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Application of Multi-Gene Stacking Strategies in Citrus Pest Resistance Breeding: From Theory to Practice

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Abstract Techniques such as gene aggregation, marker-assisted selection (MAS), and CRISPR-based genome editing have shown promising results in improving citrus resistance to pests and diseases. Gene polymerization has been successfully used to develop long-lasting resistant lines that are resistant to a variety of biological stresses, the combination of early-maturing transgenic plants with MAS has accelerated the breeding process and greatly reduced the time required to develop resistant varieties, and the CRISPR/Cas system has also played an important role in breeding citrus varieties that are resistant to various pathogens. The combination of advanced genetic engineering techniques and traditional breeding methods has the potential to produce robust citrus varieties that can withstand the challenges of multiple pests. Continued research and optimization of these strategies are essential for sustainable citrus production and improved crop resilience. This study highlights the significant advances in genetic engineering and biotechnology methods in the development of insect resistant citrus varieties, and aims to explore the application of polygenic stacking strategies in citrus pest resistance breeding, focusing on the theoretical basis and practical implementation of these strategies to improve the resistance of citrus to various pests.

Keywords Multi-gene stacking; Citrus pest resistance; Genetic engineering; Marker-assisted selection; CRISPR/Cas systems

1 Introduction

Citrus cultivation is a cornerstone of the global agricultural economy, with citrus fruits being among the most widely produced and consumed fruits worldwide. Citrus species, including oranges, lemons, limes, and grapefruits, are cultivated in over 130 countries, predominantly in tropical and subtropical regions (Sun et al., 2019). The citrus industry not only provides essential nutrients to millions of people but also supports the livelihoods of countless farmers and workers involved in its production, processing, and distribution. The economic significance of citrus is underscored by its substantial contribution to the agricultural GDP of many countries, making it a vital sector for both local and international markets (Poles et al., 2020).

Despite its economic importance, citrus cultivation faces significant challenges, particularly from pests and diseases. Pests such as the Asian citrus psyllid, which spreads Huanglongbing (HLB), and pathogens causing citrus canker and citrus tristeza virus (CTV), have severely impacted citrus yields and fruit quality (Sendín and Filippone, 2018; Endo et al., 2020). These biotic stresses not only reduce the productivity of citrus orchards but also increase production costs due to the need for pest management and disease control measures. Traditional breeding methods to develop pest-resistant citrus varieties are often hampered by the long juvenility period, high heterozygosity, and complex reproductive biology of citrus species (Rauf et al., 2013; Sendín and Filippone, 2018).

In response to these challenges, multi-gene stacking strategies have emerged as a promising approach to enhance pest resistance in citrus. This technique involves the simultaneous introduction of multiple resistance genes into a single plant, thereby providing broad-spectrum and durable resistance against a range of pests and diseases (Dormatey et al., 2020; Li et al., 2020). Advances in genetic engineering and molecular breeding techniques, such as marker-assisted selection (MAS) and genome editing, have facilitated the development of multi-gene stacking systems. These systems have been successfully applied in other crops, such as rice, to confer resistance to multiple pests and diseases, demonstrating their potential applicability in citrus breeding (Sun et al., 2019; Li et al., 2020).

This study aims to provide a comprehensive overview of the application of multi-gene stacking strategies in citrus pest resistance breeding. By synthesizing current research and advancements in this field, the study seeks to highlight the theoretical foundations, practical implementations, and future prospects of multi-gene stacking in citrus. The significance of this study lies in its potential to inform and guide future research and breeding programs aimed at developing pest-resistant citrus varieties. By addressing the challenges and opportunities associated with multi-gene stacking, this study aims to contribute to the sustainable improvement of citrus cultivation, ensuring the long-term viability and productivity of this economically important crop.

2 Background on Pest Resistance Breeding

2.1 Traditional breeding methods for pest resistance

Traditional breeding methods for pest resistance in citrus have primarily relied on classical techniques such as introduction, selection, and hybridization. These methods involve the production of variability in the plant population followed by the selection of desirable types. Historically, the evolutionary selection of resistant types resulted in land races, and with the rediscovery of Mendelian laws, breeding for resistance continued as a major approach to increasing total yield (Wenzel et al., 1985). However, these conventional methods are often bottlenecked by inadequate and lengthy breeding procedures, making them less efficient in addressing the rapid emergence of new pest threats (Rauf et al., 2013).

2.2 Advances in genetic engineering and molecular breeding

The advent of genetic engineering and molecular breeding has revolutionized the field of pest resistance breeding. Techniques such as genome editing, transgenesis, and marker-assisted selection (MAS) have been employed to introduce resistance genes into citrus varieties more efficiently. For instance, genome editing in citrus via transgenesis has successfully induced resistance to Citrus bacterial canker in sweet orange and grapefruit using the resistance gene *CsLOB1* (Salonia et al., 2020). Additionally, fast-track breeding systems integrating early flowering transgenic plants with MAS have significantly shortened the breeding duration for disease resistance, as demonstrated in the incorporation of citrus tristeza virus (CTV) resistance into citrus germplasm (Endo et al., 2020). These biotechnological approaches have shown tremendous potential in improving citrus against various diseases, making the breeding process faster and more precise (Dutt et al., 2020).

2.3 Concept and advantages of multi-gene stacking in breeding programs

Multi-gene stacking, also known as gene pyramiding, involves the assembly of multiple resistance genes into a single plant variety to provide broad-spectrum and durable resistance against various pests and diseases. This strategy has been successfully employed in other crops, such as rice, where a transgene stacking system was used to develop rice lines with multi-resistance to glyphosate, borers, brown planthopper, bacterial blight, and rice blast (Li et al., 2020). The advantages of multi-gene stacking in breeding programs include enhanced resistance durability, reduced risk of resistance breakdown, and improved agronomic traits. In citrus, gene stacking through techniques like CRISPR-based genome editing holds promise for generating varieties resistant to multiple pathogens, thereby ensuring sustainable crop improvement (Sun et al., 2019; Dormatey et al., 2020). The integration of multiple resistance genes can also help overcome the limitations of single-gene resistance, which often leads to the rapid evolution of virulent pathogen strains (Yang et al., 2011).

By leveraging the advancements in genetic engineering and molecular breeding, multi-gene stacking strategies can significantly enhance the efficiency and effectiveness of pest resistance breeding in citrus, providing a robust solution to the challenges posed by biotic stresses.

3 Theoretical Foundations of Multi-Gene Stacking

3.1 Definition and principles of multi-gene stacking

Multi-gene stacking, also known as gene pyramiding, involves the introduction of multiple genes into a single organism to confer a combination of desirable traits, such as pest resistance, disease resistance, and herbicide tolerance (Figure 1). This strategy is more effective than single-gene approaches because it targets multiple pathways or mechanisms within the pest or pathogen, thereby reducing the likelihood of resistance development (Gressel et al., 2017; Shehryar et al., 2019; Salim et al., 2020). The principles of multi-gene stacking include the selection of genes that target the same pest species, ensuring synchronous expression in the same tissues, and avoiding cross-resistance among the stacked genes (Gressel et al., 2017).

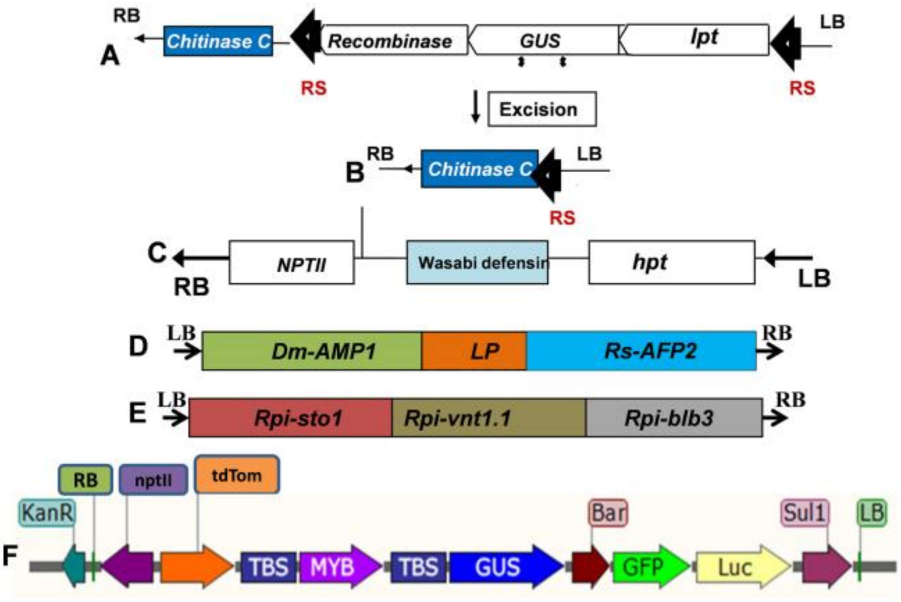

Figure 1 Schematic representation of Tranformation for gene stacking (Adopted from Shehryar et al., 2019)
Image caption: A-C, Sequential transformation of two disease resistance genes. a and b Marker-free transgenic potato plants were produced by MAT vector system. c The Marker-free transgenic potato plants were re-transformed with wasabi defensin gene. d *Dm-AMP1* and *Rs-AFP2* genes were connected by linker peptide on same plasmid between leftand right borders. e Three genes, *Rpi-sto1*, *Rpi-vnt1.1* and *Rpiblb3* were transformed simultaneously in potato. f In GAANTRY system more than three genes could be transformed simultaneouslyin plants. *GUS*, beta-glucuronidase. *hpt*, hygromycin phophotransferase. *nptII*, neomycin phophotransferase. LP, linker peptideregion isolated from the seeds of *Impatiens balsamina*. *Dm-AMP1*, Antimicrobial proteins from *Dahlia merckii*. *Rs-AFP2*, Antimicrobial proteins from *Raphanus sativus*. *Rpi-sto1*, Resistance gene for *Phytophthora infestans* from *Solanum stoloniferum*. *Rpi-vnt1.1*, *Rpi* from *S. venturii*. *Rpi-blb3*, *Rpi* from *S. bulbocastanum*. *TBS* transformation booster sequence, *MYB* CsMybA, *Bar* bialaphos resistance, *GFP* enhanced green fuorescent, *Luc* frefy luciferase, *Sul1* sulfadiazine resistance (Adopted from Shehryar et al., 2019)

3.2 Methods and technologies for gene stacking

Several advanced biotechnological methods are employed for gene stacking, including CRISPR/Cas9, TALENs, and zinc finger nucleases (ZFNs). These technologies enable precise editing and integration of multiple genes into the plant genome. CRISPR/Cas9: This technology allows for targeted gene editing by creating double-strand breaks at specific locations in the DNA, which can then be repaired by the cell's natural repair mechanisms, incorporating the desired genes. TALENs: Transcription activator-like effector nucleases (TALENs) are engineered proteins that can be designed to bind to specific DNA sequences, enabling targeted gene insertion or modification. ZFNs: Zinc finger nucleases are another class of engineered proteins that facilitate targeted genome editing. They have been used in combination with modular ‘trait landing pads’ (TLPs) to enable precise and efficient stacking of multiple traits in crops (Ainley et al., 2013). These technologies have revolutionized the field of genetic engineering by providing tools for precise and efficient gene stacking, thereby enhancing the durability and effectiveness of pest resistance in crops.

3.3 Selection of genes for stacking based on pest resistance mechanisms

The selection of genes for stacking is critical and is based on understanding the pest resistance mechanisms. Genes are chosen to target different aspects of the pest's biology, such as metabolic pathways, detoxification processes, and reproductive mechanisms. For instance, genes encoding for proteins that interfere with the pest's digestive system or reproductive cycle can be stacked to provide a multi-faceted defense (Liu et al., 2011; Salim et al., 2020).

Additionally, the genes selected for stacking should have complementary modes of action to avoid cross-resistance. For example, combining genes that produce different types of toxins can be more effective than using multiple genes that produce the same toxin (Gressel et al., 2017; Salim et al., 2020). The goal is to create a robust defense system that pests find difficult to overcome, thereby extending the effectiveness of the resistance traits.

In conclusion, multi-gene stacking is a powerful strategy in citrus pest resistance breeding, leveraging advanced genetic technologies and a deep understanding of pest resistance mechanisms to develop durable and effective pest-resistant crops. By carefully selecting and combining multiple genes, researchers can create crops that are better equipped to withstand pest pressures and reduce the reliance on chemical pesticides.

4 Genetic Basis of Pest Resistance in Citrus

4.1 Key genes involved in citrus pest resistance

The genetic basis of pest resistance in citrus involves several key genes that have been identified and utilized in breeding programs. One notable example is the resistance gene *CsLOB1*, which has been successfully used to induce resistance to citrus bacterial canker in sweet orange and grapefruit through genome editing techniques (Salonia et al., 2020). This gene plays a crucial role in enhancing the plant's defense mechanisms against bacterial pathogens. Additionally, other genes such as those involved in the synthesis of secondary metabolites and structural proteins contribute to the overall pest resistance in citrus.

4.2 Interaction of these genes with pest biology

The interaction between pest resistance genes and pest biology is complex and multifaceted. For instance, the *CsLOB1* gene in citrus not only provides resistance to bacterial canker but also interacts with the pest's ability to infect and proliferate within the host plant (Salonia et al., 2020). The effectiveness of these genes often depends on their expression levels, the timing of expression, and the specific tissues in which they are active. Moreover, the presence of multiple resistance genes can create a more robust defense system, making it harder for pests to overcome the plant's defenses. This multi-gene approach can delay the evolution of pest resistance, as pests would need to simultaneously adapt to multiple defense mechanisms (Gressel et al., 2017).

4.3 Strategies for identifying and validating resistance genes

Identifying and validating resistance genes in citrus involves several strategies. One common approach is the use of new plant breeding techniques (NPBTs) such as genome editing and cisgenesis, which allow for precise modifications of the plant genome to introduce or enhance resistance traits (Salonia et al., 2020). These techniques require a thorough understanding of the genes controlling specific traits and their interactions with pest biology. Another strategy is the use of transgene stacking systems, which involve the assembly of multiple resistance genes into a single construct and their introduction into the plant genome via transformation methods. This approach has been successfully used in other crops, such as rice, to develop multi-resistance varieties (Li et al., 2020). The validation of these genes typically involves field trials and laboratory assays to assess their effectiveness against target pests and their impact on plant health and yield.

In summary, the genetic basis of pest resistance in citrus is underpinned by key resistance genes such as *CsLOB1*, which interact with pest biology to provide effective defense mechanisms. Strategies for identifying and validating these genes include the use of NPBTs and transgene stacking systems, which have shown promise in enhancing pest resistance in citrus and other crops (Gressel et al., 2017; Li et al., 2020; Salonia et al., 2020).

5 Application of Multi-Gene Stacking in Citrus Breeding

5.1 Design and construction of gene stacks for citrus pest resistance

The design and construction of gene stacks for citrus pest resistance involve the strategic combination of multiple genes that confer resistance to various pests and diseases. This approach leverages advanced genetic engineering techniques to introduce and express multiple resistance genes within a single citrus plant. For instance, the use of CRISPR/Cas9 technology has been instrumental in modifying susceptibility genes such as *CsLOB1* to confer resistance to citrus canker (Jia et al., 2017). Additionally, the integration of resistance genes from related species into commercial citrus varieties has been achieved through fast-track breeding systems, significantly reducing the breeding time (Endo et al., 2020). The selection of appropriate genes and their effective combination is crucial for developing robust pest-resistant citrus varieties.

5.2 Techniques for introducing gene stacks into citrus plants

Several techniques are employed to introduce gene stacks into citrus plants, each with its own set of advantages and challenges. Agrobacterium-mediated transformation is a widely used method for genetic transformation in citrus, allowing for the stable integration of multiple genes. Physical methods such as biolistics and magnetofection have also been explored for their potential to introduce gene stacks (Conti et al., 2021). Moreover, the use of inducible promoters and site-specific recombination systems has improved the efficiency of transformation and regeneration protocols, which are critical for the successful application of new breeding techniques (Poles et al., 2020). The development of transfer-DNA-free base-edited citrus plants represents a significant advancement, enabling the generation of edited, non-transgenic plants with desirable traits (Alquézar et al., 2022).

5.3 Case studies of successful multi-gene stacking in citrus breeding

Several case studies highlight the successful application of multi-gene stacking in citrus breeding. One notable example is the use of CRISPR/Cas9 technology to edit the *CsLOB1* gene in Duncan grapefruit, resulting in lines with significantly reduced canker symptoms (Figure 2) (Jia et al., 2017). Another example is the fast-track breeding system that incorporated CTV resistance from trifoliate orange into citrus germplasm, achieving significant resistance in a much shorter time frame compared to conventional breeding methods (Figure 3) (Endo et al., 2020). Additionally, the overexpression of flower meristem-identity genes in *APETALA1* citrus plants has enabled rapid evaluation of transgenic traits, demonstrating the feasibility of stable transgene stacking in early-fruiting transgenic trees (Cervera et al., 2009). These case studies underscore the potential of multi-gene stacking strategies to enhance pest resistance in citrus breeding.

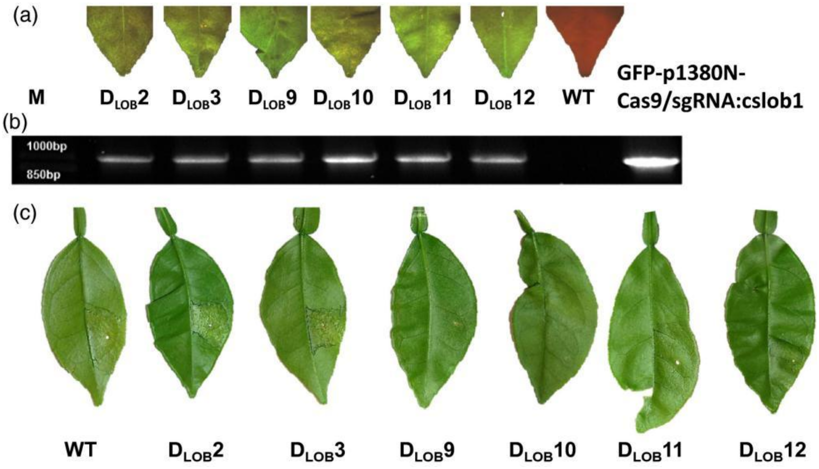

Figure 2 Analysis of GFP-p1380N-Cas9/sgRNA:cslob1-transformed Duncan grapefruit (Adopted from Jia et al., 2017)

Image caption: (a) six GFP-p1380N-Cas9/sgRNA:cslob1-transformed Duncan grapefruit plants (DLOB2, DLOB3, DLOB9, DLOB10, DLOB11 and DLOB12) were GFP positive. The wild-type grapefruit plant did not show GFP. (b) The six transgenic lines contain Cas9/sgRNA as indicated by PCR amplification using primers 35SP-5-P1 and NosP-3-P2. Plasmid GFP-p1380N-Cas9/sgRNA:cslob1 was used as a positive control. M, 1 kb DNA ladder; WT, wild type. C. The six *CsLOB1*-modified lines showed differential resistance to Xcc. At 4 days postinoculation with Xcc (5×10^8 CFU/mL) using needleless syringe, canker symptoms were observed on normal grapefruit, DLOB2 and DLOB3, but absent or reduced on DLOB9, DLOB10, DLOB11 and DLOB12 (Adopted from Jia et al., 2017)

6 Field Trials and Performance Evaluation

6.1 Overview of methodologies for field trials

Field trials are essential for evaluating the performance of genetically modified (GM) citrus varieties, particularly in terms of pest resistance. These trials typically involve the cultivation of GM citrus plants in natural or semi-natural environments where they are exposed to various pests and environmental conditions. The methodologies for field trials can be broadly categorized into several key steps. Selection of test sites: Test sites are chosen based on their historical pest pressure and environmental conditions that are representative of commercial citrus-growing regions (Sun et al., 2019; Salonia et al., 2020). Experimental design: Randomized complete block designs (RCBD) or split-plot designs are commonly used to minimize variability and ensure robust statistical analysis (Gressel et al., 2017; Li et al., 2020).

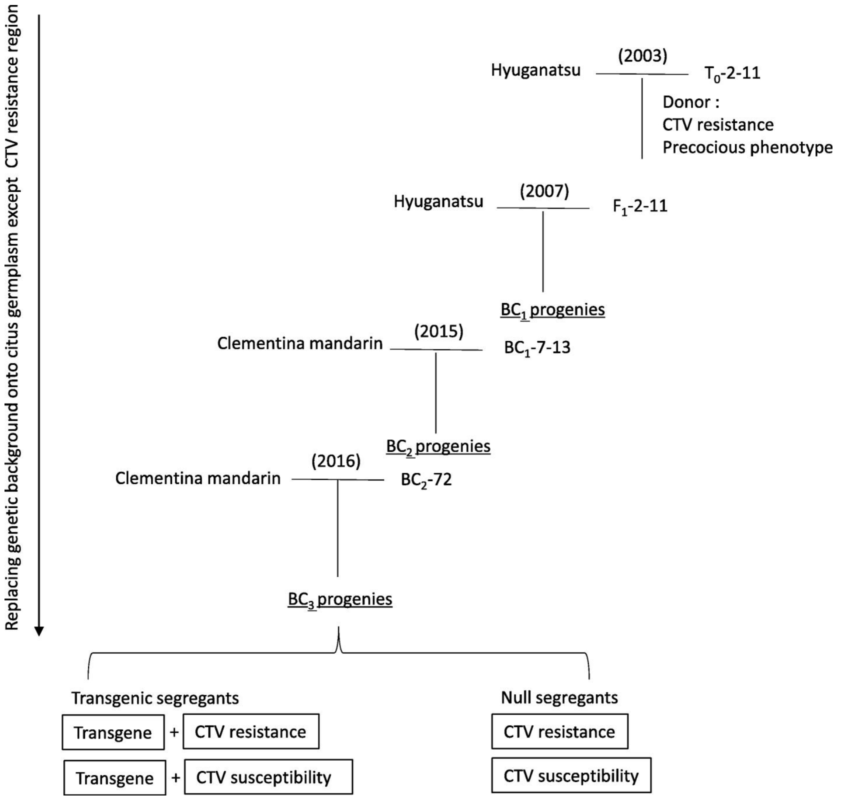

Figure 3 Scheme of fast-track breeding system to introduce CTV resistance into citrus germplasm (Adopted from Endo et al., 2020)
Image caption: Precocious transgenic trifoliate orange that overexpresses *CiFT* (T_0-2-11) was used as a primary pollen parent for the CTV resistance donor. BC progenies with T-DNA integrated regions generally flower around 3 weeks after planting. F_1-2-11, BC_1-7-13, and BC_2-72 were selected as the pollen parents with DNA markers, to possess CTV resistance and transgenes. In 2016, the flowering times of the seed parent and transgenic seedling were coordinated, to promote the breeding from the BC_2 to the BC_3 generation, in a single year (Adopted from Endo et al., 2020)

Planting and maintenance: GM citrus plants are planted alongside non-GM controls. Standard agronomic practices are followed, including irrigation, fertilization, and pest management, to ensure that any observed differences are due to genetic modifications (Sun et al., 2019; Salonia et al., 2020). Data collection: Regular monitoring and data collection are conducted to assess pest incidence, plant health, growth parameters, and yield. Advanced techniques such as remote sensing and digital imaging may also be employed for more precise measurements (Minamikawa et al., 2017). Statistical analysis: Data are analyzed using statistical software to determine the significance of observed differences between GM and non-GM plants. Metrics such as pest infestation levels, plant vigor, and yield are compared (Gressel et al., 2017; Li et al., 2020).

6.2 Key field trials and their outcomes

Several key field trials have been conducted to evaluate the performance of GM citrus varieties with stacked pest resistance genes. Trial 1: A study on the development of multi-resistance rice using a transgene stacking system demonstrated significant improvements in resistance to multiple pests and diseases. The GM rice showed enhanced resistance to glyphosate, borers, brown planthopper, bacterial blight, and rice blast, with higher yields compared to the non-GM control (Li et al., 2020). Although this study was on rice, the methodologies and outcomes are highly relevant to citrus pest resistance breeding. Trial 2: Field trials on citrus varieties engineered for resistance to bacterial canker using the *CsLOB1* gene showed promising results. The GM citrus plants exhibited significantly lower disease incidence and better overall health compared to non-GM controls (Salonia et al., 2020).

Trial 3: Another study focused on the use of CRISPR/Cas9 technology to develop citrus varieties resistant to huanglongbing (HLB). The field trials indicated that the GM citrus plants had reduced HLB symptoms and improved fruit quality (Sun et al., 2019). Trial 4: Research on stacked transgenic traits for pest and herbicide resistance highlighted the importance of using multiple genes targeting the same pest species. The field trials demonstrated that such stacking could delay the evolution of pest resistance and improve overall pest management (Gressel et al., 2017). Trial 5: A genome-wide association study (GWAS) and genomic selection (GS) in citrus breeding populations showed that these advanced genomic tools could enhance the detection of beneficial traits and improve the accuracy of selecting pest-resistant varieties (Minamikawa et al., 2017).

6.3 Criteria and metrics for evaluating pest resistance in genetically modified citrus

Evaluating pest resistance in GM citrus involves several criteria and metrics. Pest incidence and severity: The primary metric is the incidence and severity of pest infestations. This includes counting the number of pests per plant and assessing the extent of damage (Sun et al., 2019; Li et al., 2020). Plant health and vigor: Metrics such as plant height, leaf area, and overall vigor are used to assess the health of GM plants compared to non-GM controls (Gressel et al., 2017; Salonia et al., 2020). Yield and fruit quality: Yield parameters, including the number of fruits per plant and total fruit weight, are critical. Fruit quality traits such as size, juiciness, and acidity are also evaluated (Minamikawa et al., 2017). Resistance durability: Long-term studies are conducted to assess the durability of pest resistance over multiple growing seasons. This includes monitoring for any signs of pest adaptation or resistance breakdown (Gressel et al., 2017). Environmental impact: The impact of GM citrus on non-target organisms and overall ecosystem health is also considered. This includes studies on beneficial insects, soil health, and biodiversity (Sun et al., 2019; Salonia et al., 2020). By adhering to these methodologies and criteria, researchers can effectively evaluate the performance of GM citrus varieties and their potential for commercial use in pest resistance breeding programs.

7 Challenges and Limitations

7.1 Technical challenges in gene stacking and transformation

The application of multi-gene stacking strategies in citrus pest resistance breeding faces several technical challenges. One of the primary issues is the complexity of the citrus genome, which includes high heterozygosity, polyembryony, and long juvenility phases, making traditional breeding methods laborious and time-consuming (Sendín and Filippone, 2018; Salonia et al., 2020). Genetic transformation techniques, such as Agrobacterium tumefaciens-mediated transformation, have been employed to introduce resistance genes like *Bs2* for citrus canker resistance. However, the efficiency of these transformations and the subsequent regeneration of plants remain significant bottlenecks (Sendín and Filippone, 2018; Salonia et al., 2020). Additionally, optimizing protocols for genome editing tools like CRISPR/Cas9 and CRISPR/Cpf1 systems is crucial for achieving precise and efficient gene stacking (Sun et al., 2019).

7.2 Ecological and environmental considerations

The ecological and environmental impacts of deploying genetically modified citrus varieties with stacked resistance genes must be carefully evaluated. There is a potential risk of unintended effects on non-target organisms and the broader ecosystem. For instance, the introduction of multiple resistance genes could lead to changes in pest populations and the emergence of new pest species or strains that can overcome the resistance (Li et al., 2020). Moreover, the long-term sustainability of these genetically modified crops in diverse environmental conditions needs thorough assessment to ensure they do not negatively impact biodiversity or lead to ecological imbalances (Sun et al., 2019).

7.3 Regulatory and safety issues

Regulatory frameworks for genetically modified organisms (GMOs) vary significantly across different countries, posing a challenge for the widespread adoption of multi-gene stacked citrus varieties. Ensuring compliance with these regulations requires extensive safety assessments, including evaluating potential allergenicity, toxicity, and environmental impact (Sun et al., 2019; Salonia et al., 2020). The development of marker-free systems is also essential to address public concerns and regulatory requirements regarding the presence of selectable marker genes in transgenic plants (Salonia et al., 2020). Additionally, the approval process for GMOs can be lengthy and costly, further complicating the commercialization of these advanced breeding technologies.

7.4 Economic and scalability challenges

The economic feasibility and scalability of multi-gene stacking strategies in citrus breeding are significant concerns. The initial costs of developing genetically modified citrus varieties, including research, development, and regulatory approval, are substantial (Sun et al., 2019; Salonia et al., 2020). Furthermore, the scalability of these technologies to produce sufficient quantities of transgenic plants for commercial use is challenging. The long juvenility phase of citrus plants means that it takes several years before the benefits of the genetic modifications can be realized in terms of fruit production and pest resistance (Salonia et al., 2020). Additionally, the adoption of these technologies by farmers may be hindered by the high costs and the need for specialized knowledge and infrastructure to manage genetically modified crops (Sendín and Filippone, 2018).

In summary, while multi-gene stacking strategies hold great promise for enhancing citrus pest resistance, several challenges and limitations must be addressed. These include technical difficulties in gene stacking and transformation, ecological and environmental considerations, regulatory and safety issues, and economic and scalability challenges. Addressing these challenges will require continued research, collaboration, and innovation in the field of citrus breeding.

8 Future Directions and Perspectives

8.1 Emerging trends and technologies in multi-gene stacking

The field of citrus pest resistance breeding is rapidly evolving with the advent of new plant breeding techniques (NPBTs) and genetic engineering technologies. One of the most promising trends is the use of CRISPR/Cas9 for targeted genome editing, which has shown significant potential in conferring resistance to diseases such as citrus canker and Huanglongbing (HLB) (Jia et al., 2017; Sun et al., 2019; Salonia et al., 2020; Conti et al., 2021). The development of improved CRISPR systems, such as CRISPR/Cpf1, further enhances the precision and efficiency of these genetic modifications (Sun et al., 2019). Additionally, the integration of multiple resistance genes through hybridization and genetic transformation is becoming increasingly feasible, allowing for the stacking of genes that confer broad-spectrum resistance. This approach has been successfully demonstrated in other crops, such as potatoes, and holds great promise for citrus as well (Rogozina et al., 2021).

8.2 Integration with other pest management strategies

While genetic engineering and multi-gene stacking offer powerful tools for developing pest-resistant citrus varieties, their effectiveness can be further enhanced when integrated with other pest management strategies. For instance, combining genetically modified resistant varieties with traditional methods such as biological control, cultural practices, and the use of insecticides can provide a more comprehensive and sustainable approach to pest management. Understanding the mechanisms of resistance, such as the overexpression of detoxification genes in pests like the Asian citrus psyllid, can also inform the development of more targeted and effective pest control measures (Tian et al., 2019). Moreover, the use of marker-assisted selection (MAS) in conjunction with genetic engineering can accelerate the breeding process and ensure the incorporation of desirable traits (Endo et al., 2020).

8.3 Long-term vision and potential breakthroughs in citrus pest resistance breeding

Looking ahead, the long-term vision for citrus pest resistance breeding involves the creation of highly resilient citrus varieties that can withstand a wide range of biotic and abiotic stresses. This will likely involve the continued refinement of genome editing technologies and the development of more efficient transformation and regeneration protocols (Poles et al., 2020; Huang et al., 2022). The integration of genomic selection (GS) and genome-wide association studies (GWAS) can also play a crucial role in identifying and selecting for traits that contribute to pest resistance and overall fruit quality (Minamikawa et al., 2017). Additionally, the exploration of novel promoters and marker-free systems will be essential for the development of commercially viable genetically modified citrus varieties (Conti et al., 2021). Ultimately, the goal is to achieve a sustainable and resilient citrus industry that can thrive in the face of emerging pest and disease threats.

9 Concluding Remarks

The application of multi-gene stacking strategies in citrus pest resistance breeding has shown significant promise in enhancing disease resistance and improving agronomic traits. Multi-gene stacking, or transgene stacking, involves the introduction of multiple genes into a plant to confer resistance to various pests and diseases. This approach has been more effective than single-gene technology in providing durable resistance and improving crop yield. In citrus, genetic engineering techniques such as CRISPR/Cas9 have been successfully employed to develop disease-resistant varieties, particularly against bacterial canker and huanglongbing. The use of new plant breeding techniques (NPBTs) has also been highlighted as a crucial method for overcoming traditional breeding limitations and achieving improved resistance to biotic and abiotic stresses.

For researchers, the findings underscore the importance of continuing to explore and refine multi-gene stacking techniques. The development of efficient transgene stacking systems and the optimization of protocols for plant regeneration are critical areas for future research. Breeders can leverage these advanced genetic engineering tools to develop citrus varieties with enhanced resistance to multiple pests and diseases, thereby reducing the reliance on chemical pesticides and improving crop yield and quality. Policymakers should consider the regulatory frameworks surrounding the use of genetically modified organisms (GMOs) and NPBTs. Clear guidelines and support for the adoption of these technologies can facilitate their integration into agricultural practices, ultimately contributing to sustainable agriculture and food security.

Further research is needed to address the challenges associated with multi-gene stacking, such as the potential for evolved resistance in pests and the need for marker-free systems. Interdisciplinary collaboration between geneticists, plant pathologists, agronomists, and policymakers is essential to advance the development and implementation of these technologies. By working together, these stakeholders can ensure that the benefits of multi-gene stacking strategies are fully realized, leading to more resilient and productive citrus crops. Additionally, exploring the integration of conventional breeding with genetic engineering approaches can provide a comprehensive strategy for achieving durable resistance in citrus.

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Conflict of Interest Disclosure

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