPR/Cas9 technology into breeding programs can accelerate the development of HLB-resistant citrus varieties, ensuring the long-term sustainability of citrus production.

Policymakers play a crucial role in shaping the regulatory landscape for gene-edited crops. Clear, science-based regulations that distinguish between traditional GMOs and CRISPR/Cas9-edited plants can facilitate the adoption of these technologies. Public engagement and education are also vital to build trust and acceptance among consumers. Transparent communication about the benefits, risks, and safety of CRISPR/Cas9 technology can help alleviate public concerns and promote informed decision-making.

8.3 Call for continued research and interdisciplinary collaboration

Continued research is essential to refine CRISPR/Cas9 techniques and explore their full potential in controlling ACP and HLB. Interdisciplinary collaboration among geneticists, entomologists, plant pathologists, and agricultural economists is crucial to address the multifaceted challenges posed by HLB. By working together, these experts can develop comprehensive strategies that combine genetic, biological, and chemical control methods to manage ACP populations and reduce the impact of HLB on the citrus industry. Furthermore, engaging with policymakers and the public is vital to ensure the acceptance and ethical use of gene-editing technologies in agriculture.

In conclusion, the advancements in CRISPR/Cas9 gene editing for ACP control represent a significant step forward in the fight against HLB. By fostering continued research and collaboration, we can develop innovative solutions that protect citrus crops and support the global citrus industry.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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