

## Gene Stacking Strategies to Enhance the Durability of Bt Crops

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**Abstract** As global agriculture faces the compounded challenges of increasing pest pressures, environmental stressors, and limited resources, enhancing the durability of genetically modified crops, particularly Bt crops, is crucial for ensuring food security and promoting sustainable agricultural development. This study analyzes gene stacking strategies aimed at enhancing the durability of Bt crops, reviews the development history of Bt crops and their significant role in pest management, and explores how gene stacking enhances crop durability through various mechanisms while effectively reducing the development of pest resistance. Through the analysis of multiple successful case studies, this study highlights the significant impact of gene stacking strategies in improving the durability of Bt crops and their clear advantages over single-trait strategies. Additionally, it discusses the technical and other challenges encountered during implementation and proposes new directions for the development of gene stacking technology to further enhance the durability of Bt crops, improve overall crop health and yield, and contribute to sustainable agricultural development.

**Keywords** Bt crops; Gene stacking; Durability; Pest management; Sustainable agriculture

### 1 Introduction

The advent of genetically modified (GM) crops expressing *Bacillus thuringiensis* (Bt) toxins has revolutionized agricultural pest management by providing an effective and environmentally friendly alternative to chemical insecticides. Bt crops, which produce insecticidal proteins derived from the bacterium *Bacillus thuringiensis*, have been widely adopted globally due to their ability to control a broad spectrum of insect pests, thereby reducing crop losses and minimizing the need for chemical pesticide applications (MacRae et al., 2005; Pardo-López et al., 2013; Koul, 2020). However, the sustainability of Bt crops is threatened by the potential for insect pests to develop resistance to Bt toxins, necessitating the development of strategies to enhance the durability of these crops (Marroquin et al., 2000).

Bt crops are genetically engineered to express one or more Cry proteins, which are toxic to specific insect pests. These proteins function by binding to receptors in the insect gut, forming pores that disrupt gut cells and ultimately lead to the insect's death (Pardo-López et al., 2013). The widespread adoption of Bt crops, such as Bt cotton and Bt maize, has significantly reduced the reliance on chemical insecticides, leading to economic and environmental benefits (Koul, 2020). However, the continuous exposure of insect populations to Bt toxins exerts selective pressure, which can lead to the evolution of resistance in target pest species (Zhao et al., 2005).

The durability of Bt crops is crucial for maintaining their long-term efficacy and sustainability. Resistance management strategies are essential to delay the onset of resistance and prolong the effectiveness of Bt crops. One such strategy is gene stacking, or pyramiding, which involves incorporating multiple Bt genes encoding different Cry proteins into a single plant. This approach aims to provide a broader spectrum of pest control and reduce the likelihood of resistance development (Pardo-López et al., 2013; Koul, 2020). However, the concurrent use of single-gene and pyramided Bt plants can accelerate resistance if pests are exposed to similar toxins from both plant types (Zhao et al., 2005). Therefore, understanding and optimizing gene stacking strategies is vital for enhancing the durability of Bt crops.

This study evaluates the current gene stacking strategies employed to enhance the durability of Bt crops. By summarizing the mechanisms of action and resistance associated with different Bt toxins, assessing the effectiveness of various gene stacking approaches in delaying resistance development, identifying potential challenges and limitations in the implementation of gene stacking strategies, and providing recommendations for future research and regulatory policies, the study aims to support the sustainable use of Bt crops. By synthesizing existing literature on gene stacking strategies, this study provides a comprehensive understanding of best practices for enhancing the durability of Bt crops, ensuring their continued success in agricultural pest management.

## **2 Concept of Gene Stacking**

### **2.1 Definition and principles**

Gene stacking refers to the process of introducing multiple genes of interest into a single plant genome to combine various desirable traits. This technique is increasingly popular in biotechnology for enhancing crop performance by integrating multiple traits such as insect resistance, herbicide tolerance, and stress resilience. The resulting plants, known as biotech stacked or simply stacked crops, exhibit a combination of traits that provide a significant genetic and agronomic boost, enabling them to thrive in challenging farming conditions (Shehryar et al., 2019; Aparna et al., 2021).

The principles of gene stacking involve the precise integration of multiple transgenes into specific genomic locations to ensure stable and high-level expression without disrupting native gene functions. This can be achieved through various methods, including gene pyramiding, recombinase-mediated integration, and the use of designed nucleases for targeted DNA double-strand breaks and subsequent repair (Hou et al., 2014; Petolino and Kumar, 2016; Srivastava and Thomson, 2016). These techniques allow for the sequential addition of genes, creating a cumulative effect that enhances the overall performance and durability of the crops.

### **2.2 Historical development**

The concept of gene stacking has evolved significantly over the past few decades. Initially, the focus was on single-gene transformations to confer specific traits such as herbicide resistance or pest tolerance. However, the limitations of single-gene approaches, particularly in addressing complex traits like yield and stress tolerance, led to the development of multi-gene stacking strategies (Collier et al., 2018).

Early methods of gene stacking involved conventional breeding techniques to combine multiple traits, but these were often time-consuming and inefficient. Advances in genetic engineering and biotechnology have since revolutionized the field, enabling more precise and efficient methods of gene stacking. Techniques such as the use of recombinases, designed nucleases, and *Agrobacterium*-mediated transformation have paved the way for the development of high-efficiency gene stacking systems (Hou et al., 2014; Srivastava and Thomson, 2016; Collier et al., 2018). These advancements have made it possible to introduce multiple genes into crops, resulting in enhanced disease resistance, improved yield, and greater adaptability to environmental stresses (Shehryar et al., 2019; Zhao et al., 2023).

### **2.3 Types of gene stacking**

Gene stacking can be categorized into several types based on the methods used and the traits targeted. One common approach is gene pyramiding, which involves the sequential addition of genes to achieve a cumulative effect. This method is particularly effective for enhancing resistance to pests and diseases by combining multiple resistance genes into a single crop variety (Yang et al., 2011; Fuchs, 2017).

Another type of gene stacking involves the use of recombinase-mediated integration, where specific recombination sites are introduced into the plant genome to facilitate the precise insertion of multiple genes. This method allows for the repeated addition of genes at the same genomic location, ensuring stable expression and minimizing the risk of gene silencing (Hou et al., 2014; Srivastava and Thomson, 2016). Additionally, designed nucleases can be used to create targeted DNA double-strand breaks, enabling the precise integration of transgenes and the creation of stacked products with high-level and stable expression (Petolino and Kumar, 2016).

### 3 Methods of Gene Stacking

#### 3.1 Traditional breeding techniques

Traditional breeding techniques have long been employed to enhance crop traits by combining desirable genes from different parent plants. One of the primary methods used in traditional breeding is marker-assisted selection (MAS), which allows for the identification and selection of plants that carry specific genes of interest. This technique has been particularly effective in gene pyramiding, where multiple genes conferring resistance to biotic and abiotic stresses are stacked together to create more resilient crop varieties (Dormatey et al., 2020). For instance, MAS has been successfully used to develop crops with improved tolerance to drought and salinity, which are critical for sustainable agricultural production in the face of climate change (Shailani et al., 2020).

Despite its effectiveness, traditional breeding has limitations, particularly in the time required to achieve desired results and the complexity of combining multiple traits. The process can be slow and labor-intensive, often taking several generations to achieve the desired gene combinations. Additionally, traditional breeding is limited by the genetic diversity available within the species, making it challenging to introduce novel traits that are not present in the gene pool (José et al., 2020). These limitations have driven the development of more advanced techniques, such as genetic engineering and biotechnological methods, to overcome these challenges and achieve more precise and efficient gene stacking.

#### 3.2 Genetic engineering approaches

Genetic engineering has revolutionized the field of crop improvement by enabling the direct manipulation of plant genomes to introduce desirable traits. One of the key techniques in genetic engineering for gene stacking is the use of transgenic technology, which allows for the insertion of multiple genes into a plant's genome. This method has been used to create crops with enhanced resistance to pests, diseases, and environmental stresses (Halpin, 2005). For example, the GuanNan Stacking (GNS) system (Figure 1) utilizes Type IIS restriction enzyme-mediated Golden Gate cloning and Gateway recombination to assemble multiple gene expression cassettes into a single binary vector, facilitating the creation of transgenic plants with stacked traits (Qin et al., 2022).

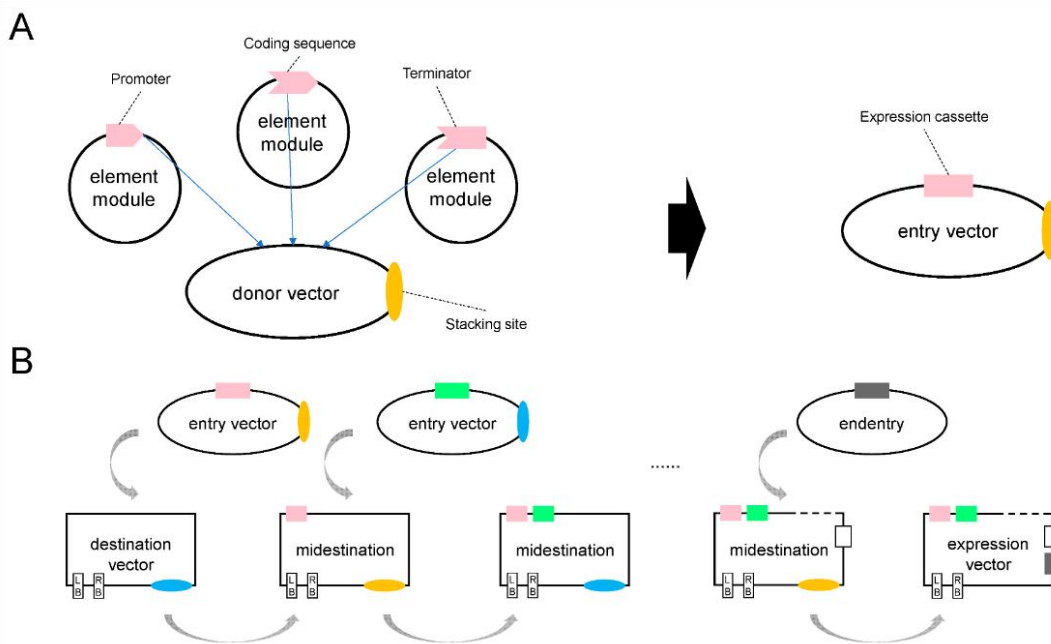


Figure 1 Basic principles of the GNS system (Adapted from Qin et al., 2022)

Image caption: A: Assembly of an entry vector using the backbone of the donor vector and three element modules, each containing components of the expression cassette, namely the promoter, coding sequence (CDS), or terminator; B: Stacking process using the target vector and entry vector (Adapted from Qin et al., 2022)

Another approach in genetic engineering is the use of site-specific recombination systems, such as the Cre-lox system, which allows for the precise integration of multiple genes into specific genomic locations. This method ensures the co-inheritance of the stacked genes, providing stable expression of the desired traits across generations (Srivastava, 2018). The high efficiency and precision of recombinase-mediated gene stacking make it a powerful tool for developing crops with complex trait combinations, such as improved tolerance to both drought and salinity. These advancements in genetic engineering have significantly expanded the possibilities for gene stacking, enabling the creation of crops with multiple, stacked traits that are difficult to achieve through traditional breeding alone.

### **3.3 Advanced biotechnological methods**

Advanced biotechnological methods have further enhanced the capabilities of gene stacking by introducing more sophisticated techniques for gene integration and expression. One such method is the use of modular gene stacking systems, which allow for the assembly of large, complex gene constructs that can be introduced into plants in a single transformation event. These systems, such as the GNS system, utilize advanced cloning techniques to create multi-gene expression cassettes that can be efficiently integrated into the plant genome (Qin et al., 2022). This approach not only simplifies the process of gene stacking but also increases the likelihood of successful integration and expression of the stacked genes.

Another promising biotechnological method is the use of genome editing tools, such as CRISPR/Cas9, to precisely modify plant genomes and introduce multiple genes at specific locations. This technique allows for the targeted insertion of genes, reducing the risk of off-target effects and ensuring the stable expression of the stacked traits (Schaart et al., 2016). Genome editing has been used to create crops with enhanced resistance to diseases and environmental stresses by stacking multiple resistance genes into a single locus (Shailani et al., 2020). The precision and flexibility of genome editing make it an ideal tool for gene stacking, enabling the development of crops with complex trait combinations that are tailored to specific agricultural needs.

## **4 Mechanisms of Enhancing Durability**

### **4.1 Multiple modes of action**

One of the primary strategies to enhance the durability of Bt crops is through the incorporation of multiple modes of action. This involves stacking different genes that produce various insecticidal proteins, each targeting different aspects of pest physiology. For instance, the development of transgenic rice expressing a fusion protein of Cry1Ab and Vip3A has shown significant resistance to major rice pests such as the Asiatic rice borer and rice leaf folder, without compromising agronomic performance (Xu et al., 2018). This approach ensures that pests are less likely to develop resistance simultaneously to multiple toxins, thereby prolonging the effectiveness of the Bt crops.

Moreover, the combination of different Bt genes, such as *Cry1Ab* and *Cry2A*, in a single crop variety has demonstrated higher efficacy against pests compared to single-gene varieties. This gene stacking strategy not only enhances pest control but also maintains the yield and quality of the crops under field conditions (Yang et al., 2011). By employing multiple modes of action, the durability of Bt crops can be significantly improved, reducing the reliance on chemical insecticides and promoting sustainable agricultural practices.

### **4.2 Reducing resistance development**

Reducing the development of resistance in pest populations is crucial for maintaining the long-term effectiveness of Bt crops. One effective method is gene pyramiding, which involves stacking multiple resistance genes within a single plant. This strategy has been shown to suppress the emergence of virulent pathogen isolates and enhance the durability of resistance genes (Djian-Caporalino et al., 2014; REX Consortium, 2016). For example, pyramiding different resistance genes in pepper and lettuce crops has been experimentally proven to control root-knot nematodes more effectively than other strategies such as cultivar mixtures or gene rotation.

Additionally, rotating and stacking resistance genes can slow the evolution of virulent pathogen strains. A model predicting the effects of different rotational management strategies indicated that rotating cultivars with different



resistance genes could lead to longer periods of disease suppression, although it may also select for highly virulent strains (Cr  t   et al., 2020). Therefore, combining gene pyramiding with other integrated pest management practices can provide a robust approach to delay resistance development and sustain the efficacy of Bt crops.

### 4.3 Enhancing plant health and yield

Enhancing plant health and yield is another critical aspect of improving the durability of Bt crops. Gene stacking not only confers resistance to pests but also improves overall plant performance under various stress conditions. For instance, the simultaneous introduction of multiple genes such as *AVP1*, *OsSIZ1*, and *Fld* in creeping bentgrass has resulted in significantly better growth (Figure 2) and tolerance to abiotic stresses like drought, salinity, and heat (Zhao et al., 2023). This demonstrates that gene stacking can lead to superior plant performance and resilience, contributing to higher yields and better crop quality.

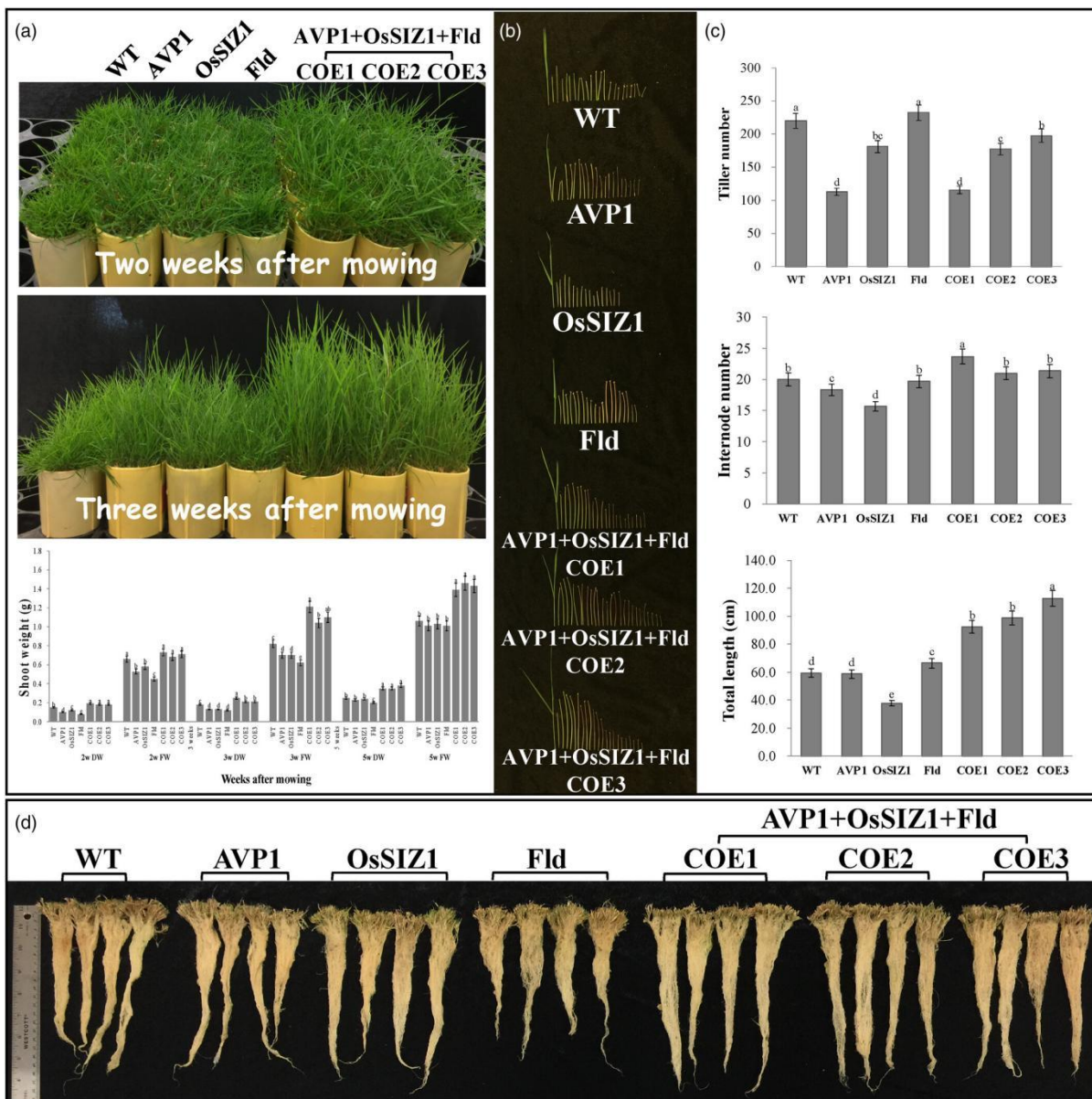


Figure 2 Comparison of growth and development between transgenic plants expressing *AVP1*, *OsSIZ1*, and *Fld* and wild-type control plants (Adapted from Zhao et al., 2023)

Image caption: (a) Plant growth status at 2 and 3 weeks after pruning; (b) Internode structure of the longest tiller in each plant; (c) Summary of the total number of tillers, total number of internodes of the longest tiller, and total length; (d) Root development of 13-week-old plants (Adapted from Zhao et al., 2023)

Zhao et al. (2023) compared the growth and development of transgenic plants (TG) expressing three transgenes (*AVPI*, *OsSIZ1*, and *Fld*) and single transgene (*AVPI*, *OsSIZ1*, or *Fld*) with non-transgenic wild-type (WT) control plants. The results showed that the expression of the three transgenes significantly enhanced plant growth performance, including higher biomass and stronger root development. The transgenic plants also exhibited significantly better tillering, internode number, and internode length compared to the wild-type control plants. Moreover, the multi-gene engineering approach significantly improved plant growth and development, providing a new strategy for increasing crop yield.

Furthermore, the integration of Bt genes into crops has shown substantial benefits in terms of yield increase and reduction in insecticide usage. Bt crops like Bt corn and cotton have demonstrated high levels of protection from major insect pests, leading to increased yields and reduced reliance on chemical insecticides. By enhancing plant health and yield through gene stacking, the overall sustainability and productivity of agricultural systems can be improved, ensuring food security and environmental conservation.

## **5 Case Studies of Successful Gene Stacking**

### **5.1 Examples from major Bt crops**

Gene stacking has been widely adopted in major Bt crops such as maize, cotton, and rice to enhance resistance against pests and diseases. For instance, in rice, the introduction of two *Bacillus thuringiensis* (Bt) genes through sexual crossing has shown significant success. The resulting transgenic rice lines exhibited higher resistance to pests like the striped stem borer and leafhoppers compared to single-gene lines, demonstrating the commercial potential of this approach (Yang et al., 2011). Similarly, in maize, the use of targeted genome editing techniques such as zinc finger nucleases (ZFNs) has enabled the precise integration of multiple herbicide resistance traits, resulting in crops that can withstand various herbicides and thus offer better weed management (Ainley et al., 2013).

In cotton, the combination of Bt genes has been shown to provide durable resistance against a range of pests. Field trials conducted over several years have demonstrated that stacked Bt cotton varieties not only offer enhanced pest resistance but also maintain agronomic and phenotypic characteristics similar to their single-trait counterparts, thereby ensuring no compromise on yield or quality (Figure 3) (José et al., 2020). These examples underscore the effectiveness of gene stacking in major Bt crops, providing a robust strategy for pest and disease management.

José et al. (2020) studied the agronomic and volunteer plant characteristics of cotton stacks (Cotton stacks 1-4) and single events. The results showed that, compared to the control group, the cotton stack materials exhibited significant differences in agronomic traits. The stack materials outperformed single genetic engineering materials and conventional controls in certain traits, such as plant height and yield. Tests conducted at different locations further supported these findings, indicating that gene stacking technology has the potential to improve cotton yield and agronomic traits. This study provides important experimental data and theoretical support for genetic engineering breeding in cotton.

### **5.2 Comparative analysis of stacked vs. single traits**

Comparative studies between stacked and single-trait genetically modified (GM) crops have consistently shown the superiority of stacked traits in terms of pest resistance and overall crop performance. For instance, a comprehensive study involving soybean, maize, and cotton revealed that stacked GM crops exhibited negligible differences in agronomic and phenotypic characteristics compared to their single-trait counterparts. This indicates that stacking does not introduce additional risks but rather enhances the benefits of GM crops (José et al., 2020).

Moreover, the durability of resistance conferred by stacked traits is significantly higher than that of single traits. In rice, for example, the pyramiding of two Bt genes resulted in higher resistance levels and better field performance against pests compared to single-gene lines (Yang et al., 2011). Similarly, the use of gene stacking in maize through targeted genome editing has shown that stacked traits can be precisely integrated and maintained

across generations, providing a reliable and efficient approach to trait management (Ainley et al., 2013). These findings highlight the advantages of stacked traits over single traits in enhancing crop resilience and performance.

### 5.3 Lessons learned

The successful implementation of gene stacking strategies in Bt crops offers several valuable lessons. The integration of multiple genes can significantly enhance the durability of pest and disease resistance, reducing the likelihood of resistance development in pests. This has been demonstrated in various crops, including rice and maize, where stacked traits have provided more robust and long-lasting resistance compared to single traits (Yang et al., 2011; Ainley et al., 2013).

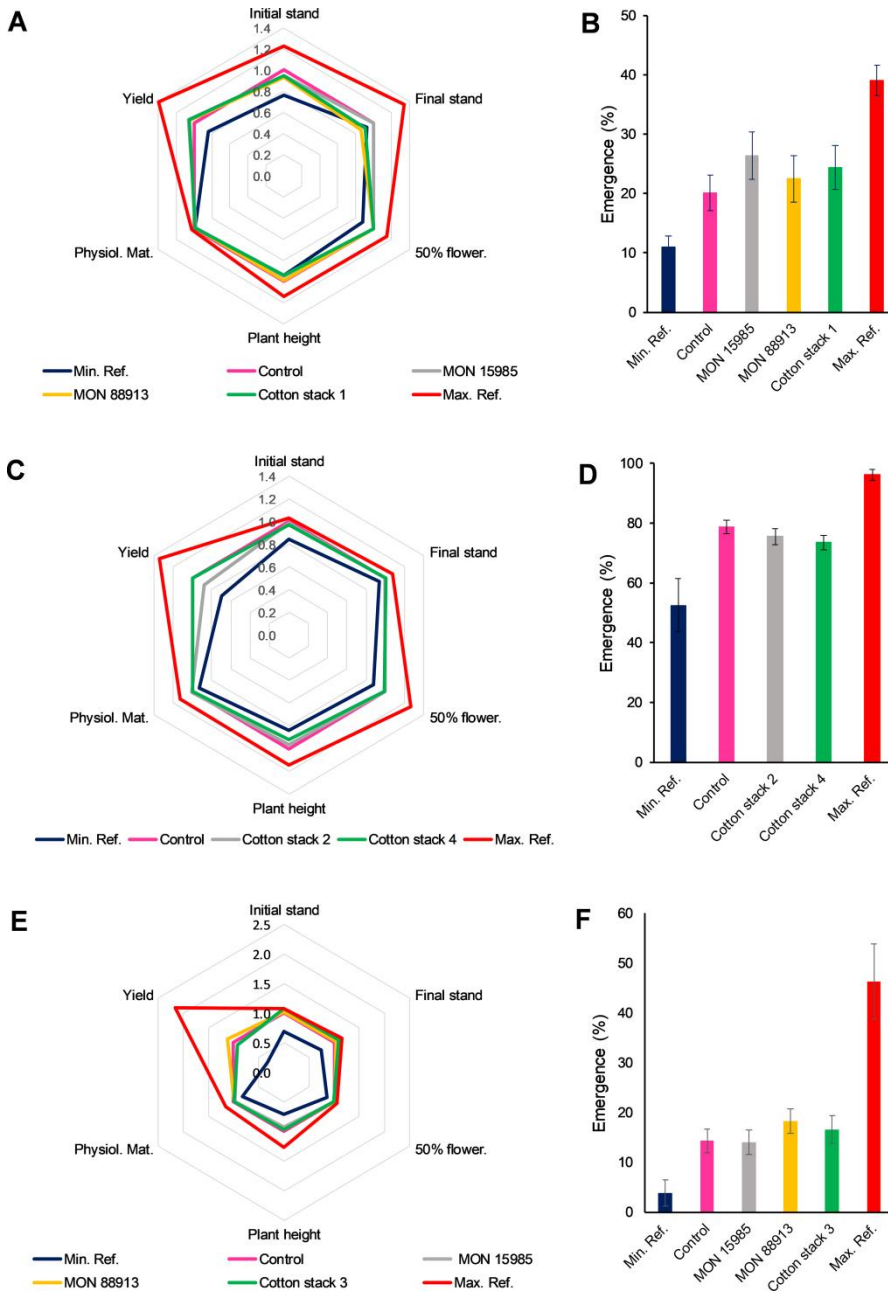


Figure 3 Evaluation of agronomic traits and emergence rates in cotton stacks and single events (Adapted from José et al., 2020)  
 Image caption: A, C, and E show the agronomic traits of different cotton materials (stacked, single, conventional control, and commercial reference materials), including initial plant number, final plant number, plant height, days to 50% flowering, physiological maturity, and seed cotton yield; B, D, and F present the emergence rates of different materials (Adapted from José et al., 2020)

The use of advanced techniques such as targeted genome editing can facilitate the precise and efficient stacking of traits, ensuring that the desired characteristics are consistently expressed in the resulting crops. This approach not only improves the effectiveness of gene stacking but also simplifies the breeding process, making it more manageable and scalable (Ainley et al., 2013).

The comparative analysis of stacked and single-trait crops underscores the importance of regulatory frameworks that recognize the safety and benefits of stacked traits. The evidence from field trials and compositional analyses supports the need for updated regulatory guidelines that streamline the approval process for stacked GM crops, thereby promoting their adoption and commercialization (Bell et al., 2018; José et al., 2020). These lessons provide a roadmap for future research and development in gene stacking, paving the way for more resilient and sustainable agricultural practices.

## **6 Challenges and Limitations**

### **6.1 Technical and biological challenges**

Gene stacking, while promising, faces several technical and biological challenges. One of the primary technical hurdles is the difficulty in achieving stable expression of multiple genes in transgenic plants. The expression of multiple genes can lead to unpredictable interactions and reduced efficacy of individual genes, as seen in the case where the expression of the *cry1C* gene decreased after gene stacking in rice (Yang et al., 2011). Additionally, the development of transgenic plants with multiple stacked genes is complex and time-consuming, often requiring advanced molecular techniques and extensive field testing to ensure stability and effectiveness (Halpin, 2005).

Biologically, the evolution of pest resistance remains a significant challenge. Despite the use of gene stacking to delay resistance, pests can still develop resistance to multiple Bt toxins over time. This is particularly concerning given the high adaptability of pests and the potential for cross-resistance, where resistance to one toxin confers resistance to another (Ainley et al., 2013). Moreover, the effectiveness of gene stacking can be influenced by environmental factors and the genetic background of the host plant, which can affect the expression and performance of the stacked genes (Xu et al., 2018).

### **6.2 Regulatory and ethical issues**

The deployment of Bt crops with stacked genes also raises several regulatory and ethical issues. Regulatory frameworks for genetically modified organisms (GMOs) vary widely across different countries, and obtaining approval for crops with multiple transgenes can be more complex and time-consuming than for single-gene modifications. This can delay the commercialization and adoption of such crops, limiting their potential benefits. Additionally, there are concerns about the long-term environmental impacts of releasing transgenic plants with stacked genes, including potential effects on non-target organisms and biodiversity (REX Consortium, 2016).

Ethically, the use of gene stacking in Bt crops raises questions about the control and ownership of genetic resources. The development and commercialization of these crops are often dominated by large biotech companies, which can lead to issues of access and equity, particularly for smallholder farmers in developing countries (Dormatey et al., 2020). There is also the broader ethical debate about the use of genetic engineering in agriculture, with some stakeholders advocating for more natural and sustainable farming practices over the use of GMOs (Bailey-Serres et al., 2019).

### **6.3 Economic and market considerations**

From an economic perspective, the development and deployment of Bt crops with stacked genes involve significant costs. The research and development process is expensive, and the regulatory approval process can add further financial burdens. These costs are often passed on to farmers, who may face higher seed prices for transgenic crops with stacked genes compared to conventional or single-gene varieties (Shailani et al., 2020). Additionally, the market acceptance of GMOs varies, with some regions and consumers being more resistant to genetically modified products, which can limit the market potential for these crops (Crété et al., 2020).



Market considerations also include the potential for market monopolies, where a few large companies control the majority of the market for Bt crops with stacked genes. This can reduce competition and innovation, potentially leading to higher prices and reduced choices for farmers. Furthermore, the reliance on a limited number of Bt crops with stacked genes can increase the risk of widespread pest resistance, which could undermine the long-term sustainability and economic viability of these crops (Ainley et al., 2013).

## **7 Future Directions in Gene Stacking**

### **7.1 Emerging technologies**

The future of gene stacking in Bt crops is poised to benefit significantly from emerging technologies, particularly advancements in genome editing tools such as CRISPR/Cas9. This technology has revolutionized the field of genetic engineering by enabling precise modifications to DNA sequences, which can be leveraged to stack multiple beneficial genes in crops. CRISPR/Cas9's versatility and efficiency make it an ideal tool for creating crops with enhanced traits, such as increased resistance to pests and diseases, improved nutritional content, and greater tolerance to environmental stresses (Belhaj et al., 2015; Arora and Narula, 2017; Eş et al., 2019). Additionally, the development of DNA-free delivery methods, such as the use of CRISPR ribonucleoproteins (RNPs) and viral vectors, offers a promising approach to avoid regulatory hurdles associated with transgenic crops, thereby facilitating the adoption of gene-stacked Bt crops (Ma et al., 2020; Rao and Wang, 2021).

Another emerging technology is the use of base editing and prime editing, which allow for more precise and targeted modifications without introducing double-strand breaks. These technologies can be used to fine-tune the expression of stacked genes, ensuring that each gene contributes optimally to the desired traits. The ability to control gene expression spatially and temporally using inducible systems, such as light-inducible Cas9, further enhances the potential of gene stacking strategies by providing a means to activate or deactivate genes in response to specific environmental cues (Nihongaki et al., 2018). These advancements collectively pave the way for more sophisticated and durable Bt crops.

### **7.2 Potential for new traits**

The potential for new traits in Bt crops through gene stacking is vast. By combining multiple genes that confer different types of resistance, it is possible to create crops that are not only resistant to a broader range of pests but also exhibit enhanced resistance to diseases and environmental stresses. For instance, stacking genes that confer resistance to both insects and fungal pathogens can provide a more comprehensive defense mechanism, reducing the reliance on chemical pesticides and fungicides (Cai et al., 2020; Wang et al., 2021). Additionally, the integration of genes that enhance drought tolerance and nutrient use efficiency can lead to crops that are more resilient to climate change and capable of thriving in suboptimal growing conditions (Arora and Narula, 2017; Bao et al., 2019).

Moreover, gene stacking can be used to improve the nutritional quality of Bt crops. By incorporating genes that enhance the production of essential vitamins, minerals, and other beneficial compounds, it is possible to develop crops that not only protect against pests but also contribute to better human health. For example, the CRISPR/Cas9 system has been used to enhance the nutritional content of horticultural food crops, demonstrating the potential for similar applications in Bt crops (Wang et al., 2021). The ability to stack multiple traits in a single crop variety opens up new possibilities for creating multifunctional crops that address both agricultural and nutritional challenges.

### **7.3 Collaboration and innovation**

Collaboration and innovation are crucial for advancing gene stacking strategies in Bt crops. Interdisciplinary collaboration between geneticists, plant biologists, agronomists, and bioinformaticians can accelerate the development and deployment of gene-stacked crops. Collaborative efforts can facilitate the sharing of knowledge, resources, and technologies, leading to more efficient and effective gene stacking approaches. For instance, partnerships between academic institutions and industry can drive the translation of research findings into practical applications, ensuring that new technologies reach farmers and benefit agricultural production (Sun et al., 2016; Eş et al., 2019).

Innovation in gene stacking also requires a supportive regulatory environment that encourages the development and adoption of new technologies. Policymakers and regulatory agencies need to work closely with scientists and industry stakeholders to establish clear guidelines and frameworks that address safety and ethical concerns while promoting innovation. Public-private partnerships can play a key role in this process by fostering an environment of trust and collaboration. Additionally, engaging with farmers and other end-users is essential to ensure that gene-stacked Bt crops meet their needs and are adopted widely (Bao et al., 2019; Eş et al., 2019). By fostering a culture of collaboration and innovation, the potential of gene stacking to enhance the durability and effectiveness of Bt crops can be fully realized.

## **8 Implications for Sustainable Agriculture**

### **8.1 Environmental benefits**

Gene stacking strategies in Bt crops offer significant environmental benefits by reducing the reliance on chemical insecticides. The deployment of transgenic crops expressing multiple Bt genes has been shown to effectively control a broad spectrum of insect pests, thereby minimizing the need for chemical interventions. For instance, transgenic rice expressing two Bt genes demonstrated high efficacy against major pests like the striped stem borer and leaffolders, which can lead to a reduction in insecticide use and associated environmental contamination (Yang et al., 2011). Additionally, the use of Bt crops has been associated with the conservation of beneficial arthropods, such as predators and parasitoids, which play a crucial role in natural pest control. Studies have shown that Bt crops do not adversely affect these non-target species, thereby supporting conservation biological control and enhancing the overall ecological balance (Romeis et al., 2019).

Moreover, the reduction in chemical insecticide applications due to the adoption of Bt crops can lead to lower levels of pesticide residues in the environment, contributing to improved soil and water quality. This is particularly important in regions where intensive agriculture has led to significant environmental degradation. By integrating Bt crops with other sustainable agricultural practices, such as crop rotation and biological pest control, the environmental footprint of agricultural production can be further minimized, promoting long-term ecological sustainability (Anderson et al., 2019; Gassmann and Reisig, 2022).

### **8.2 Socioeconomic impacts**

The socioeconomic impacts of gene stacking strategies in Bt crops are multifaceted, encompassing increased agricultural productivity, economic benefits for farmers, and enhanced food security. The development of transgenic crops with stacked Bt genes has been shown to provide durable resistance against a wide range of insect pests, leading to higher crop yields and reduced crop losses. For example, transgenic rice expressing a fusion protein of Cry1Ab and Vip3A exhibited high resistance to major rice pests without compromising agronomic performance, which can translate to increased profitability for farmers (Xu et al., 2018).

Furthermore, the adoption of Bt crops can lead to significant cost savings for farmers by reducing the need for chemical insecticides and associated labor costs. This economic benefit is particularly pronounced in smallholder farming systems, where the cost of insecticides can be a substantial burden. Additionally, the increased stability and predictability of crop yields provided by Bt crops can enhance food security, particularly in regions prone to pest outbreaks and food shortages (Zhang et al., 2012; Catarino et al., 2016). However, it is important to consider the potential socioeconomic challenges, such as the risk of pest resistance development and the need for continuous monitoring and management strategies to ensure the long-term sustainability of Bt technology (Manyangarirwa et al., 2006).

### **8.3 Role in integrated pest management**

Gene stacking strategies play a crucial role in integrated pest management (IPM) by providing a robust and sustainable approach to pest control. The incorporation of multiple Bt genes in transgenic crops can delay the evolution of pest resistance, thereby extending the efficacy of Bt technology. For instance, the pyramiding of Bt genes in rice has been shown to provide effective control of multiple pest species, reducing the likelihood of resistance development and enhancing the overall resilience of the pest management system (Yang et al., 2011; Salim et al., 2018).

In the context of IPM, Bt crops can be integrated with other pest control methods, such as biological control, crop rotation, and the use of refuges, to create a diversified and sustainable pest management strategy. The use of Bt crops can reduce the reliance on chemical insecticides, thereby minimizing the risk of secondary pest outbreaks and promoting the conservation of natural enemies (Anderson et al., 2019; Romeis et al., 2019). Additionally, the integration of Bt crops with other IPM practices can enhance the overall effectiveness and sustainability of pest management programs, ensuring long-term agricultural productivity and environmental health (Gassmann and Reising, 2022).

By leveraging the benefits of gene stacking strategies and integrating them with comprehensive IPM plans, farmers can achieve more sustainable and resilient agricultural systems. This holistic approach not only addresses the immediate challenges of pest control but also contributes to the broader goals of sustainable agriculture and environmental conservation.

## **9 Concluding Remarks**

The systematic review on gene stacking strategies to enhance the durability of Bt crops has highlighted several key findings. Transgene stacking, which involves the introduction of multiple genes into crop plants, has been shown to confer higher and more durable resistance to insects and diseases compared to single-gene technology. This approach has been particularly effective in managing resistance to *Bacillus thuringiensis* (Bt) toxins, which are crucial for pest control in transgenic crops. Additionally, the study has underscored the importance of combining resistance genes with other agricultural practices to slow down the evolution of virulent pathogen genotypes. The use of linker peptides, such as LP4/2A, has been identified as a reliable method for producing gene stacking in crops like maize and tobacco, enhancing both pest resistance and herbicide tolerance.

Gene stacking is of paramount importance for the sustainability and effectiveness of Bt crops. By incorporating multiple resistance genes, gene stacking can significantly delay the development of resistance in pest populations, thereby extending the useful life of Bt crops. This strategy not only improves pest resistance but also enhances crop yield and reduces the need for chemical pesticides, contributing to more sustainable agricultural practices. Moreover, gene stacking can be combined with other resistance management strategies, such as crop rotation and the use of quantitative resistance genes, to further enhance the durability of Bt crops. The ability to stack genes for multiple traits, including herbicide tolerance and disease resistance, makes this approach highly versatile and beneficial for modern agriculture.

Future research should focus on optimizing gene stacking techniques to overcome the current technical challenges associated with the expression and manipulation of multiple genes in plants. There is a need for more empirical studies to compare different gene deployment strategies and their long-term effects on pest resistance and crop yield. Additionally, exploring the potential of combining gene stacking with other innovative technologies, such as RNA interference (RNAi) and marker-assisted selection (MAS), could provide new avenues for enhancing crop resistance and sustainability. Research should also investigate the ecological impacts of gene stacking, including its effects on non-target organisms and overall biodiversity, to ensure that this approach contributes to environmentally friendly agricultural practices. Finally, developing more efficient and reliable methods for gene stacking, such as the use of advanced linker peptides, will be crucial for the successful implementation of this strategy in a wider range of crops.

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## **Conflict of Interest Disclosure**

The author affirms 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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