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Emerging technological developments to address pest resistance in Bt cotton

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Abstract

Cotton plays a crucial role in shaping Indian economy and rural livelihoods. The cotton crop is prone to numerous insect pests, necessitating insecticidal application, which increases production costs. The advent of the expression of *Bacillus thuringiensis* (Bt) insecticidal protein in cotton has significantly reduced the burden of pest without compromising environmental or human health. After the introduction of transgenic cotton, the cultivated area expanded to 22 million hectares, with a 64% increase in adoption by farmers worldwide. Currently, Bt cotton accounts for 93% of the cultivated cotton area in India. However, extensive use of Bt cotton has accelerated resistance development in pests like the pink bollworm. Furthermore, the overreliance on Bt cotton has reduced the use of broad-spectrum pesticides, favouring the emergence of secondary pests with significant challenges. This emphasizes the urgent necessity for developing novel pest management strategies. The high-dose and refuge strategy was initially effective for managing pest resistance in Bt cotton, but its implementation in India faced challenges due to misunderstandings about the use of non-Bt refuge crops. Although gene pyramiding was introduced as a solution, combining mono toxin also led to instances of cross-resistance. Therefore, there is a need for further exploration of biotechnological approaches to manage insect resistance in Bt cotton. Advanced biotechnological strategies, such as sterile insect release, RNA interference (RNAi)-mediated gene silencing, stacking Bt with RNAi, and genome editing using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR-Cas), offer promising tools for identifying and managing resistance genes in insects. Additionally, CRISPR-mediated gene drives and the development of novel biopesticides present potential avenues for effective pest management in cotton cultivation. These innovative approaches could significantly enhance the sustainability and efficacy of pest resistance management in Bt cotton.

Keywords Bt Cotton, Gene pyramiding, RNAi, Modified toxin, Genome editing, Plant derived insecticidal protein, Gene drive

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Background

Cotton is a major cash crop that provides fibre for the textile industry as well as biofuels. Globally, around 150 countries are involved in the cotton sector, with an annual output exceeding USD 60 billion. India leads the world in cotton acreage, with approximately 11.91 million hectares, accounting for 36% of the global area (Central Institute for Cotton Research, 2022). Even though India ranks first in the cotton area, it occupies the second position in total lint production, next to China, due to the predominant cultivation of cotton (>75%) under rainfed conditions. Besides, climate change and sudden outbreaks of pests and diseases are also attributed to lower productivity. Over 166 insect pests were recorded in cotton (Rajendran et al., 2018). Among them, sucking pests (thrips, aphids, jassids, and whitefly) and chewing pests (pink bollworms, spiny bollworms, spotted bollworms, and American bollworms) cause direct harm to crops and increase production costs, besides acting as carriers of many disease pathogens (Rajendran et al., 2018; Popp et al., 2013). The cultivation of transgenic cotton has significantly increased on a large scale since its introduction in 1996. After the introduction of transgenic cotton, the cultivated area expanded to 22 million hectares, with a 64% increase in adoption by farmers worldwide. Despite the advantages, resistance in insects to transgenic cotton is a major problem in transgenic cotton regions all over the world (Srikanth et al., 2019). Conventional breeding methods are effective in transferring the resistant traits but require considerable time and resources. A new era of insect control options has opened up with the advent of agricultural biotechnology (Kumari et al. 2022). While widespread adoption of Bt cotton worldwide has yielded advantages like effective pest control and economic benefits, It also puts a lot of pressure on target cotton pests to evolve resistance to Bt toxin, reducing its effectiveness. Therefore, this review summarizes how Bt cotton is adopted globally, the evolution of insect resistance against Bt cotton, the factors affecting the expression of Bt protein concentration, the strategies that are practiced for increasing the durability of resistance, and related biotechnological mechanisms for improving the insect resistant management in Bt cotton.

Bt cotton adoption and the battle against insect resistance

The introduction of Bt cotton, which is resistant to bollworms, has been widely adopted in many cotton-producing countries, demonstrating notable success in pest management. Bt cotton was first commercially introduced in the USA in 1996, rapidly gaining popularity and covering 15% of cotton-producing areas by 1997, 37% by 2001, and 85% by 2019. In China, research on transgenic crops began in the early 1990s, with the first field tests conducted in 1994. China commercially introduced Bt cotton in 1997, with the cotton producing area expanding from 1.5 million hectares to 3.5 million hectares by 2001 (Zhang, 2000).

China has successfully managed insect resistance in Bt cotton by adopting dual pest management strategies, namely 'natural refuges' and 'seed mix refuges'. Over approximately 20 years, these resistance management strategies have yielded different results. In developed countries such as the United States, resistance management measures have effectively controlled the development of cotton pest resistance. Similarly, China has also been successful, whereas countries like India have encountered challenges in Bt cotton pest resistance management (Quan et al., 2023).

In India, the commercial use of Bt cotton began in 2002 with the approval of three Bt cotton-hybrids developed by Monsanto, marking a pivotal shift in agricultural practices. The area under Bt cotton increased from 0.56 million hectares to 3.7 million hectares between 2004 and 2007, and production rose from 1.56 million bales to 3.56 million bales between 2001 and 2011. The introduction of Bollgard II (*Cry1Ac*+*Cry2Ab*) led to an 8% increase in adoption, indicating significant acceptance in India's agricultural landscape (Razzaq et al., 2023). However, since 2015, the pink bollworm (*Pectinophora gossypiella*) has gradually increased its survival rate on Bollgard II cotton and developed field-level resistance to Bollgard I in central India (Naik et al., 2018). Laboratory bioassays initially detected field-evolved resistance of pink bollworm to *Cry1Ac* in Gujarat in 2008, with subsequent resistance identified in Maharashtra and Madhya Pradesh by 2010 (Naik et al. 2018). Despite increased attacks by the pink bollworm, farmers have continued to find Bt cotton more profitable because of a lack of alternatives. By 2019, Bt cotton accounted for 94% of total cotton cultivation in India, which covers nearly 13 million hectares, equivalent to 41% of the global cotton area (International Cotton Advisory Committee (ICAC), 2021). Bt hybrids are cultivated in more than 90% of India's cotton area, with insecticide costs per hectare significantly lower than in other cotton-producing countries (ICAC, 2021).

Brazil, initially impacted by the cotton boll weevil in the 1970s and 1980s, adopted transgenic cotton extensively in the 1990s, significantly improving its cotton production. By 2023, Brazil had maintained its status as the world's second-largest cotton exporter, with exports totaling 1.618 million tonnes and generating USD 3.07 billion in revenue. Similarly, Mexico introduced Bt cotton in 1996, and by 2008, 96% of its cotton fields were planted with this kinds of variety. Argentina, an early adopter since 1998, witnessed widespread adoption of Bt cotton among small farms and families, resulting in

lower pesticide costs and higher yields, positioning the country as a global leader in Bt cotton production (Razzaq et al., 2023). However, in countries like India, the increase in Bt cotton acreage has accelerated resistance development in the pink bollworm due to its oligophagous nature. The excessive reliance on Bt cotton has also led to decreased use of broad-spectrum insecticides, causing secondary pests to emerge as significant threats, necessitating the adoption of novel pest-management strategies. The introduction of Bt cotton at the global was mentioned in Fig. 1.

Climate change on Bt efficacy

The production of Bt protein in sensitive plant organs at the appropriate phases of plant development is important for maintaining the efficacy of Bt cotton against target pests. However, several studies indicated that the expression levels of Bt protein varied during the cotton growing season resulting in varying insecticidal efficacies (Liu et al., 2019). In Bt cotton, various environmental conditions such as high temperatures with low relative humidity results in the reduction of Bt toxin content in leaves (Khan et al., 2022; Khan et al., 2023). Similarly, an increase in soil salinity (electrical conductivity above 9.1 $dS{\cdot}m^{-1}$) results in a significant reduction of Bt protein in leaves as well as squares (Wang et al., 2018). Under drought stress conditions, the Bt-toxin content decline correlated with the crop resistance against bollworms (Ullah et al., 2008). However, the application of high nitrogen fertilizer results in a 14% increase of Bt toxins in leaves (Chen et al., 2017a, b), and the application of plant growth regulators like gibberellic acid (GA_3) increases Bt toxin concentration in squares, resulting in lower bollworm number and hazard rate with higher yield (Xiang et al., 2019). The initial research suggested that variations in insecticidal protein concentrations were responsible for fluctuations in Bt crop efficiency. Subsequent studies indicated that down-regulated expression of Bt genes did not always lead to decreased levels of Bt insecticidal proteins, and even when the protein levels were lower, the

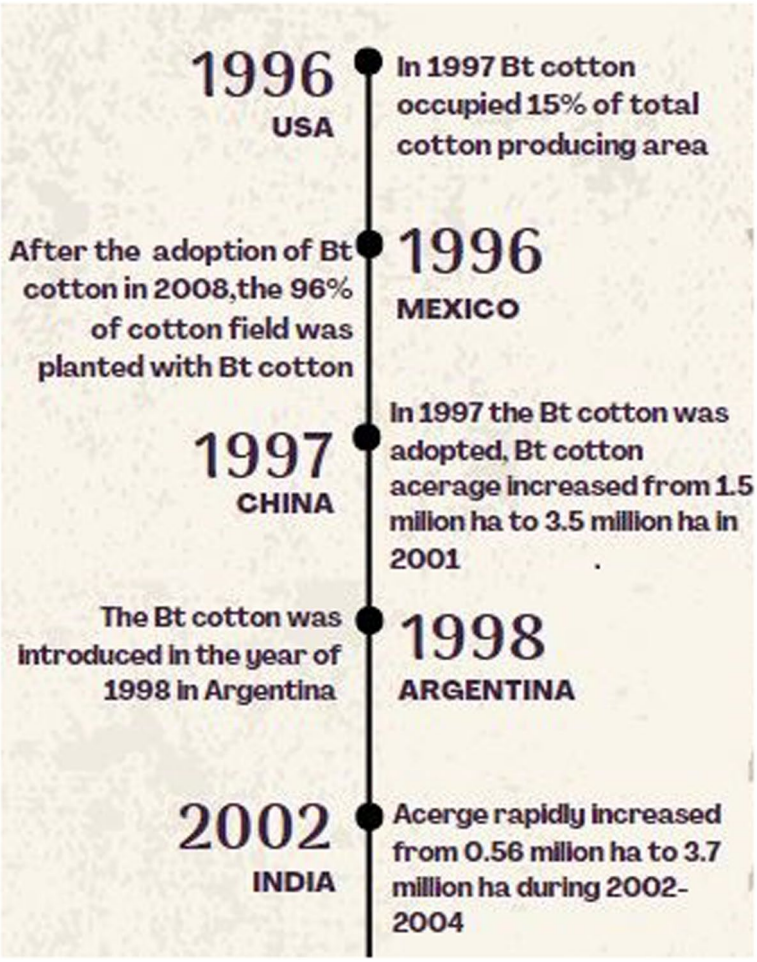

Fig. 1 The global adoption timeline of Bt cotton

effectiveness of Bt crops against target pests was not consistently reduced. Because the changing environmental conditions did not directly affect the pest resistance efficacy of Bt crops, they triggered a series of physiological changes in both plants and insects, which may result in decreased efficacy of Bt protein.

Genetics of insecticide resistance in the face of climate change

The performance of Bt crops under the changing environmental conditions involves a three-way interaction, where both the plants and the insect pests are influenced by individual or combined environmental stress factors, as well as by the interactions between the plant and the pest (Girón-Calva et al., 2020). The changing environmental conditions have a direct impact on the evolutionary dynamics of insect populations. For insects, rising temperatures can accelerate metabolic rates, potentially impacting detoxification processes that break down insecticides within their bodies. This enhanced metabolic rate may result in faster detoxification and removal of insecticides, which makes insecticides less effective in pest control (Aleem et al., 2023). Climate change can apply selective pressure on some genetic variations or alleles associated with insecticide resistance which may lead to higher survival and reproductive advantages for insects in altered climatic conditions (Zafar et al., 2020).

Factors contribute to insect resistance against Bt toxin at the insect level

Understanding the genetics and molecular mechanisms behind insecticide resistance is essential for devising effective strategies to control insect pests and prevent the emergence of resistance (Hamza et al., 2023). The genetic changes can arise through a variety of mechanisms, including mutations, gene amplification, and changes in gene expression, resulting in metabolic pathway modifications and target site insensitivity (Zafar et al., 2020).

Gene expression modulation and mutations in receptor binding sites of insects are major reasons for Bt gene resistance decrease. Membrane-bound cadherin and ATP-binding transporters are reported as crucial receptors for Cry proteins to kill insects (Fabrick et al., 2023; Liao et al., 2022). The recessive resistance in *Helicoverpa armigera, P. gossypiella*, and *H. virescens*, to Cry1Ac is closely associated with mutations that impair a cadherin protein (Li et al., 2019). *P. gossypiella* developed resistance to Cry1Ac toxin due to the mutations in the cadherin transmembrane protein, which impacts cellular trafficking (Wang et al., 2019). The *Cadherin-86C* (*cad-86C*) gene is involved in Cry1A resistance in other lepidopterans and it is a feasible target of Cry1Ac selection in *H. zea*. However, the mechanism of resistance is unclear (Fritz et al., 2020). In the case of *SfCad*, it does not play a role in the mechanism of action of Cry1Ab or Cry1Fa toxins in *Spodoptera frugiperda* (Zhang et al., 2020). Cadherin gene silencing in *H. armigera* larvae did not significantly affect Cry2Ab toxicity (Naing et al., 2023). ABC transporters could just be an additional binding site on the membrane surface, which would increase the concentration of toxins locally and speed up pore insertion because of the concentration effect. There is a theory that ABCC2 acts similarly to cadherin to promote the formation of the pre-pore oligomer (Ocelotl et al., 2017; Heckel et al., 2021). Pink bollworm resistance to Cry2Ab is associated with a mutation in the ABC transporter gene *PgABCA2* (Mathew et al., 2018). ABCA2 is required for Cry2Ab toxicity in *Trichoplusia ni* and mutation of ABCA2 results in resistance to Cry2Ab. (Yang et al., 2019). Cotton bollworms were highly resistant to the Cry1Ac toxin due to a combination of mutations in cadherin and ABCC2, as well as due to low levels of expression of ABCC3 (Zhang et al., 2021). The efficiency of Cry2Aa insecticidal activity against *H. armigera* larvae significantly decreased when the genes encoding these proteins were silenced with specific siRNAs, with CAD- and ALP2-silenced larvae showing similar reductions in mortality (41.67% and 43.06%, respectively) and APN4-silenced larvae exhibiting a more substantial reduction (61.11%) compared with the controls. These results reveal that CAD, APN4, and ALP2 are involved in the mechanism of action of Cry2Aa in *H. armigera*, playing an important functional role in the toxicity of the Cry2Aa toxin (Zhao et al., 2017). Diagnosing and monitoring of resistant alleles in insects are important in managing pesticide resistance. The qPCR results for Cry1C tolerant or susceptible strains of *S. littoralis* revealed significant upregulation of the cytochrome P450 (*CYP*) gene in the tolerant strain, while the expression levels of *Try, ALP*, and *Cad* were significantly downregulated. The *APN* relative mRNA expression showed no significant differences between the two strains which indicates the importance of *Try, ALP*, and *Cad* in Cry1C insecticidal activity in concerning *S. littoralis* (Khalil et al., 2021). Apart from genetic mutations, metabolic detoxification, and cross resistance, the behavioural adaptation of insects may change their feeding behaviour to avoid consuming Bt crops, which also leads to insecticide resistance. Delaying the evolution of insecticide resistance can be achieved by proposing innovative management strategies and by developing efficient monitoring techniques based on the identification of insecticide resistance genes, resistance mechanism, and developing molecular markers for the identification of resistance genes with integrated pest

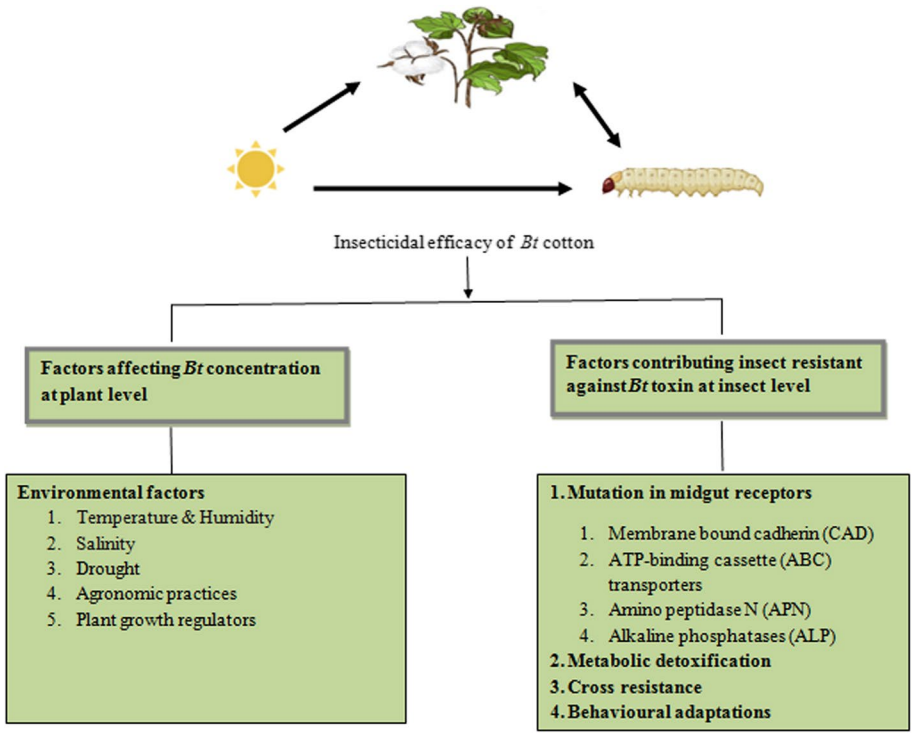

Fig. 2 Various factors influencing insecticidal efficacy of Bt cotton, including the interaction among plants, insect pests, and changing environmental conditions

management strategies. Various factors influencing the insecticidal efficacy of Bt cotton were listed in Fig. 2.

Strategies for managing pest resistance in Bt cotton

As a result of insect populations developing resistance to Bt toxins, several research teams have focused on understanding these mechanisms and developing practical solutions for controlling pest damage in Bt cotton fields to maintain sustainable cotton production (Zhang et al., 2021; Tabashnik et al., 2020; Tang et al., 2024). Currently, strategies such as cotton pyramided with two or more Bt toxin genes that act through different mechanisms, refuge strategy, and release of sterile insects are being used to delay the resistance development in target pests of cotton. Consequently, the potential of biotechnology should be explored for managing resistance against insect pests in Bt cotton. The biotechnological strategies, *viz.,* gene silencing by RNAi, pyramiding *Bt* gene with RNAi, genome editing using clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins (CRISPR-Cas) could be used as a tool to identify resistant genes in insects against Bt cotton, CRISPR mediated gene drives and other biopesticides could be explored for effective management of cotton pests.

High dose and refuge strategy

The high-dose and refuge method was the first effective strategy for managing target-pest resistance to Bt toxin development that was researched and implemented. The strategy primarily relies on mating between susceptible and resistant individuals generated in Bt and non-Bt (refuge) host plants (Fig. 3). The combination of high dose Bt cotton and refuge strategy provides durable resistance against insect pests. The refuge plants provide enough susceptible insects to dilute the resistant population and the heterozygous inviduals will be killed by high dose of Bt cotton.

This strategy delays the accumulation of resistant pest populations. In comparison to border refuge, planting refuge alternatively with Bt cotton also results in higher levels of Cry1Ac expression. However, implementing structured refuge planting poses challenges for farmers due to operational complexities and various other factors (Dimase et al., 2020). Refuges might be non-Bt cultivars of the same plant species or non-Bt plants of different species, which are also referred to as "natural refuges" (Guan et al., 2023). The Yellow River Region and Northwest Region of China adopted the "natural refuge" plan, incorporating non-Bt crops such as corn, soybean, vegetables, peanuts, and other host crops for managing cotton bollworm (polyphagous), which resulted in the avoidance

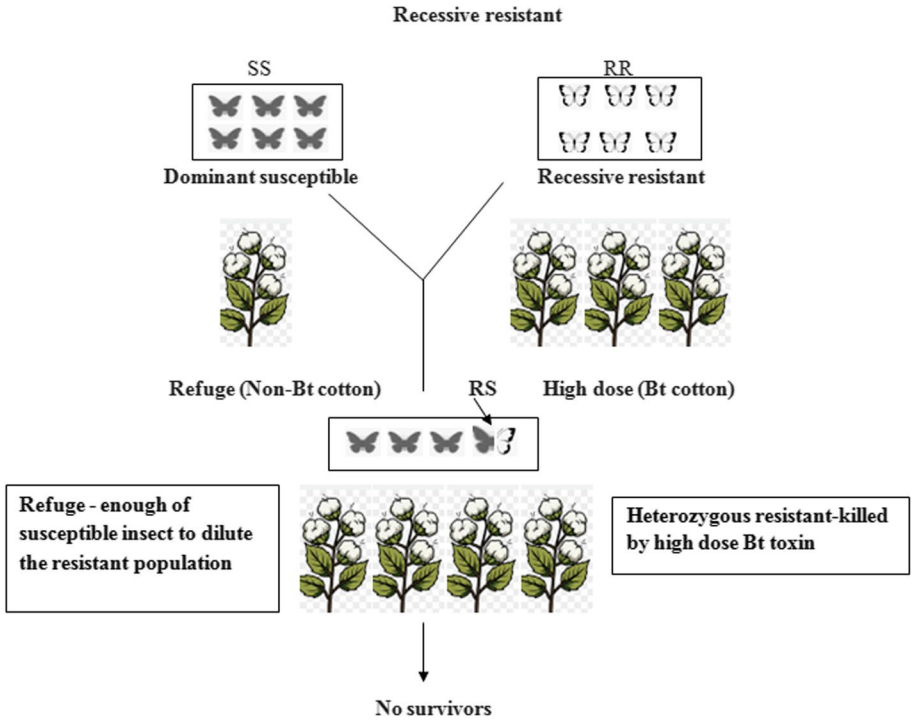

Fig.3 High dose & refuge strategy for insect resistance management in Bt cotton

of practical resistance to Cry1Ac for more than 20 years (Quan et al., 2023).

Utilizing F_2 generation hybrids (crosses between Bt and non-Bt cotton) to generate a refuge comprising 25% non-Bt cotton seeds helps to further postpone the development of resistance in monophagous pests like pink bollworm (Quan et al., 2023). In India, a significant factor contributing to the resistance of pink bollworms to Bt cotton is inadequate availability of refuge areas. By effectively implementing the refuge strategy and mixing of Bt and non-Bt seeds, the insect resistance in Bt cotton can be efficiently controlled.

Gene pyramiding

Gene pyramiding, which involves stacking multiple genes in a single crop to target the same insect pest species, is a highly effective approach to developing transgenic plants with enhanced pest resistance and improved crop yield. Compared with single-toxin transgenic varieties, gene pyramiding offers superior control over different insect species. The biggest challenge to long-term sustainability of Bt technology remains the emergence of resistance to Bt toxins in target pest populations (Jurat-Fuentes et al., 2021). Genetically pyramided crops that produce multiple Bt toxins, such as Cry or Vip insecticidal proteins, aim to prevent resistance from evolving resistance. Bt cotton producing Cry1Ac or Cry1Ac and Cry2Ab proteins have been proven to be efficient against pink bollworms, which provide almost 100% insect resistance compared with the control (Tabashnik et al., 2012). The introduction of *vip3AcAa* and *cry1Ac* genes into cotton increased larval mortality rates for *S. litura, S. exigua*, and *Agrotis ipsilon*, suggesting *vip3AcAa* as a viable alternative for gene pyramiding in integrated pest management (Chen et al., 2017b).The pyramided approach may be useful in extending the *Bt* gene durability for controlling *H. zea*, a pest that is naturally less susceptible to Cry proteins (Santiago-González et al., 2022). *Vip3Aa*-resistant *H. zea* was completely or almost completely killed by pyramided Bt crops, where *H. zea* has not evolved resistance to *Cry1* and *Cry2* toxins. These findings suggest that in many US regions, dealing with *Vip3Aa* resistance could reap advantages from the pyramiding approach (Kennedy et al., 2023). The synthetic transgene *Cry3Bb1* and *Cry3* were introduced into the Eagle-2 genotype to evaluate the effectiveness of *Cry*-gene stacking for pest management and reducing insect resistance in transgenic cotton. Over three days, the transgenic M1 plants exhibited 60% resistance to pink bollworm and 75% to army bollworm, compared with the negative control. In the M2 transgenic plants containing only *Cry3Bb1*, the mortality was observed to be 40% against pink bollworms, whereas 45% mortality was observed against army bollworms. In the case of M3 transgenic plants containing single gene *Cry3*, which showed 20% and 30% resistance to pink and army bollworms, respectively. These results highlight the

efficacy of pyramided *Cry* genes in managing cotton-chewing pests (Zafar et al., 2022). Similarly, the *Cry* gene expression level was threefold higher in the pyramided FBs-222 cotton lines bearing the *Cry11* and *Cry1H* genes than in the non-transgenic plants. Insect bioassays demonstrated that transgenic cotton achieved 90% mortality against pink bollworms and 80% against army bollworms (Razzaq et al., 2023). By integrating multiple genes with unique modes of action, this strategy provides durable and broad-spectrum resistance against various insect pests. However, continuous pest pressure may still lead to the evolution of resistance in herbivores.

One major concern of gene pyramiding is the possibility of developing cross-resistance, which occurs when a pest population is selectively exposed to one Bt toxin and can lower susceptibility to other toxins (Tabashnik et al., 2013). Cross-resistance arises in Bt toxins with comparable amino acid sequences and binding sites in insects' midgut. Cross-resistance among Bt toxins in the pyramids is linked to comparable amino acid sequences in domain II, as demonstrated in a study of 80 pests by Welch et al. (2015) Asymmetric cross-resistance existed between the Cry1Ac and Cry2Ab toxins in the investigated strains of pink bollworm (Ma et al., 2020). The Cry2Ab resistant *H. armigera* had high cross-resistance to Cry1Ac, Cry1Fa, Cry1Aa, and Cry2Aa toxins, but low cross-resistance to Cry1Ab and Cry1Ca, no cross-resistance to abamectin and spinetoram, and negative cross-resistance to Vip3Aa toxin (0.14-fold) (Tang et al., 2024). So, applying only gene pyramiding techniques is insufficient to address the problem of resistance evolution of Bt genes. As a result, it will be important to use various combinations of tactics shortly, such as RNA interference with gene pyramiding techniques (Salim et al., 2020).

Modified toxins

There is a broad agreement that pyramiding genes for resistance is a useful strategy. However, a significant challenge in gene pyramiding is the emergence of cross-resistance due to the use of mono toxin. To address this issue, integrating modified Bt proteins, engineered for increased potency and specificity, become essential. Protein engineering plays a pioneer role in managing Cry resistance by enhancing Cry toxins' stability, binding affinity to pest midgut receptors, and their ability to oligomerize or perforate membranes. (Fu et al. 2024) engineered *B. thuringiensis* Cry2Ab toxin through site-specific mutation, aiming to improve protein efficacy by increasing oligomerization and pore-formation abilities while maintaining proteolytic activation similar to wild-type Cry2Ab. The results revealed that variant L183I had enhanced insecticidal activity against *Plutella xylostella*. In order to combat insect resistance to the natural Bt toxins Cry1Ab and Cry1Ac, the genetically engineered Bt toxins Cry1AbMod and Cry1AcMod were developed by Tabashnik et al. (2011). The efficacy of modified toxins against resistant strains of *Ostrinia nubilalis* and *P. xylostella* was > 350 times higher than that of native Bt toxins. By combining these advanced proteins with gene pyramiding, we can enhance efficacy against a broader range of pests and significantly delay resistance development.

Domain exchange

Combining domains from various cry proteins might generate potent toxins with unique targets. The recombinant Bt construct was designed by exchanging domain I from *cry1Ac* and domain II & III *cry9Aa* gene. The efficacy of recombinant toxin against *H. armigera* was found around five fold higher than native cry1Ac protein (Shah et al., 2017). The use of chimeric toxins, which are created by domain swapping, domain rearrangement, and other methods, prevents the insects from developing resistance to cry toxins. Transgenic cotton containing *cry1AcF* (created through domain swapping) and *cry2Aa* (developed via codon modification) is employed to control *H. armigera* infestation, achieving up to 100% larval mortality (Karthik et al., 2021).

Fusion protein

A fusion protein typically refers to a genetically engineered protein that combines elements of different insecticidal proteins to enhance effectiveness against target pests. These fusion proteins are often created by combining domains or segments of Bt insecticidal proteins with other proteins or domains to create a novel toxin with improved insecticidal properties (Xu et al., 2018). The sequences from *cry2Aa* and *cry2Ac* genes were combined to produce a novel *cry2AX1* gene which was found effective against *H. armigera* in transgenic cotton (Sakthi et al., 2015). A chimeric protein's safety for human or animal intake can be assessed with the safety of its donor proteins (Wang et al., 2018). Domain shuffling and sequence swapping are successfully implemented to form new toxin combinations with improved insecticidal activity against new or resistant pests (Syed et al., 2020). However, this field should be explored further for insect resistance management in transgenic cotton.

Releasing of sterile insect

The efficacy of Bt technology diminishes with widespread resistance to pests. Classical biological control releases imported natural enemies, while the sterile insect technique targets and suppresses local insect populations threatening agriculture or human health (Mustafa et al., 2022). In 2002, Oxitec company developed a sterile

insect technique that demonstrated its effectiveness in field trials of mosquito strains conducted in Malaysia, Brazil, and the Cayman Islands. A large number of targeted pests were bred, rendered sterile by radiation, and then released to compete with the wild population, consequently reducing the number of their progeny. By employing similar methods, introducing sterile pink bollworm moths in Bt cotton fields emerged as a practical substitute for the prescribed refuge strategy in the prevention of resistance to Bt cotton. These sterile moths engaged in mating with the infrequent resistant insects, resulting in no progeny, thus effectively stalling the evolution of Bt resistance (Wu et al., 2014). Incorporating the "release of sterile pest" strategy into a comprehensive eradication program led to a reduction of over 99% in the *P. gossypiella* in a Bt-cotton field. This outcome indicates the effectiveness of the approach in delaying the development of resistance in pink bollworms to Bt toxins (Tabashnik et al., 2010). A synergistic reduction of pest populations was observed with the use of sterile insect releases and transgenic Bt cotton, as evidenced by computer models and 21 years of field data from Arizona, USA. The program started in 2006, and led to a remarkable decrease, from over 2 billion pests in 2005 to zero by 2013 (Tabashnik et al., 2021). Sterility in insects can be attained through either irradiation or genetic modification techniques. The implementation of the sterile insect technique in target pest control, presents a promising strategy that not only mitigates resistance issues but also offers a sustainable solution for managing pest populations effectively.

RNAi interference

RNA interference (RNAi) is a biological process that suppresses gene expression through the introduction of double-stranded RNA (dsRNA) molecules, which are complementary to specific target genes (Munawar et al., 2023). Dicer enzyme processes these dsRNA molecules, cleaving them into small RNA molecules known as short interfering RNAs (siRNAs). These siRNAs are then incorporated into the RNA-induced silencing complex (RISC), which unwinds the double-stranded siRNA and uses one strand as a guide to locate and bind to the complementary mRNA. The RISC then cleaves the mRNA, preventing its translation into protein (Christiaens et al., 2022). RNAi has rapidly emerged as a powerful reverse genetics tool for enabling the study of gene function, regulation, and interactions at both cellular and organism levels. It has also demonstrated potential in pest management (Ren et al. 2019). The initial instance of lepidopteran management through RNAi was observed in tobacco plants, achieved by specifically targeting the cytochrome P450 monooxygenase gene (*CYP6AE14*) of *H. armigera* which involves in detoxification of plant defences compounds and in developing insecticide resistance (Mao et al., 2007). The recent advancements in cotton insect pest management utilizing RNAi were listed in Table 1.

A delivery method integrating rough-surface hollow mesoporous silica (RHMS) was created to transport *CYP6CY13* dsRNA with the chemical insecticide imidacloprid for effective control of *A. gossypii.* The

Table 1 Application of RNA interference in cotton insect pest management

Target insect	Target genes	RNAi crop	Effects of RNAi on the target insect	References
H. armigera	*HaHR3*	Cotton	Higher larval mortality and deformities of pupation and adult eclosion	Han et al. (2017)
Aphis gossypii	*UGT344B4 /UGT344C7*	Cotton	Significantly reduced the detoxification against insecticide	Chen et al. (2019)
H. armigera	*HaJHAMT, HaPTTH, HaPBAP, HaHR3, HaAP-4, HaEHP*	Cotton	Mortality ranging from 60% to 90%, reduced larval weight, phenotypic deformities and delayed pupation	Jaiwal et al. (2020)
Anthonomus grandis	*CHS2*, *vitellogenin* (*Vg*), *ETHr*	Cotton	Mortality was observed up to 70% and insect development was severely impaired which resulting in malformation in first and third instars larvae	Ribeiro et al. (2022)
A. gossypii	*CYP6CY3*	Cotton	Increased mortality of the nymphs to insecticides	Zhang et al. (2023)
A. gossypii	*CYP6CY14, CYP6DC1*	Cotton	Higher mortality rate	Ullah et al. (2023)
Earias vittella	*JHAMT, CHS, AMN, CAD, AMY V-ATPase*	Cotton	ds*CHS* resulted in a significant reduction in the percent pupation and adult emergence	Sandal et al. (2023)
S. littoralis	*SlAQP2 SlAQP3*	Cotton	Larval and pupal mortality, deformed pupae and adults and prolonged development	Khalil et al. (2023)
Bemisia tabaci	*CYP6CX3*	Cotton	Cyantraniliprole *resistance mechanism of B. tabaci was identified*	Wen et al. (2024)

effectiveness of the complex against *A. gossypii* was tested in pots, with a notable improvement of 19.95% within a span of 5 days (Lv et al., 2023). However, the efficiency of RNAi in insect resistance is based on choosing the ideal target gene(s) for silencing, target insect, vector construct designing, and the mode of dsRNA delivery. Several methods for delivering dsRNA have been effectively employed to manage resistance against insect pests, which include microinjection, administering dsRNA through an artificial diet, and host delivered RNAi (Saakre et al., 2023). Ingestion, feeding the pest on artificial diets that contain dsRNA, is the most common method among the RNAi delivery approach. In microinjection, the dsRNA can be administered directly into insect tissues, including the hemocoel. The cells can then take up the injected dsRNA and trigger RNAi. Microinjection of dsRNA is a promising method for controlling insect pests. However, challenges such as target gene mutations and the delivery of dsRNA to insect populations in field settings can limit its effectiveness. Transgenic plants (host delivered RNAi) can serve as a continuous source of dsRNA for pest control, though the development and regulatory approval of such crops is often time-consuming and costly. Each method holds its advantages and limitations (Munawar et al., 2023). The application of RNAi was anticipated to offer control over a broader array of insect pests, particularly those of the sap-sucking insects that transgenic crops have failed to control. Furthermore, this technique presents novel opportunities for implementing environment friendly insect pest management strategies in agriculture. However, RNAi also has limitations in insect resistance management. One of the major challenges is mutation and over expression in target genes. Additionally, concerns about off-target effects and activation of alternative pathways must also be considered.

Pyramiding Bt gene with RNAi

Farmers are increasingly adopting Bt pyramids which generate multiple toxins to combat the same pest. However, the effectiveness and long-term sustainability of these pyramids are compromised by cross-resistance and antagonism among Bt toxins, thus necessitating the exploration of alternative management strategies (Hackett & Bonsall. 2016). RNAi can be combine with Bt toxins to produce more effective and durable resistance against insect pests (Tabashnik & Carrière. 2017) (Fig. 4).

RNAi employs small dsRNA to reduce target gene expression at a particular sequence. To accomplish safe and effective pest control with RNAi, the goal is to limit the expression of genes that encode proteins required by pests but not other organisms. Potential targets for RNAi include genes encoding proteins that manufacture or transport juvenile hormone (JH) (Li et al., 2022). Ni et al., (2017) developed two types of transgenic cotton plants producing dsRNA to interfere with the JH metabolism of the global *H. armigera*. They tested larvae from a Bt-resistant and a susceptible strain of *H. armigera* on seven types of cotton: two controls, Bt cotton, two types of RNAi cotton (targeting juvenile hormone acid methyltransferase (JHAMT) or juvenile hormone-binding protein, and two pyramids (Bt cotton plus each type of RNAi). Both types of RNAi cotton were effective against Bt-resistant insects. Bt and RNAi worked independently against the susceptible strain of *H. armigera*. The transgenic crop coupled with multiple gene pyramiding and

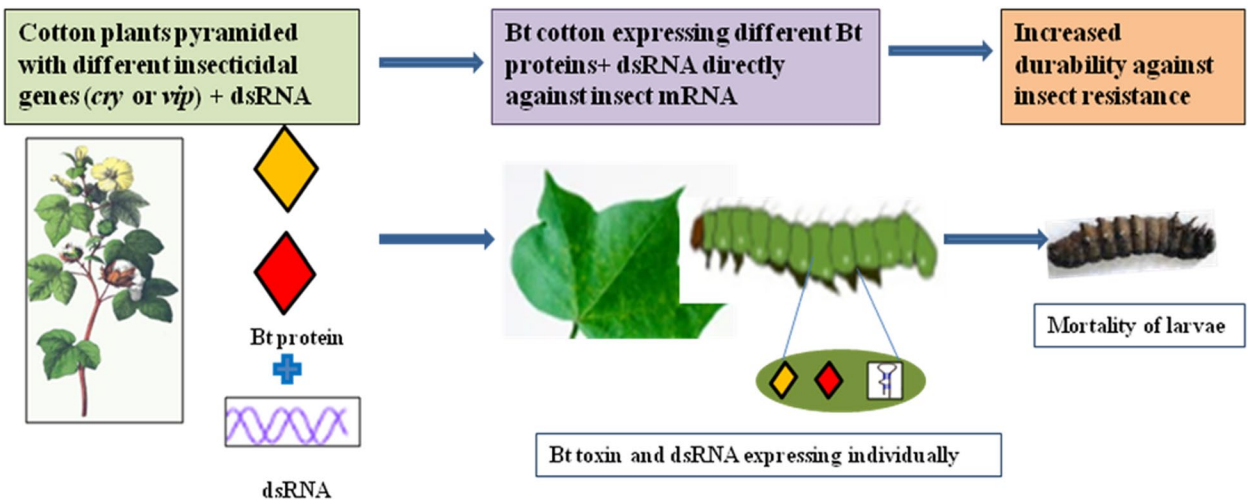

Fig.4 Multiple *Bt* gene pyramiding and silencing (Pyramiding *Bt* gene + RNAi)

silencing, along with a refuge strategy, was proposed for the effective control of fall armyworm, which is advantageous to delay or prevent the natural spread of resistance alleles (Ren et al. 2019; Zafar et al., 2020).

Genome editing (CRISPR-Cas9)

CRISPR-Cas, an RNA-guided endonuclease system, initially developed as an adaptive immune mechanism in bacteria. In an engineered CRISPR-Cas system, the Cas protein is modified to target precise locations using a single guide RNA (sgRNA). Comprising approximately 20 nucleotides, the sgRNA is designed according to specific target sequences. It binds to the target DNA through complementary base pairing and knocks down or knocks out the target gene. The distinguishing characteristic of CRISPR technology lies in its programmable and predictable ability to bring DNA, RNA, and proteins into proximity (Li et al., 2021., Khan et al., 2023). Dependence on a single solution, such as Bt cotton, has resulted in increasing insect resistance. Additionally, existing insect-resistant cotton strains do not protect against all cotton pests. This highlights the imminent need to research novel biotechnological techniques like CRISPR for broader and long-lasting pest resistance (Khalid & Amjad. 2023). CRISPR-Cas can directly edit the genomes of pests by knocking out genes responsible for resistance to Bt toxins. This targeted approach can reduce resistance development and improve the efficacy of Bt cotton. Additionally, the knock-out mutation caused by CRISPR-Cas tools helps in identifying and validating genes involved in resistance mechanisms. By understanding these genes, researchers can develop new strategies to overcome insect resistance. The recent insights in identifying resistance genes utilizing CRISPR-Cas9 in cotton pests were listed in Table 2.

The utilization of CRISPR-Cas9 gene editing has proven to be efficient in identifying genes wherein mutations can confer resistance to Bt toxins (Fabrick et al., 2023). Despite the fast progress of CRISPR-Cas9-based genome editing, researchers are facing challenges like off-target effects, finding better ways to deliver the gene of interest, and enhancing editing efficiency. Even though these challenges exist, both RNAi and CRISPR-Cas9 have the potential to revolutionize farming. Because of the importance, current attention must be focused on enhancing CRISPR-Cas9-based pest management technology.

CRISPR-mediated gene drive

Genetic control strategies involve genetically modifying pests to achieve two main goals: either reducing the number of pests in a population or replacing harmful pests with less damaging ones. Gene drives, a subset of these tactics, are selfish genetic components that are transmitted across to offspring at super Mendelian (>50%) frequencies through sexual reproduction. Gene drive elements can originate from naturally occurring selfish genetic elements or be engineered from completely synthetic designs (Legros et al., 2021). The CRISPR-mediated gene drive spreads a genetic modification through a population at a faster rate than normal inheritance. After a gene drive is inserted into an organism, its offspring will inherit the gene drive on one chromosome and a regular gene from the other parent. Early in development, the CRISPR component cuts the regular copy and the repair process uses the gene drive as a template. This results in the offspring having two copies of the modified gene (Scott et al., 2018).

Gene drive-based control strategies are amenable in *Bemisia tabaci* because it has haplo-diploid sex determination and it is an introduced pest, local control of this pest is effective in insect pest management (Esvelt et al., 2014). Releasing gene-driven insects in regions where one pest species dominates can lead to complex

Table 2 Application of CRISPR-Cas9 in insect pest resistant management

Target insect	Target genes	Effects of CRISPR on the target insect	Reference
H. armigera	*Glutathione S-transferase* (*GST* cluster gene)	Knockout of the *GST* cluster gene significantly increased the sensitivity to *lambda*-cyhalothrin	Jin et al. (2023)
H.armigera, S.litura	*GmUGT*	Over-expression of the *GmUGT* gene is functionally involved in imparting resistance to leaf-chewing pests	Zhang et al. (2022a, b)
P. gossypiella	*PgABCA2*	Disruptive mutations in *PgABCA2* increased the frequency of resistance to Cry2Ab	Fabrick et al. (2021)
H. zea	*HzABCA2*	Knockout of *ABCA2* confers resistance to Cry2Ab	Fabrick et al. (2022)
B. tabaci	*lncD09, lncA07*	Knockout mutation of *lncD09 and lncA07* confers the importance of jasmonic acid against sucking pest resistance	Zhang et al. (2022a, b)
H. armigera	*HaCAD-KO, HaABCC2-M, HaABCC3-M*	Very high levels of resistance were observed to Cry1Ac	Liao et al. (2022)
S. frugiperda	*PBAN*	The specific inhibition of *PBAN* in females has a major impact on mating	Ashok et al. (2023)

changes in pest dynamics in neighbouring areas where multiple pest species are present. A similar situation occurred in China when the widespread use of Bt cotton reduced populations of pink bollworm, but caused an increase in plant bug populations (Baltzegar et al., 2018). The long-distance dispersal ability of the lepidopteran insect pest, *S. frugiperda*, increases the likelihood of it crossing geographic borders and leads to challenges in monitoring, regulation, and ensuring biosafety measures in adopting gene drive for pest control (Legros et al., 2021). In theory, future gene drives could be made to activate only on target insects when certain components of diet are present on a particular crop variety. So, this new paradigm is complicated at the same time it holds enormous promise for future pest management efforts.

Other biopesticides for the management of insect pests of cotton

Biopesticides are sourced from natural materials like plants, microorganisms, and certain minerals. Unlike synthetic pesticides, they control pests through non-toxic mechanisms, specifically targeting pests while minimizing negative impacts on the environment, beneficial organisms, and human health. Shahid et al. (2023) broadly classified these biopesticides as bio-derived chemicals, microbial biopesticides, and plant-incorporated protectants. Bio-derived chemicals originate from natural substances like plant extracts or pheromones and function as repellents, attractants, or disruptors of pest behaviour. Microbial biopesticides employ microorganisms like bacteria, fungi, and viruses to manage pests through diverse mechanisms, such as direct infection or toxin production. Plant-incorporated protectants involve genetically modifying plants to produce insecticidal proteins, ensuring continuous pest control. The best-known example of plant-incorporated pesticides is Bt crops that were genetically modified to express a protein toxic to specific pests. A key advantage of plant-incorporated pesticides is their specificity. The pesticidal proteins target specific pests while being safe for beneficial organisms and humans, allowing precise pest control and minimizing non-target effects compared to broad-spectrum chemical pesticides (Razzaq et al. 2023).

Despite the advantages, the widespread application of Bt δ-endotoxins in agricultural fields diminishes their insecticidal efficacy. However, the second-generation Bt gene *Vip3Aa*, which does not compete for midgut receptor sites with known cry proteins, which maybe a promising alternative. Plant lectins target the carbohydrate components of pests, such as glycoproteins, glycolipids, and glycoconjugates, to disrupt insect metabolism. Because of this feature, plant lectins can be used as defence proteins against phytophagous pests (Singh et al. 2023). By incorporating *Vip3Aa* along with the *Allium sativum* leaf agglutinin gene, transgenic cotton has successfully developed durable resistance against important chewing and sucking insects (Din et al., 2021).

Lectins as insecticidal protein

Mannose-binding lectin, a collectin family of proteins that includes *Galanthus nivalis* agglutinin (GNA) isolated from snowdrop plant, *Allium sativum* agglutinin isolated from garlic, and *Narcissus pseudonarcissus* lectin isolated from wild daffodils, plays a critical function in plant protection against sap-sucking insects (Upadhyay et al., 2012). The transgenic cotton lines harbouring insect gut-binding lectins have shown notable resistance to sucking and chewing pests (Vanti et al., 2018). The sap-sucking homopteran insects are often tolerant to Bt toxin but it reveals susceptibility towards various plant lectins (Paul & Das. 2021). Lectins isolated from soybean and white kidney bean were found to be effective in reducing larval weight and pupation in cotton leafworm, *S. littoralis* Boisd. (Mohsen et al. 2021). A codon-modified lectin GNA gene (*ASGNA*) was transiently expressed in cotton to determine its effectiveness as an insect-resistance gene against cotton aphids. The findings demonstrate that *ASGNA* had strong insecticidal efficacy against sap-sucking insects (He et al., 2022). The *Nicotiana tabacum* agglutinin domain binds to mannose N-glycans and glucose-N-acetyl oligomers. NICTABA-related lectins have been found to limit the growth of cotton leafworm larvae (*S. littoralis*) (Singh et al., 2023). Lectins from the leguminous plants *Glycine max* and *Phaseoulus vulgaris* were found to significantly inhibit α-amylase and total protease enzyme activity in larvae of spiny bollworm (Metayi et al., 2024). Currently, transgenic crops are genetically modified to produce Bt toxins are not effective against pests that feed on the phloem of plants. By targeting sucking insect pests like aphids, plant lectins have the potential to be a promising entomotoxic option. In addition, lectin-encoding genes from lectin-producing plants can be manipulated through genetic engineering techniques to provide protection against insect pests on non-lectin-producing agricultural plants.

Plant protease inhibitors

Protease inhibitors (PIs) can be classified as serine, cysteine, aspartic, or metalloprotease inhibitors based on the action mechanism of proteolytic enzymes and the active amino acids present in their active site (Haq et al., 2004). The serine protease inhibitors were found effective against cotton boll weevil (*Anthomonas grandis*) in 2004 (Franco et al., 2004). To enhance *Nicotiana alata* proteinase inhibitor effectiveness against *Heicoverpa*

armigera, Dunse et al. (2010) experimented with the combination of *solanum tuberosum* potato type I inhibitor (StPin1A), a potent inhibitor of *H. punctigera* and *H. armigera* chymotrypsins. The artificial diet containing both inhibitors significantly inhibited larval growth, a result not achieved by either inhibitor alone. In China, genetically modified cotton varieties utilize Bt toxins as insecticidal proteins to combat lepidopteron larvae, with the addition of the cowpea trypsin inhibitor gene (*CpTI*) as a secondary transgene to enhance protection. This combination of genes marks was the only instance of commercial deployment of a proteinase inhibitor transgene, with *Bt/CpTI* cotton cultivated on more than 0.5 million hectares in 2005 (Gatehouse., 2011). Jadhav et al. (2016) demonstrated a dose-dependent inhibition of insect growth and development in response to *Capsicum annuum* protease inhibitors-7 (*CanPI-7*). *CanPI-7* feeding is more effective and potent against neonates of *H. armigera* compared with to third-instar larvae. When caterpillars were fed three different concentrations of *CanPI-7* by mixing in diet, on the 16th day, there was significant inhibition of insect gut proteolytic activity in larvae fed on moderate dose (0.015%) compared with those fed on 0.03% (high) and 0.01% (low) concentrations. However, the plant-derived protease inhibitors have seen significant failure in recent years due to a lack of knowledge concerning insect physiology and biochemistry (Singh et al., 2020). Sometimes PIs that perform well during in vitro inhibition assays do not perform well in subsequent bioassays. Advanced techniques and strategies for next-generation pest management could be achieved by stacking PIs with additional insecticidal proteins, plastid engineering, recombinant proteinase inhibitors, RNAi-based treatments, and genome editing via CRISPR-Cas9 technology.

Genetically enhancing plant defense mechanisms

Plant resistance to herbivores is mostly dependent on induced defenses, which also regulate interactions between phytophagous arthropods and plants through signaling pathways and genes. Notably, the JA, salicylic acid (SA), and ethylene signaling pathways play critical roles in mediating the induced defense responses of plants (Chu et al., 2017). (Mo et al. 2024) showed that overexpression of jasmonate ZIM-domain (JAZ) protein GhJAZ24 confers resistance to cotton bollworm and fall armyworm. However, this overexpression also led to sterility in transgenic cotton by recruiting TOPLESS and histone deacetylase 6. They have developed an induced JAZ approach to address the sterility coupled with *GhJAZ24* overexpression. The induced JAZ transgenic cotton maintained fertility and showed insecticidal activity against cotton bollworm and fall armyworm. This shows that the induced JAZ-based approach for generating alternative insecticidal proteins with distinctive mechanisms of action, thus holding immense potential for future crop engineering. The elevated activities of plant chitinases could reduce aphid populations because plant chitinases are pathogenesis related proteins and are induced by pathogens as well as by pest attack (Rajendran et al., 2011). Zhong et al., (2021) cloned the cotton chitinase gene *GhChi6* and studied its role in providing aphid resistance in transgenic *Arabidopsis*. In the *GhChi6* transgenic *Arabidopsis* line, the levels of *AtEDS1*, *AtPAD4*, and *AtEDS5* (SA synthesis related genes) within the SA signaling pathway were higher than in wild-type plants. In contrast, reduced expression levels of *AtLOX2* (JA synthesis-related gene) in the JA signaling pathway and *AtEIN2* (ethylene signaling pathway receptor gene) in the ethylene signaling pathway compared with wild-type plants were observed. These findings suggest that the cotton chitinase gene *GhChi6* influences the plant's defense response to aphid attack, providing valuable insights for developing strategies to enhance cotton resistance to aphids.

Regulatory and ethical aspects

New scientific advancements like genetic engineering (CRISPR-Cas9), RNAi, gene drive, and the introduction of other insecticidal proteins offer promising chances to control pests in cotton. Yet, they also raise concerns about rules and ethics that need careful consideration. It is necessary to analyze the current regulatory frameworks to understand public perception and implications for biosecurity and biodiversity, and it is critical to manage these elements responsibly (Mackelprang & Lemaux 2020).

Future perspective

The future of pest resistance management in Bt cotton is closely tied to advancements in biotechnology. As pest resistance to Bt toxins continues to evolve, innovative biotechnological approaches are essential to sustain the effectiveness of Bt cotton. Advancements in developing new *Bt* gene and discovering of novel toxins through genomic and proteomic techniques will enhance the effectiveness and longevity of Bt cotton. Improved RNAi delivery systems, such as nanoparticle-based carriers, can boost the stability and uptake of RNAi in pests. Combining RNAi with Bt toxins offers a synergistic effect, enhancing pest control and mitigating resistance. Future strategies may involve using CRISPR-mediated gene drives to introduce susceptible alleles into pest populations, potentially reversing resistance and precisely controlling or eradicating pests. Genomic selection and gene

editing technologies will accelerate the development of cotton varieties with broad-spectrum and durable resistance, reducing reliance on Bt toxins alone. Single-cell RNA sequencing (scRNA-seq) is gaining significant interest from both scientists and industry professionals for its ability to uncover the molecular mechanisms behind key biological processes in cotton, such as fibre initiation, development, somatic embryogenesis, and responses to environmental stresses. Considering its potential, scRNA-seq should also be investigated for its applications in enhancing insect pest management in cotton (Pan et al., 2024). Developing smart integrated pest management systems that integrate biotechnological innovations with real-time pest monitoring and predictive modelling will allow for more precise and sustainable pest management strategies.

Conclusion

Transgenic crops producing insecticidal proteins from the bacterium *Bacillus thuringiensis* are extensively cultivated worldwide. However, the effective management of insect resistance remains a significant challenge. Various strategies like pyramided cotton with two or more distinct *Bt* toxin genes, refuge strategy, and releasing of sterile insects were proposed initially. But the effective control of insect pests in Bt cotton could be achieved by combining biotechnological advancements like gene silencing by RNAi, modified toxins, CRISPR mediated gene drives and other plant derived insecticidal proteins in Bt cotton background.

In conclusion, the emerging biotechnological advancements in insect-resistant management represent a promising frontier in the cultivation of Bt cotton. While these advancements hold immense potential in addressing pest management issues and it is imperative to maintain a balanced approach that considers environmental sustainability, farmer livelihoods, and regulatory frameworks. By harnessing the power of biotechnology responsibly, we can further enhance the resilience and productivity of Bt cotton cultivation, ultimately contributing to sustainable agriculture.

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