Cotton Genomics and Genetics 2024, Vol.15, No.4, 207-217
http://cropscipublisher.com/index.php/cgg

Research Report **Open Access**

Utilizing Genome Sequencing for Enhanced Pest Resistance in Cotton

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Cotton Genomics and Genetics, 2024, Vol.15, No.4 doi: 10.5376/cgg.2024.15.0020
Received: 11 Jul., 2024
Accepted: 18 Aug., 2024
Published: 26 Aug, 2024

Preferred citation for this article:
Zhu S.J., and Luo M.T., 2024, Impact of polyploidy on the evolution and diversity of cotton, Cotton Genomics and Genetics, 15(4): 207-217 (doi: 10.5376/cgg.2024.15.0020)

Abstract This study aims to explore genetic and genomic strategies for enhancing pest resistance in cotton. By examining recent advancements in genome sequencing and editing technologies, the study seeks to identify key genetic traits and methodologies that can be leveraged to develop pest-resistant cotton varieties. The study highlights several significant discoveries in the field of pest resistance in cotton. Genome editing technologies, particularly CRISPR/Cas9, have shown immense potential in engineering durable resistance against insect pests and pathogens by altering effector-target interactions and knocking out host-susceptibility genes. Additionally, genome-wide association studies (GWAS) have identified specific loci and genes, such as GhnsLTPsA10, that play crucial roles in coordinating disease and insect resistance in cotton. The study also discusses the challenges posed by the rapid evolution of resistance in pests, such as the cotton bollworm, and the need for continuous monitoring and development of new resistance management strategies. The findings underscore the importance of integrating advanced genome sequencing and editing technologies in cotton breeding programs to enhance pest resistance. These genetic strategies not only promise to improve crop yields and reduce reliance on chemical insecticides but also contribute to sustainable agricultural practices. Future research should focus on fine-tuning these technologies and addressing the regulatory and social acceptance challenges associated with genetically modified crops.

Keywords Genome sequencing; Pest resistance; Cotton; Genetic breeding; Agricultural biotechnology

1 Introduction

Cotton (*Gossypium* spp.) is a vital crop globally, serving as a primary source of natural fiber for the textile industry. However, cotton cultivation faces significant challenges due to various pests and pathogens that can severely impact yield and quality. The development of pest-resistant cotton varieties is crucial to ensure sustainable production and economic stability in cotton-growing regions(Sun et al., 2023).

Cotton cultivation is widespread, with major production areas in countries such as China, India, the United States, and Pakistan. Despite its economic importance, cotton is highly susceptible to a range of biotic stresses, including insect pests like bollworms and aphids, and diseases such as Verticillium wilt and Fusarium wilt. Traditional pest management strategies, including chemical pesticides, have been employed extensively but pose environmental and health risks and can lead to the development of resistant pest populations (Chen et al., 2021).

The introduction of genetically modified cotton, particularly *Bacillus thuringiensis* (Bt) cotton, has revolutionized pest management by reducing reliance on chemical pesticides and enhancing crop protection. However, the efficacy of Bt cotton has diminished over time due to the evolution of resistance in target pest populations (Zafar et al., 2020). Therefore, there is a pressing need to develop new strategies and technologies to enhance pest resistance in cotton. Advances in genomics and biotechnology, such as genome-wide association studies (GWAS), CRISPR/Cas9-mediated gene editing, and the development of transgenic cotton lines expressing multiple Cry toxins, offer promising avenues for improving pest resistance (Katta et al., 2020).

This study aims to provide a comprehensive overview of the current state of research on utilizing genome sequencing for enhanced pest resistance in cotton (Siddiqui et al., 2019). The study will cover various aspects, including the identification of resistance loci and genes through GWAS, the development of transgenic cotton lines with improved pest resistance, and the application of advanced biotechnological tools such as CRISPR/Cas9 for functional genomics studies. By synthesizing findings from multiple studies, this study seeks to highlight the potential of genome sequencing and related technologies in addressing the challenges of pest resistance in cotton cultivation and to identify future research directions for sustainable cotton production (Grover et al., 2021).

2 Advancements in Cotton Genome Sequencing

2.1 Overview of cotton genome projects

Cotton (Gossypium spp.) is a vital crop globally, and its genome has been the focus of extensive research to improve pest resistance. Various genome projects have been initiated to map and understand the genetic basis of resistance to pests and diseases. For instance, a genome-wide association study (GWAS) identified significant loci associated with resistance to Verticillium wilt and Fusarium wilt in cotton, providing valuable insights into the genetic factors underlying these resistances (Chen et al., 2021). Additionally, the development of multi-parent advanced generation inter-cross (MAGIC) populations has facilitated the identification of quantitative trait loci (QTLs) for resistance to various pests and diseases, further advancing our understanding of cotton genomics (Zhu et al., 2022).

2.2 Techniques and technologies in genome sequencing

The advent of high-throughput sequencing technologies has revolutionized cotton genome research. Techniques such as genome-wide association studies (GWAS), transcriptome analysis, and metabolomics have been employed to identify key genes and metabolic pathways involved in pest resistance. For example, 1H-NMR spectroscopy has been used to profile metabolites in Bt and non-Bt cotton varieties, identifying key compounds responsible for insect resistance (Shami et al., 2023). Additionally, the use of Agrobacterium-mediated transformation and genome walking technology has enabled the development and characterization of transgenic cotton varieties with enhanced resistance to pests (Katta et al., 2020).

2.3 Milestones in cotton genomic research

Significant milestones have been achieved in cotton genomic research, particularly in the development of pest-resistant varieties. The identification of key resistance loci and genes through GWAS and QTL mapping has been a major breakthrough. For instance, the discovery of a major QTL for Fusarium wilt resistance in a MAGIC population represents a significant advancement in breeding resistant cotton varieties (Zafar et al., 2020). Furthermore, the development of transgenic cotton varieties expressing multiple Cry genes has shown effective resistance against major pests like Helicoverpa armigera and Spodoptera litura, marking a critical step towards sustainable pest management in cotton (Zafar et al., 2020). The integration of advanced genomic tools and techniques continues to drive progress in enhancing pest resistance in cotton, ensuring the crop's resilience and productivity (Tahir et al., 2021).

3 Genomic Insights into Pest Resistance

3.1 Identification of pest resistance genes

The identification of pest resistance genes in cotton has been significantly advanced through various genomic approaches (Wilding, 2018). For instance, a genome-wide association study (GWAS) identified several resistance loci in elite cotton varieties, with a particular focus on the GhnsLTPsA10 gene, which plays a crucial role in mediating resistance to both fungal pathogens and insects by regulating metabolic flux (Chen et al., 2021). Additionally, the development of transgenic cotton varieties, such as Narasimha, which incorporate multiple Cry genes, has demonstrated enhanced resistance to lepidopteran pests (Katta et al., 2020). Another study utilized a combination of GWAS, QTL-seq, and transcriptome sequencing to identify candidate genes related to Verticillium wilt resistance, highlighting the importance of transcription factors and flavonoid biosynthesis in pest resistance (Zhao et al., 2021).

3.2 Genetic variability and pest resistance

Genetic variability is a key factor in the development of pest-resistant cotton varieties. Research has shown that different cotton lines exhibit significant genetic variation in their resistance to pests. For example, a study on the rkn1 locus in *Gossypium hirsutum* revealed that genetic differences between homologous chromosome segments contribute to varying levels of resistance to root-knot nematodes (Wang et al., 2020). Furthermore, the use of

CRISPR/Cas9 technology has enabled the creation of a high-throughput mutagenesis library, targeting numerous insect-resistance-related genes, thereby uncovering genetic variability that can be harnessed for breeding pest-resistant cotton6. The identification of natural genetic variation in pest resistance genes, such as those involved in detoxification and systemic acquired resistance, further underscores the importance of genetic diversity in enhancing pest resistance (Galanti et al., 2023).

3.3 Molecular mechanisms of pest resistance

The molecular mechanisms underlying pest resistance in cotton involve complex interactions between various genes and metabolic pathways. The GhnsLTPsA10 gene, for example, mediates resistance by influencing phenylpropanoid metabolism (Chen et al., 2021), which affects the balance of flavonoid and lignin biosynthesis, crucial for defense against both fungal pathogens and insects (Figure 1). The overexpression of Cry genes in transgenic cotton has been shown to induce effective resistance against specific insect pests by producing proteins that are toxic to these pests2. Additionally, the role of transcription factors such as CncC:Maf in regulating the expression of detoxification genes highlights the importance of transcriptional regulation in pest resistance9. The use of CRISPR/Cas9 technology has also provided insights into the genetic basis of insecticide resistance, revealing how specific gene modifications can enhance resistance by altering key molecular pathways7. In summary, the integration of genomic tools and technologies has significantly advanced our understanding of pest resistance in cotton. By identifying key resistance genes, exploring genetic variability, and elucidating molecular mechanisms, researchers are better equipped to develop cotton varieties with enhanced resistance to pests, thereby improving crop yield and sustainability (Sun et al., 2021).

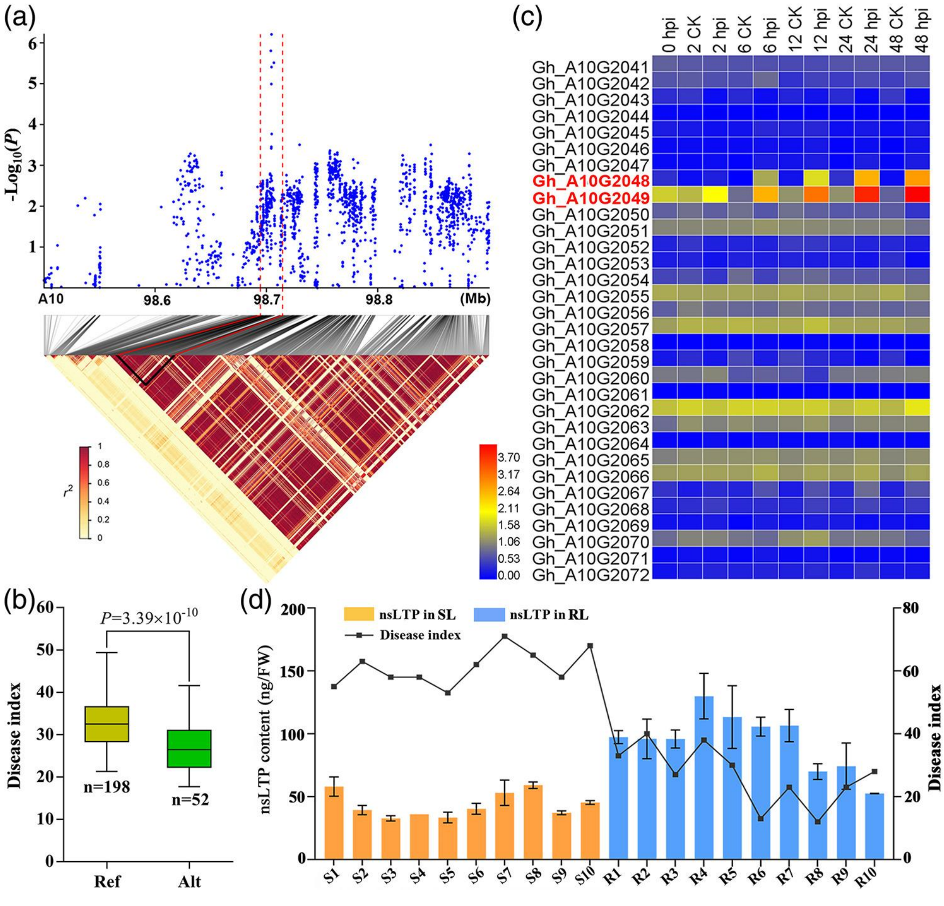

Figure 1 GhnsLTPsA10 is significantly associated with cotton resistance to *V. dahliae* (Adapted from Chen et al., 2021)

Image caption: (a) Local Manhattan plot (top) and LD heat map (bottom), red dotted lines indicated the candidate region; (b) Box plots for disease index, based on the significantly associated single-nucleotide polymorphism on At10; (c) Expression pattern of the 51 candidate genes, the boxes in the figure represent log2(1+FPKM) values (mean values of three biological replicates); red and blue indicate high and low transcript levels; (d) The relationship between nsLTP content and VW resistance in 10 resistant and 10 susceptible cotton cultivars (Adapted from Chen et al., 2021)

Chen et al. (2021) found that the gene *GhnsLTPsA10* is significantly associated with resistance to Verticillium dahliae in cotton. Their study utilized a local Manhattan plot and LD heat map to identify candidate regions, and expression patterns of 51 candidate genes were analyzed. The gene expression levels were measured in terms of log2(1+FPKM) values, revealing differential expression patterns. Furthermore, the study highlighted a significant relationship between non-specific lipid transfer protein (nsLTP) content and resistance to VW, as demonstrated in both resistant and susceptible cotton cultivars. This association underscores the potential of *GhnsLTPsA10* in enhancing disease resistance in cotton breeding programs.

4 Genetic Engineering for Pest Resistance

4.1 Traditional breeding vs. genetic engineering

Traditional breeding methods have long been employed to enhance pest resistance in crops, including cotton. These methods involve selecting and cross-breeding plants with desirable traits over multiple generations. However, traditional breeding is often time-consuming and limited by the genetic variability available within the species. Additionally, it can be challenging to achieve specific resistance traits without also introducing unwanted characteristics (Mishra et al., 2021).

In contrast, genetic engineering offers a more precise and efficient approach to developing pest-resistant crops. Techniques such as CRISPR/Cas9 allow for targeted modifications at specific genomic locations, enabling the introduction or alteration of genes associated with pest resistance without the extensive time and effort required for traditional breeding (Bisht et al., 2019). This precision reduces the likelihood of introducing undesirable traits and accelerates the development of resistant varieties2 8.

4.2 CRISPR/Cas9 and other genome editing tools

CRISPR/Cas9 has emerged as the most prominent genome editing tool due to its simplicity, efficiency, and versatility. This system uses a guide RNA to direct the Cas9 enzyme to a specific DNA sequence, where it creates a double-strand break. The cell's natural repair mechanisms then introduce mutations or incorporate new genetic material at the targeted site, enabling precise genetic modifications1 3 6. Other genome editing tools include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases. While these tools have been used successfully in various applications, they are generally more complex and less efficient than CRISPR/Cas9. For instance, ZFNs and TALENs require the design of specific protein-DNA interactions, which can be labor-intensive and costly (Mushtaq et al., 2019). Recent advancements in CRISPR technology, such as the development of CRISPR/Cas12a (formerly known as Cpf1) and base editors, have further expanded the potential applications of genome editing in crop improvement. These tools offer additional flexibility and precision, enabling more sophisticated genetic modifications to enhance pest resistance (Langner et al., 2018).

4.3 Case studies: successful engineering of pest-resistant cotton

Several case studies have demonstrated the successful application of genome editing technologies to develop pest-resistant cotton. For example, researchers have used CRISPR/Cas9 to knock out specific genes in cotton plants that are susceptible to insect pests, resulting in enhanced resistance (Figure 2) (Tyagi et al., 2020). This approach has been particularly effective in targeting genes involved in the plant's defense mechanisms, such as those encoding for effector proteins that interact with pest pathogens (Nascimento et al., 2023). Another successful case involved the modification of cotton plants to express synthetic immune receptors, which can recognize and respond to a broad spectrum of insect pests. This strategy has shown promise in providing durable and broad-spectrum resistance, reducing the reliance on chemical pesticides and contributing to sustainable agricultural practices. Moreover, genome editing has been employed to modify the genomes of insect pests themselves, creating gene drives that reduce pest populations or counteract resistance to insecticides. This innovative approach has the potential to complement plant-based resistance strategies, offering a multifaceted solution to pest management in cotton cultivation (Douris et al., 2020). In summary, the integration of genome editing technologies, particularly CRISPR/Cas9, has revolutionized the development of pest-resistant cotton. These tools offer unprecedented precision and efficiency, enabling the rapid and targeted enhancement of resistance traits, which is crucial for sustainable agricultural production in the face of evolving pest challenges.

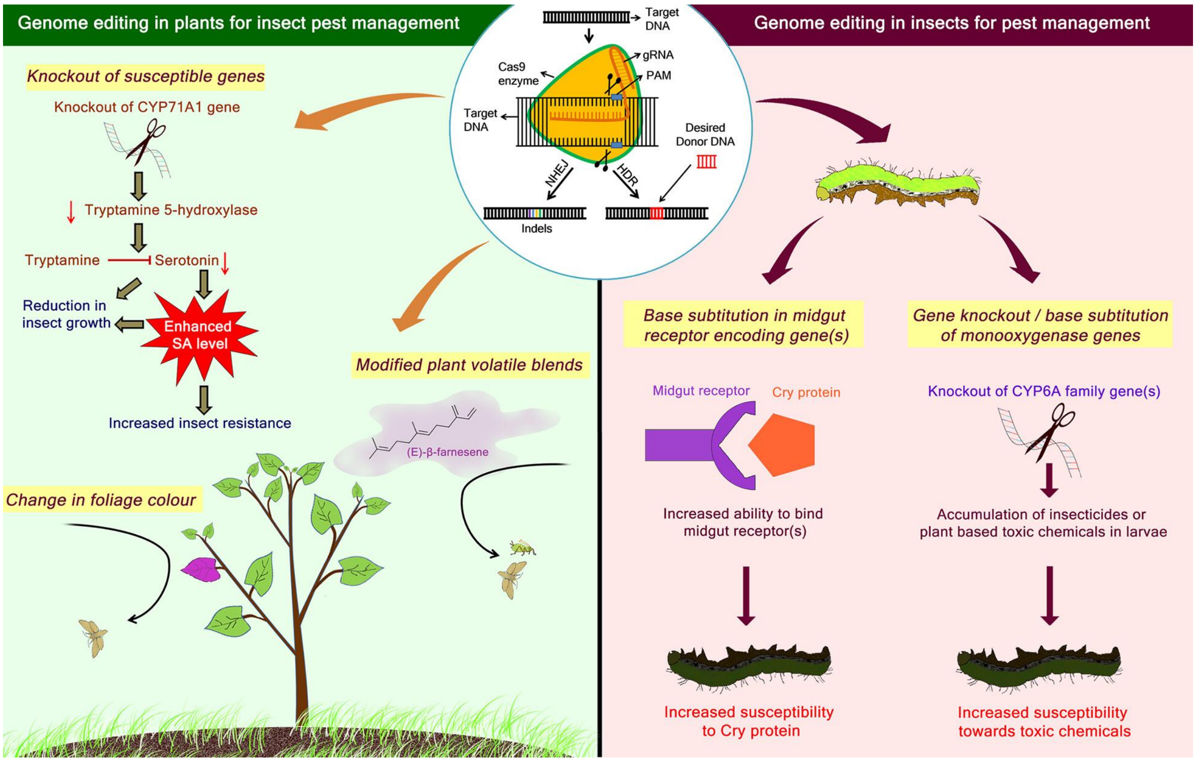

Figure 2 Strategies of CRISPR-based gene editing in plants and insects for pest management (Adopted from Tyagi et al., 2020)

Image caption: Genome editing in plants for resistance against insect pests has been demonstrated by knocking down susceptible genes, modification of plant volatile blends, and changing foliage color. Editing in insects for susceptibility toward plants can be achieved by modification of Cry protein binding receptors and knockdown of detoxification enzymes (Adopted from Tyagi et al., 2020)

Tyagi et al. (2020) found that CRISPR-based gene editing can significantly enhance pest management strategies by targeting both plants and insects. In plants, knocking out susceptible genes, such as CYP71A1, increases resistance to pests by enhancing salicylic acid (SA) levels and modifying plant volatile blends. This results in reduced insect growth and increased resistance. Additionally, altering foliage color can deter pests. In insects, editing techniques, such as base substitution in midgut receptor genes and knocking out detoxification enzyme genes, can increase susceptibility to plant toxins and Cry proteins. These modifications lead to higher binding affinity of Cry proteins to midgut receptors and accumulation of toxic chemicals in larvae, thus improving pest control effectiveness.

5 Functional Genomics and Pest Resistance

5.1 Transcriptomics and gene expression studies

Transcriptomics and gene expression studies have significantly advanced our understanding of pest resistance in cotton. For instance, the identification of GhnsLTPsA10, a nonspecific lipid transfer protein gene, has shown its role in mediating resistance to Verticillium wilt and Fusarium wilt while negatively affecting resistance to aphids and bollworms. This gene's expression varies between roots and leaves, coordinating resistance through the redirection of metabolic flux, particularly in phenylpropanoid metabolism (Chen et al., 2021). Additionally, comparative transcriptomic approaches have identified differentially expressed genes (DEGs) in resistant and susceptible cotton lines to Southern root-knot nematode (Meloidogyne incognita), highlighting genes involved in defense response, detoxification, and callose deposition (Kumar et al., 2019). These studies underscore the importance of transcriptomics in pinpointing key genes and pathways involved in pest resistance.

5.2 Proteomics and metabolomics in pest resistance

Proteomics and metabolomics provide deeper insights into the biochemical pathways that confer pest resistance. NMR-based metabolomics has identified key metabolites in Bt cotton that contribute to insect resistance, such as terpinolene, α-ketoglutaric acid, and shikimic acid. These metabolites are involved in the tricarboxylic acid cycle, shikimic acid, and phenylpropanoid pathways, which are crucial for the plant's defense mechanisms4. Furthermore, the integration of proteomics with other omics approaches has elucidated the role of various proteins and metabolites in enhancing pest resistance, thereby offering potential targets for genetic engineering (Yang et al., 2021).

5.3 Integrative omics approaches

Integrative omics approaches combine genomics, transcriptomics, proteomics, and metabolomics to provide a comprehensive understanding of pest resistance mechanisms. For example, the use of CRISPR/Cas9-mediated mutagenesis has identified several genes that enhance insect resistance by activating systemic acquired resistance (SAR) pathways involving salicylic acid and reactive oxygen species (ROS) (Figure 3) (Sun et al., 2023). Additionally, the integration of multi-omics data has facilitated the construction of models to predict complex traits, thereby aiding in the development of pest-resistant cotton varieties2. These integrative approaches are crucial for developing robust strategies for pest resistance management in cotton. By leveraging these advanced omics technologies, researchers can develop more effective and sustainable pest-resistant cotton varieties, ultimately improving crop yield and quality (Shami et al., 2023).

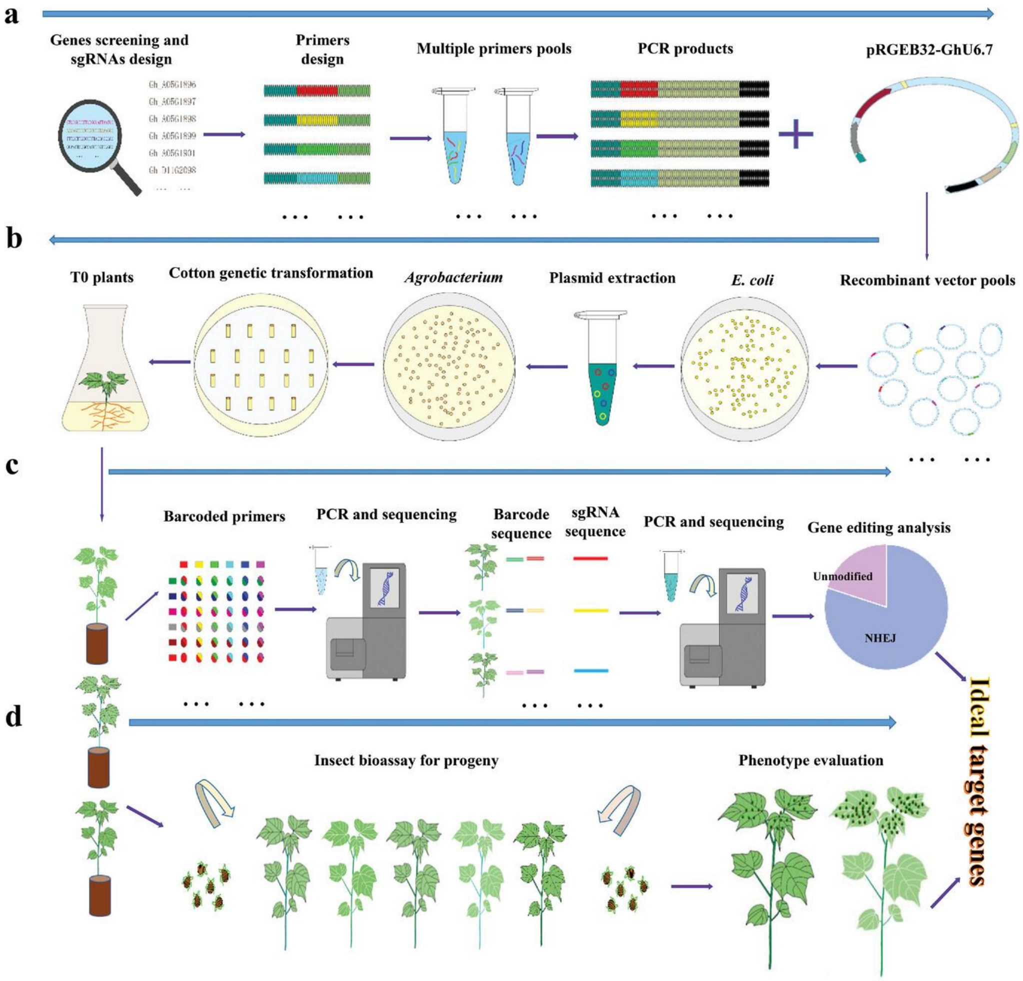

Figure 3 Schematic diagram of high-throughput mutant library construction for CRISPR/Cas9 system in cotton (Adopted from Sun et al., 2023)

Image caption: a) The construction of pooled sgRNAs library, including gene and sgRNA screening, primer designing, primers pooling, and PCR amplification; b) Genetic transformation of cotton was carried out using pooled Agrobacterium strains. A number of small-size plasmid libraries were propagated in E. coli and plasmids were extracted and mixed in equal quantities to transform to Agrobacterium. A large number of Agrobacterium colonies were pooled for cotton transformation; c) Barcode technology and high-throughput sequencing were used to identify the editing sites and editing profiles in different samples. Regenerated T0 plants usually have multiple editing sites (distinguished by colors and outlines); d) Phenotype detection and insect bioassays were combined with DNA sequencing to identify candidate genes for future research (Adopted from Sun et al., 2023)

Sun et al. (2023) found that constructing a high-throughput mutant library for the CRISPR/Cas9 system in cotton significantly streamlines genetic analysis and breeding. The process begins with the creation of a pooled sgRNAs library, followed by genetic transformation using Agrobacterium strains. Small plasmid libraries are propagated in E. coli, extracted, mixed, and used to transform Agrobacterium, which is then employed for cotton transformation. Barcode technology and high-throughput sequencing enable precise identification of editing sites and profiles across samples. The regenerated T0 plants exhibit multiple editing sites, facilitating a comprehensive analysis. By combining phenotype detection and insect bioassays with DNA sequencing, researchers can efficiently identify candidate genes for further study. This integrated approach enhances the efficiency of discovering gene functions and developing improved cotton varieties with desirable traits, such as enhanced pest resistance, thereby advancing cotton breeding programs and agricultural productivity.

6 Application of Genome Sequencing in Breeding Programs

6.1 Marker-assisted selection (MAS)

Marker-Assisted Selection (MAS) leverages molecular markers to select plants with desirable traits, significantly enhancing the efficiency of breeding programs. MAS is particularly effective when disease resistance is controlled by one or a few genes with large effects. For instance, in potato breeding, MAS has been used to identify resistance genes for nematodes and blight, utilizing kompetitive allele-specific PCR (KASP) assays developed through whole-genome resequencing. Similarly, in chickpea, linked markers for Ascochyta blight resistance were identified using genotyping-by-sequencing (GBS) methods, providing valuable resources for MAS in breeding programs (Sudheesh et al., 2021). The application of MAS in forest trees has also been highlighted, where QTL mapping studies have identified markers closely linked to disease resistance traits, thus reducing the breeding period (Younessi-Hamzekhanlu and Gailing, 2022).

6.2 Genomic selection (GS)

Genomic Selection (GS) is a breeding technology that uses genome-wide markers to predict the performance of individuals for specific traits, making it suitable for traits controlled by many genes of small effect. GS has shown higher accuracy compared to MAS, especially for quantitative disease resistance. For example, in wheat, GS models incorporating both major and minor gene markers achieved higher prediction accuracies for stripe rust resistance (Merrick et al., 2021). In rice, GS models such as genomic best linear unbiased prediction (gBLUP) and Bayesian methods demonstrated high accuracy in predicting resistance to rice blast across different isolates (Huang et al., 2019). Additionally, GS has been effectively used in maize to improve resistance to tar spot complex, with prediction accuracies enhanced by using diverse training sets and incorporating known trait-marker associations (Cao et al., 2021).

6.3 Incorporation of genomic data into breeding strategies

Incorporating genomic data into breeding strategies involves integrating MAS and GS to maximize the efficiency and accuracy of breeding programs. This integration allows for the selection of both major and minor genes, providing a comprehensive approach to disease resistance. For instance, in forest trees, combining QTL mapping and GWAS has enabled the identification of markers for MAS, while GS has accelerated the breeding process by using genome-wide markers. In maize, a stepwise implementation of MAS and GS has been suggested to improve resistance to tar spot complex, leveraging the strengths of both methods (Huang et al., 2019). Furthermore, the use of pooled whole-genome resequencing in potato has streamlined the development of KASP markers, facilitating the incorporation of genomic data into breeding programs (Younessi-Hamzekhanlu and Gailing, 2022). By utilizing genome sequencing technologies, breeding programs can achieve enhanced pest resistance in cotton and other crops, ultimately leading to more resilient agricultural systems.

7 Challenges and Future Directions

7.1 Technical and biological challenges

The application of genome sequencing and editing technologies in enhancing pest resistance in cotton faces several technical and biological challenges. One significant challenge is the complexity of the cotton genome, which is polyploid and large, making precise editing difficult (Tyagi et al., 2020). Additionally, the identification and functional validation of key resistance genes remain a bottleneck. For instance, while genome-wide association studies (GWAS) have identified several resistance loci, the functional roles of these loci are not always clear. Another challenge is the potential for unintended off-target effects, which can lead to undesirable traits or reduced fitness in the modified plants (Bisht et al., 2019). Moreover, the dynamic nature of pest populations, which can rapidly evolve resistance to new control measures, complicates the development of long-lasting resistance strategies (Chen et al., 2021).

7.2 Ethical and regulatory considerations

The deployment of genome-edited crops, including those with enhanced pest resistance, is subject to stringent regulatory frameworks and ethical considerations. Regulatory policies vary significantly across different regions, with some countries adopting more stringent measures than others. The lack of a harmonized global regulatory framework can hinder the widespread adoption of these technologies. Additionally, there are ethical concerns related to the potential ecological impacts of releasing genetically modified organisms (GMOs) into the environment. These concerns include the potential for gene flow to wild relatives and non-target effects on other organisms (Yin and Qiu, 2019). Public perception and acceptance of GMOs also play a crucial role, as societal resistance can impede the commercialization and adoption of genome-edited crops (Chen et al., 2021).

7.3 Future prospects and emerging technologies

Despite these challenges, the future prospects for utilizing genome sequencing and editing technologies to enhance pest resistance in cotton are promising. Advances in CRISPR/Cas9 and other genome editing tools continue to improve their precision and efficiency, reducing the risk of off-target effects (Rato et al., 2021). Emerging technologies such as host-induced gene silencing (HIGS) and the use of biocontrol agents (BCAs) offer additional strategies for pest management. The development of high-throughput screening methods, such as next-generation sequencing (NGS), can facilitate the rapid identification of resistance genes and the monitoring of resistance evolution in pest populations. Furthermore, the integration of genomic data from wild cotton species, such as Gossypium stocksii, can provide valuable genetic resources for breeding pest-resistant cotton varieties (Délye et al., 2020). Overall, continued research and innovation in genome editing and sequencing technologies hold great potential for sustainable pest management in cotton cultivation (Grover et al., 2021).

8 Concluding Reamrks

The utilization of genome sequencing and editing technologies has shown significant promise in enhancing pest resistance in cotton. Various studies have demonstrated the potential of these technologies in developing insect and pathogen-resistant crops. For instance, CRISPR/Cas9-based genome editing has been effectively used to engineer durable resistance against insect pests and pathogens by altering effector-target interactions and knocking out host-susceptibility genes. Additionally, genome-wide association studies (GWAS) and transcriptome sequencing have identified key resistance loci and candidate genes in cotton, which can be targeted for breeding resistant varieties. The development of transgenic cotton varieties expressing multiple Cry genes has also been successful in conferring resistance to lepidopteran pests. Furthermore, the integration of high-throughput sequencing and bioassays has provided comprehensive insights into the resistance mechanisms and allele frequencies in pest populations.

The findings from these studies have significant implications for cotton breeding and pest management. The identification of specific resistance genes and loci through genome sequencing and GWAS can facilitate marker-assisted breeding, enabling the development of cotton varieties with enhanced resistance to pests and diseases. The use of genome editing tools like CRISPR/Cas9 offers a precise and efficient method for introducing resistance traits into cotton, potentially reducing the reliance on chemical pesticides and mitigating the environmental impact of pest management. The development of transgenic cotton varieties with multiple Cry genes can provide broad-spectrum resistance to various insect pests, thereby improving crop yield and quality. Additionally, the integration of high-throughput sequencing and bioassays can aid in the early detection and management of resistance in pest populations, ensuring the long-term efficacy of pest control strategies.

The application of genome sequencing and editing technologies holds great promise for enhancing pest resistance in cotton. Future research should focus on the functional validation of identified resistance genes and the development of robust genome editing protocols for cotton. It is also essential to establish specific regulatory frameworks and conduct comprehensive risk assessments to ensure the safe deployment of genome-edited and transgenic cotton varieties. Collaborative efforts between researchers, breeders, and policymakers will be crucial in translating these scientific advancements into practical solutions for sustainable cotton production. By leveraging the power of genome sequencing and editing, we can develop resilient cotton varieties that can withstand the challenges posed by pests and pathogens, ultimately contributing to global food security and agricultural sustainability.

Acknowledgments

The authors express their gratitude to the two anonymous peer reviewers for their valuable suggestions for improving this study.

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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