

INSECT RESISTANCE MANAGEMENT IN BT CROPS: FROM HIGH-DOSE/REFUGE STRATEGIES TO TOXIN DIVERSIFICATION AND MOLECULAR MONITORING

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ABSTRACT

Bt crops have provided effective control of several important insect pests and reduced dependence on chemical insecticides, but their continued use has imposed strong selection pressure for resistance. This review examines major approaches for managing resistance to Bt crops, beginning with the high-dose/refuge strategy and extending to toxin diversification and molecular monitoring. The high-dose/refuge strategy has delayed resistance in several pest species, but its effectiveness can be reduced by inadequate toxin expression, non-recessive resistance, limited refuge compliance, and non-random mating. Gene pyramiding can improve durability when component toxins act independently and are expressed at effective levels, whereas cross-resistance can reduce its benefits. Modified Cry toxins, structurally unrelated Cry, Cyt and Vip proteins, and the exploitation of negative cross-resistance provide additional options for overcoming resistance. Non-Bt traits, including protease inhibitors and lectins, can further diversify insecticidal mechanisms. RNA interference offers a mechanistically distinct approach and has progressed to commercial use, while CRISPR/Cas9-based strategies show potential through modification of plant defense and metabolic pathways. Finally, DNA-based and genomic methods can improve early detection of resistance alleles. Overall, durable resistance management requires integration of mechanistically distinct control tactics with appropriate refuges and continuous resistance monitoring, supported by field-scale validation.

KEYWORDS: Bt crops; Insect resistance management; HDR; Modified toxins; Resistance monitoring

1. INTRODUCTION

Transgenic crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) have become an important component of insect pest management. They can provide effective protection against important insect pests while reducing the reliance on chemical insecticides (Carrière et al., 2003). However, sustained cultivation of Bt crops imposes strong selection pressure on target insect populations, allowing resistance to evolve when individuals carrying resistance alleles survive toxin exposure and reproduce. Field-evolved resistance refers to a genetically based reduction in the susceptibility of an insect population to a Bt toxin due to selection pressure under field conditions. This type of resistance has been reported in several major crop pests (Tabashnik et al., 2023).

The resistance evolution is influenced by both genetic and ecological factors. The frequency and inheritance of resistance alleles, fitness costs, mating patterns, pest dispersal, crop deployment and the availability of susceptible insects all affect the rate at which resistance develops (Gould, 1998; Tabashnik et al., 2008). These factors form the basis of insect resistance management (IRM), which aims to conserve susceptible insect populations and extend the sustainability of Bt crops.

The high-dose/refuge (HDR) strategy has served as the cornerstone of insect resistance management (IRM) for Bt crops, relying on co-cultivation of high-toxin-expressing Bt plants with non-Bt refuges to preserve susceptible alleles and enforce recessive inheritance of resistance (Gould, 1998). However, its key assumptions are frequently violated under field conditions (Tabashnik & Carrière, 2019). Failure to achieve true high-dose expression, in particular, has been directly implicated in field-evolved resistance in several major pest species (Coates et al., 2020; Kukanur et al., 2018).

Pyramided Bt crops express two or more insecticidal proteins and were developed as an alternative to single-toxin Bt crops. Pyramiding can delay resistance when the toxins have independent modes of action, provide redundant killing, and are expressed at sufficiently high levels. However, its effectiveness may be reduced by cross-resistance, inadequate expression of individual toxins, and the simultaneous cultivation of single-toxin and pyramided Bt crops (Carrière et al., 2015, 2016). This has directed attention toward structurally engineered and novel Bt toxins, including modified Cry proteins, Cyt-Cry synergists, Vip3 proteins, and chimeric constructs, which can restore toxicity in resistant insects or diversify modes of action within pyramided systems (Tabashnik et al., 2011; Torres-Quintero et al., 2018).

Non-Bt insecticidal traits such as protease inhibitors and lectins offer modes of action unrelated to Bt toxicity and can be deployed alone or stacked with Bt toxins to broaden pest spectrum and dilute selection pressure (Hamza et al., 2018; Najeebullah et al., 2025). RNA interference (RNAi) and CRISPR/Cas9-based genome editing add a

further layer, silencing essential insect genes or modifying plant defense pathways to confer resistance through mechanisms entirely unconnected to Bt toxins (Baum et al., 2007; Ni et al., 2017; Zhang et al., 2022). Alongside these tactics, resistance-monitoring systems (from bioassays to DNA-based and genomic diagnostics) remain a practical necessity: without early detection of resistance alleles, field control failures can occur before any management response is possible (Andow & Ives, 2002; Huang, 2006). No single tactic has proven sufficient on its own. This review examines the principles, successes, and limitations of the high-dose/refuge strategy and gene pyramiding; evaluates modified and novel Bt toxins, non-Bt insecticidal proteins, RNAi, and CRISPR/Cas9-based genome editing; and considers advances in molecular resistance monitoring, toward a clearer picture of how these strategies can be combined into durable insect resistance management for transgenic Bt crops.

2. Insect Resistance Management Strategies in Bt Crops

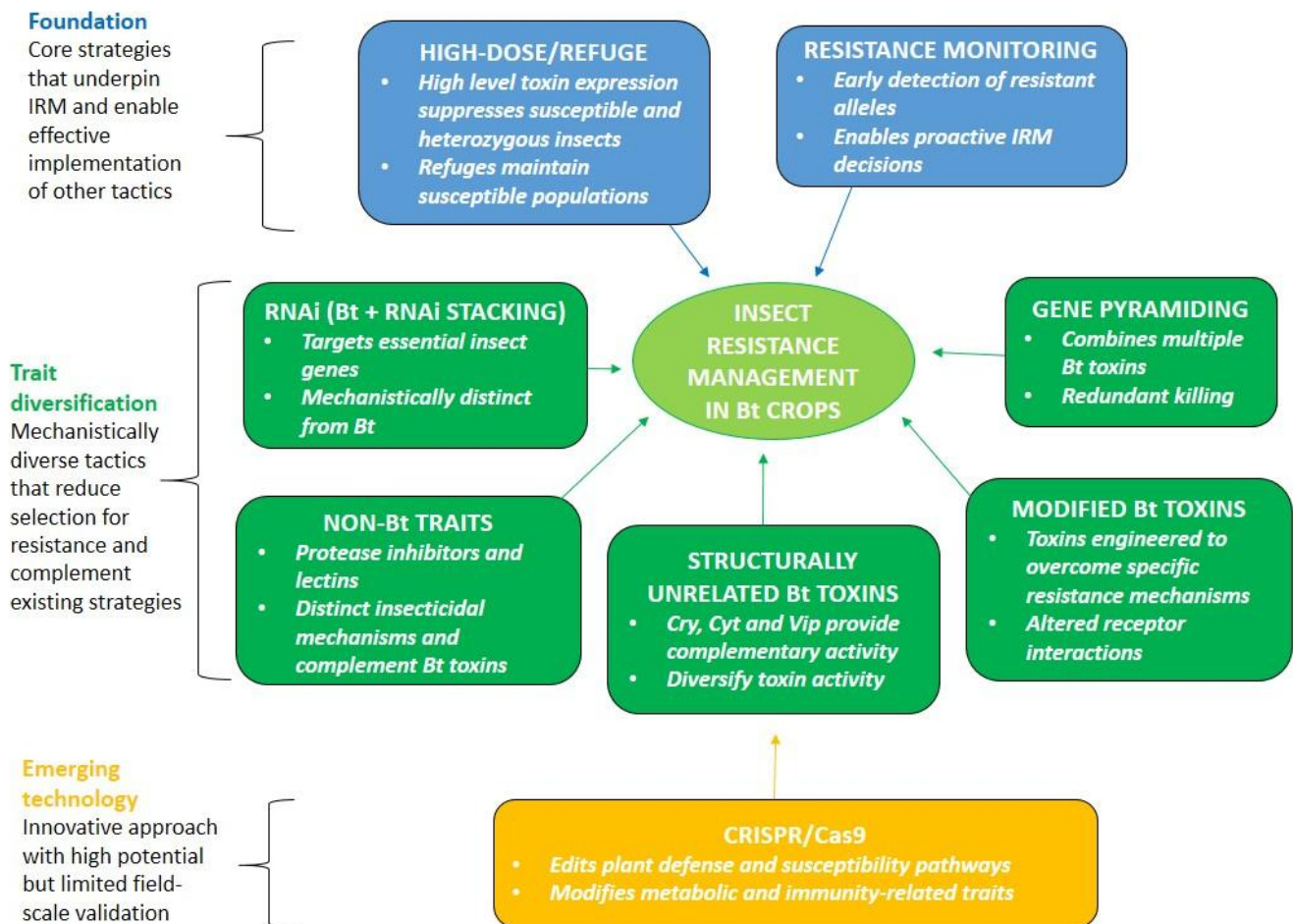


Fig. 1. Overview of Insect Resistance Management (IRM) strategies for Bt crops.

2.1. High-dose/refuge strategy (HDR)

The HDR strategy is a widely adopted insect resistance management (IRM) approach that effectively delays the evolution of resistance in Bt crops (Van den Berg, 2017) and has successfully delayed resistance for more than 25 years in key lepidopteran pests (Reisig et al., 2022). This strategy combines Bt crops expressing high levels of Cry toxins with nearby refuges of non-Bt plants to maintain susceptible insect populations within the agroecosystem (Tabashnik et al., 2008). A Bt event is considered high-dose when toxin expression reaches at least 25-fold the concentration required to kill susceptible insects or causes $\geq 99.99\%$ mortality in homozygous susceptible individuals, thereby ensuring that resistance behaves as a recessive trait (Gassmann & Reisig, 2023). The high-dose/refuge strategy relies on several key assumptions. Resistance is assumed to be recessive and controlled by a single locus with two alleles, resulting in three genotypes: RR, RS, and SS. It also assumes that resistance alleles occur at low frequencies in field populations and that resistant and susceptible adults mate randomly (Gould, 1998). Under these assumptions, refuges generate abundant susceptible insects that mate with rare homozygous-resistant individuals emerging from Bt crops, producing heterozygous offspring that are eliminated by high toxin concentrations, thereby suppressing resistance allele frequencies (Lu et al., 2024). But, in field settings, these key assumptions of the HDR strategy are often violated. Although increasing refuge area can partially compensate for these constraints, particularly under non-recessive resistance, such measures may

impose economically unacceptable yield losses (Tabashnik & Carrière, 2019). Consequently, integrated strategies combining refuges with additional control tactics, such as the release of genetically engineered insects, have been proposed to allow smaller refuges while maintaining resistance durability and crop productivity (Alphey et al., 2009). Such approaches can also enhance landscape-level pest suppression even when HDR assumptions are violated, although optimized release strategies may be required to maximize resistance management benefits (Brewer & Bonsall, 2021). For example, low-level releases of male-selecting (MS) engineered *P. xylostella* males, when combined with broccoli expressing Cry1Ac, effectively suppressed population growth and slowed the spread of Bt resistance. In this system, MS transgenes acted as species-specific control agents by preventing the survival of female progeny, thereby enhancing the durability of Bt crops while allowing reduced refuge sizes (Harvey-Samuel et al., 2015).

Refuges can be implemented in multiple forms, broadly classified as structured or unstructured. Refuges are commonly implemented as structured refuges, where non-Bt varieties of the same crop are planted separately from Bt crops. Alternatively, unstructured refuges rely on alternative host plants, including other crops or weeds near Bt fields, to maintain populations of susceptible insects (Camargo et al., 2020). Blended refuges or seed mixtures involve planting Bt and non-Bt seeds together throughout the field. This approach can accelerate resistance evolution if larvae move between Bt and non-Bt plants, thereby increasing exposure of heterozygotes to sublethal toxin doses (Pezzini et al., 2023). Natural refuge strategies are most suitable for polyphagous pests with high dispersal capacity but are ineffective for monophagous or weakly dispersing species (Van den Berg, 2017). Whether seed mixtures delay or hasten resistance depends on larval movement, the dominance of resistance and whether pyramided toxins are used, so their suitability must be assessed pest by pest (Carrière et al., 2016).

In general, the optimal size, configuration, and spatial proximity of refuges relative to Bt crops are determined by biological traits of the target pest (feeding behaviour, dispersal patterns, voltinism, population density), spatial and temporal distribution, abundance, and host suitability of non-Bt plants within the agricultural landscape, all of which critically influence IRM outcomes (Li et al., 2017; Van den Berg, 2017). Modeling studies indicate that resistance dominance and refuge proportion jointly determine resistance trajectories, with small refuges sufficient when resistance is fully recessive but substantially larger refuges required under partial dominance (Tabashnik et al., 2008). Once non-Bt plant abundance falls below the threshold needed to maintain susceptible populations, resistance evolution accelerates rapidly, underscoring the importance of spatially explicit modeling for optimizing refuge design (Ives et al., 2011).

When the high-dose criterion is not met, resistance evolution can be substantially accelerated, as insufficient toxin expression allows survival of heterozygous individuals. In such cases, pyramiding multiple Bt toxins can still delay resistance more effectively than single-toxin events, although this mitigation is often incomplete (Carrière et al., 2015). The absence of true high-dose expression has been identified as a major driver of field-evolved resistance in several transgenic crop-pest systems, including *Striacosta albicosta* to Cry1F and Cry1A.105 + Cry2Ab2 maize (Smith et al., 2017; Coates et al., 2020), and *H. armigera* to Cry1Ac expressing cotton (Kukanur et al., 2018). These cases illustrate that, even in the presence of refuges, failure to achieve high-dose expression can undermine resistance management and necessitate integration of toxin pyramids with complementary pest management strategies (Deitloff et al., 2015).

2.2. Gene pyramiding

Pyramided Bt crops express multiple toxins targeting a single pest species, thereby delaying resistance evolution, as individuals carrying resistance alleles to one toxin are effectively controlled by another (Gassmann & Reisig, 2023). This approach enhances pest control efficacy, broadens the spectrum of target species, and delays resistance evolution, which explains why most contemporary Bt crops express multiple Bt toxins (Carrière et al., 2015).

The durability of both single-toxin and pyramided Bt crops depends on several key factors, including adequate refuge availability, low initial resistance allele frequency, recessive resistance inheritance, fitness costs associated with resistance, and incomplete expression of resistance in heterozygotes (Tabashnik et al., 2013). When each toxin in a pyramid is expressed at lethal levels and cross-resistance is absent, survival is restricted to individuals carrying resistance alleles to all toxins and if resistance to each toxin is rare and recessive, survival is limited to extremely rare insects homozygous for all resistance alleles (Carrière et al., 2016). For a two-toxin pyramid to be maximally effective, each toxin should kill at least 95% of susceptible insects (Roush, 1998).

The principal advantage of pyramided Bt crops lies in their ability to reduce survival of insects heterozygous for resistance alleles through redundant killing, whereby each toxin independently eliminates nearly all susceptible individuals (Brévault et al., 2013). Zafar et al. (2022) reported transgenic cotton lines co-expressing Cry3Bb1 and Cry3, which exhibited substantially higher mortality of pink bollworm (60%) and cotton bollworm (75%) compared with lines expressing Cry3Bb1 alone (40% and 45%) or Cry3 alone (20% and 30%). Similarly, pyramided Bt crops producing Cry2 and/or Vip3Aa proteins showed complete redundant killing of Cry1-resistant *H. zea*, with Vip3Aa-containing pyramids fully eliminating both Cry1- and Cry2-resistant individuals (Santiago-González et al., 2022). Consequently, for the same refuge size, pyramided crops are expected to delay resistance evolution more effectively than single-toxin varieties (Tabashnik & Gould, 2012).

Despite their potential advantages, pyramided Bt strategies may be less effective when deployment conditions do not meet the required assumptions. Growing single-gene and dual-gene Bt varieties in close proximity can increase selection for resistance to multiple toxins (Tabashnik et al., 2013). This risk has also been demonstrated through modelling studies and experiments conducted with non-commercial Bt broccoli (Zhao et al., 2005). Empirical and

modelling work shows that pyramided cotton may fall short of the benefit expected from redundant killing (Brévault et al., 2013), and that resistance can evolve to multiple toxins even when they act at different targets (Tabashnik et al., 2013; Carrière et al., 2015). In addition, resistance evolution can be accelerated by cross-resistance among structurally related toxins; *Spodoptera frugiperda* populations resistant to Cry1Fa maize rapidly developed resistance to Cry1A.105 + Cry2Ab pyramided maize in laboratory assays, likely due to cross-resistance between Cry1Fa and Cry1A.105 (Santos-Amaya et al., 2015). Collectively, these findings highlight that selection for resistance to one toxin can effectively convert a pyramided crop into a functional single-toxin system, thereby eroding its durability (Carrière et al., 2015).

Cross-resistance and antagonistic interactions have been documented among several Bt toxin combinations and are often associated with amino acid sequence similarities, particularly within domains II and III of Cry toxins, with stronger cross-resistance observed among Cry1, Cry2, and Cry3 groups that share a conserved three-domain structure than between these toxins and structurally distinct proteins such as Vip3Aa or Cry34/35Ab (Carrière et al., 2015). Accordingly, pyramids combining structurally unrelated toxins are expected to provide the greatest resistance-management benefits.

More durable resistance management can be achieved by addressing non-ideal conditions, including limited redundant killing in pests with low susceptibility, significant cross-resistance within toxin combinations, simultaneous cultivation of single-toxin and pyramided crops, and accelerated resistance evolution driven by larval movement and gene flow in seed mixtures (Carrière et al., 2016). Sustained susceptibility of insect pests ultimately depends on maintaining high-dose expression, ensuring recessive inheritance of resistance, keeping resistance alleles rare, and integrating pyramids with adequate refuges of non-Bt host plants (Tabashnik et al., 2013).

2.3. Exploiting negative cross-resistance

Understanding cross-resistance relationships among Bt toxins is critical for designing durable Bt pyramids, as combining toxins lacking cross-resistance reduces the risk of resistance evolution. In some cases, resistance to one toxin can result in negative cross-resistance, whereby insects carrying resistance alleles become hypersusceptible to another toxin (Fig. 2), a phenomenon that can be strategically exploited for resistance management (Pittendrigh et al., 2014). For example, Cry1Ac-selected populations of the soybean looper *Chrysodeixis includens* exhibited increased susceptibility to Cry2Aa and Cry1Fa, with up to six-fold negative cross-resistance, indicating that Cry1Ac, Cry1Fa, and Cry2A are compatible components of multi-toxin pyramids (Rodrigues-Silva et al., 2019). Similarly, a bollworm strain harboring an *ABCC2* mutation showed negative cross-resistance between Cry1Ac and abamectin, highlighting the potential of leveraging resistance-associated fitness trade-offs for effective resistance management (Xiao et al., 2016). Although abamectin is not a Bt toxin, this example illustrates how resistance-associated physiological changes can create exploitable trade-offs between unrelated insecticidal modes of action.

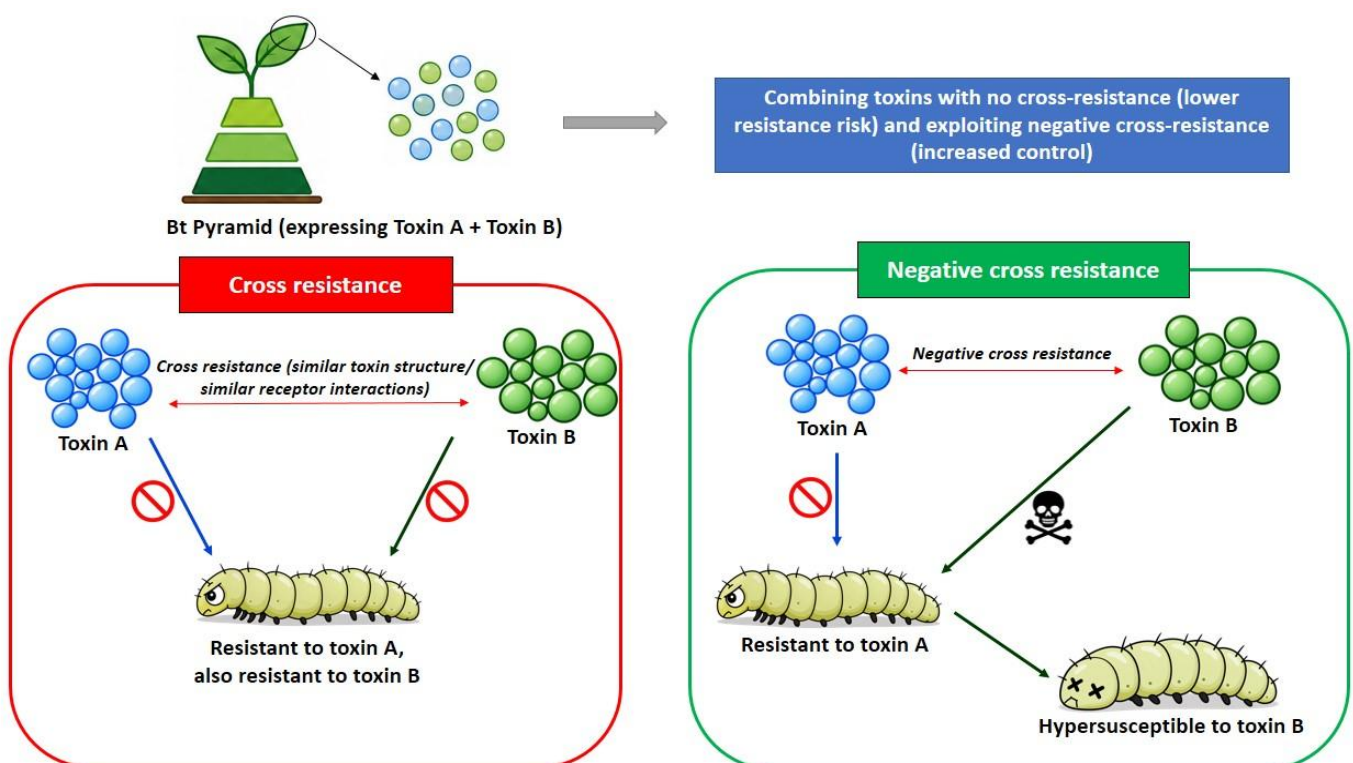


Fig. 2. Cross-resistance vs Negative cross-resistance.

2.4. Structurally unrelated Bt toxins

The deployment of novel pyramids, combined with proper refuge strategies, enhances the durability of transgenic insecticidal traits by slowing resistance evolution (Gassmann & Reisig, 2023). Kumar et al. (2021) developed, for the first time, transgenic cowpea lines expressing the *Bt cry2Aa* gene, which conferred enhanced resistance to the lepidopteran pest *Maruca vitrata*. These *cry2Aa*-expressing cowpea lines, therefore, represent a valuable genetic resource for pyramiding with existing *cry1Ab* cowpea cultivars to improve the durability of resistance against *M. vitrata*. Similarly, Tariq et al. (2022) introduced the insecticidal *cry11a12* gene into the local cotton cultivar GS-01, which showed moderate mortality but significant developmental abnormalities in *P. gossypiella* compared with those feeding on non-transgenic plants. These novel toxins can be exploited in pyramiding strategies to complement existing Cry toxins and thereby strengthen resistance management.

In addition to Cry proteins, *B. thuringiensis* produces cytolytic (Cyt) proteins encoded by *cyt* genes, which are primarily active against dipteran insects and contribute to resistance management through synergistic interactions with Cry and Vip toxins. For example, Lai et al. (2023) characterized the individual and combined activities of Cyt1Aa, Cry4Aa, Cry4Ba, and Cry11Aa against *Aedes albopictus*, and identified a Cyt1A-like protein that enhanced Cry11Aa toxicity by more than 20-fold. Although Cyt proteins are generally dipteran-specific, Cyt1Aa has also been reported to be active against the cotton leaf beetle (*Chrysomela scripta*) and to suppress resistance to Cry3Aa (Federici & Bauer, 1998). Mechanistically, Cyt proteins function as membrane-bound partners for Cry toxins; binding of Cry11Aa to Cyt1Aa facilitates Cry11Aa oligomerization and pore formation, thereby enhancing toxicity (Soberón et al., 2013). Exploiting this synergy, several hybrid toxins combining Cry, Cyt, and Vip proteins have been engineered to overcome resistance and broaden insecticidal activity (Torres-Quintero et al., 2018).

Vegetative insecticidal proteins (Vip) represent another important class of Bt toxins. Vip proteins are non-crystalline toxins produced during the vegetative growth phase of *Bacillus thuringiensis* and are initially classified into four subfamilies (Vip1-Vip4) based on amino acid sequence similarity (Estruch et al., 1996). Vip1 and Vip2 proteins primarily target coleopteran and hemipteran insects (Sattar & Maiti, 2011), whereas Vip3 proteins are highly effective against lepidopteran pests. Notably, Vip3 toxins possess modes of action and receptor binding distinct from Cry proteins, conferring activity against lepidopteran populations with reduced susceptibility to Cry toxins. Supporting their utility, Bhatti et al. (2023) developed transgenic maize inbred lines expressing a synthetic *vip3Aa86* gene for control of the maize stem borer (*Chilo partellus*), with lines showing the highest *vip3Aa86* expression exhibiting increased insect mortality. Together, these findings highlight the value of Cry, Cyt, and Vip toxins as complementary components of diversified Bt-based resistance-management strategies.

2.5. Modified Bt toxins

Modified *Bacillus thuringiensis* (Bt) toxins are engineered variants of native Cry proteins designed to overcome insect resistance by restoring or altering interactions with midgut targets (receptors). These modifications are achieved through approaches such as domain swapping, site-directed mutagenesis, truncation (Deist et al., 2014), or fusion with heterologous peptides. Such engineering can enhance binding to alternative receptors, bypass mutations in primary target receptors (e.g., cadherin or ABCC2), or improve pore-forming efficiency, thereby re-establishing toxicity in resistant insect populations. Consequently, engineered Bt toxins represent a resistance-management strategy that extends the functional lifespan of Bt crops by countering target-site mediated resistance mechanisms rather than relying solely on increased toxin dose.

Cadherin acts as a primary receptor and plays an important role in Cry toxin toxicity. Accordingly, mutations in cadherin genes have been associated with resistance to Cry1Ac in several lepidopteran pests, including *H. armigera* (Yang et al., 2007, 2009). To overcome cadherin-based resistance, genetically modified Cry1Ab and Cry1Ac toxins lacking α -helix-1 (Cry1AbMod and Cry1AcMod) were developed. These modified toxins are capable of forming oligomers and inducing pore formation *in vitro* even in the absence of cadherin (Fig. 3) (Muñoz-Garay et al., 2009). Similarly, compared with the original toxin counterparts, Cry1AbMod and Cry1AcMod exhibited enhanced toxicity against resistant strains of *P. xylostella* and *O. nubilalis*, effectively killing insects in which cadherin receptors were altered or absent (Tabashnik et al., 2011).

Beyond truncation-based strategies, rational toxin engineering has been used to alter receptor specificity. Torres-Quintero et al. (2018) engineered a hybrid Cyt1Aa-loop3 toxin by incorporating domain II loop 3 from Cry1Ab, a region involved in binding to APN, ALP, and cadherin receptors. This modification enhanced receptor interactions and increased toxicity against the lepidopteran pests *Manduca sexta* and *P. xylostella*. This result demonstrates that the insect specificity of Cyt toxins can be rationally modified by introducing receptor-binding loops from unrelated Cry toxins with different host ranges.

Fusion of Bt toxins with mechanistically distinct proteins provides another direction for resistance management. Pan et al. (2025) developed a transgenic soybean event (CAL-16) expressing a Cry1Ab-Vip3A fusion protein that caused 100% mortality in neonate larvae of *H. armigera*, *S. litura*, *Agrotis ipsilon*, *S. exigua*, and *S. frugiperda*, demonstrating broad-spectrum efficacy against major lepidopteran pests. By combining Cry1Ab and Vip3A within a single fusion protein, CAL-16 integrates two mechanistically distinct modes of action, offering a rational strategy to broaden pest control and can reduce resistance risks relative to single-toxin traits.

Chimeric Bt toxins further illustrate the utility of sequence recombination in expanding insecticidal spectra. A chimeric Bt gene, *cry2AX1*, combining sequences from *cry2Aa* and *cry2Ac*, was developed to enhance insecticidal activity against lepidopteran pests, particularly *Helicoverpa armigera* (Udayasuriyan et al., 2010; Manikandan et al., 2016). Transgenic cotton expressing *cry2AX1* showed 27.0-88.8% mortality against *H. armigera*,

demonstrating high efficacy even at low expression levels (Sakthi et al., 2015). Similarly, transgenic rice plants expressing a synthetic *cry2AX1* gene caused 20-80% larval mortality in rice leaffolder bioassays, confirming its effectiveness against multiple lepidopteran pests (Manikandan et al., 2016). Consistently, transgenic rice expressing *cry2AX1* exhibited effective resistance against the yellow stem borer (*Scirpophaga incertulas*), rice leaffolder (*Cnaphalocrocis medinalis*), and the oriental armyworm (*Mythimna separata*) (Chakraborty et al., 2016). By integrating functional elements from two or more Cry proteins, it broadens insecticidal activity and provides a mechanistically diversified Bt trait that is well suited for pyramiding and resistance management in transgenic crops.

2.6. Non-Bt traits

2.6.1. Protease inhibitors (PIs)

Protease inhibitors (PIs) are small, defense-related proteins that are constitutively expressed mainly in seeds and can be induced in vegetative tissues following wounding or herbivory (Koiwa et al., 1997). They exert insecticidal effects by forming stable, non-dissociable complexes with digestive proteinases in the insect midgut, thereby disrupting proteolysis, impairing nutrient assimilation, and ultimately reducing insect growth, survival, and fecundity (Lawrence & Koundal, 2002). PIs target a broad range of digestive enzymes, including serine, cysteine, aspartate proteases, and metallo-carboxypeptidases, leading to impaired development and increased susceptibility to natural enemies (Habib & Fazili, 2007).

Resistant populations of *S. frugiperda* feeding on Bt maize expressing Cry1F, Cry1A.105, and Cry2Ab2 in combination with soybean trypsin inhibitor (SBTI) exhibited increased mortality, reduced larval weight, and suppressed digestive trypsin activity. These findings suggest that SBTI ingestion enhances susceptibility of Bt-resistant *S. frugiperda* to Bt maize by impairing midgut protease function (Fonseca et al., 2023). Co-expression

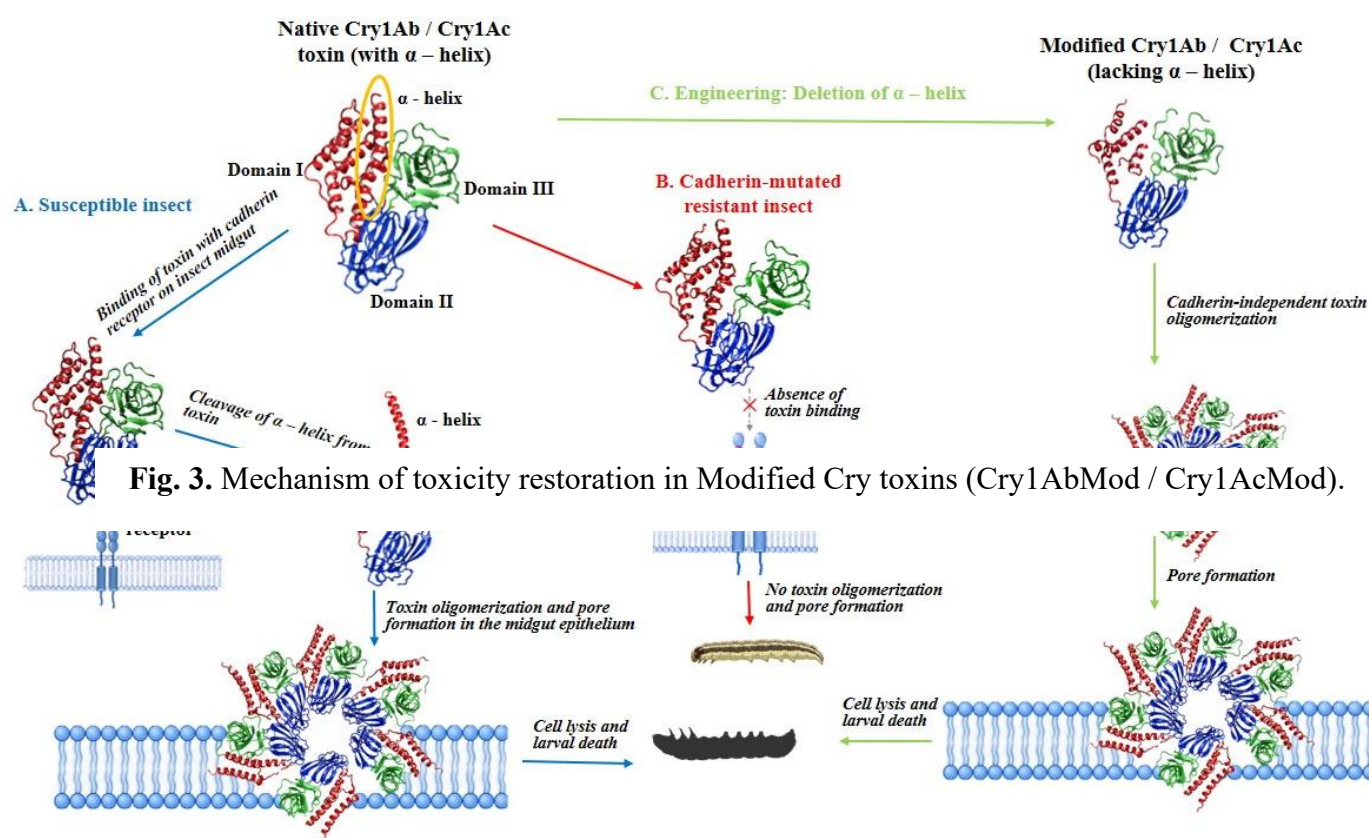


Fig. 3. Mechanism of toxicity restoration in Modified Cry toxins (Cry1AbMod / Cry1AcMod).

of barley-derived serine (BTI-CMe) and cysteine (Hv-CPI2) protease inhibitors in tomato significantly suppressed tomato pinworm (*Tuta absoluta*) by reducing larval growth, survival, and fecundity through sustained inhibition of midgut trypsin activity. This suppression was accompanied by activation of endogenous plant defenses, including induction of Protease inhibitor 2 (PIN2), increased glandular trichome density and altered volatile emission profiles (Hamza et al., 2018). These findings demonstrate the potential of protease inhibitors as complementary insecticidal traits in selected crop-pest systems

2.6.2. Lectins

Lectins represent another important class of non-Bt defense proteins used for insect resistance management. Lectins are carbohydrate-binding proteins produced by plants and other organisms that contain at least one non-catalytic domain capable of reversibly recognizing specific mono- or oligosaccharides (Peumans & Van Damme, 1995). Owing to their resistance to proteolytic degradation, lectins persist in the insect gut, where they bind glycoconjugates on midgut epithelial cells, disrupt epithelial integrity, increase membrane permeability, and facilitate the translocation of toxic substances into the haemolymph (Sprawka et al., 2015). Through these

interactions, lectins interfere with insect physiology and negatively affect growth, development, and fecundity (Shahidi-Noghabi et al., 2009). Multiple plant lectin families, including *Galanthus nivalis* agglutinin (GNA), *Nicotiana tabacum* agglutinin (NICTABA), hevein-related lectins, ricin-related lectins, amaranthins, legume lectins, and jacalins have been reported to exhibit strong insecticidal activities (Van Damme et al., 2008), and their effects across different insect orders have been comprehensively documented (Singh et al., 2023). Lectins have also been investigated as complementary traits that could diversify insecticidal mechanisms when combined with Bt or other technologies. For example, co-expression of the mannose-binding lectin PTA (*Pinellia ternata* agglutinin) with the Bt toxin Cry2Ab provided broad-spectrum control of both chewing and sucking insects. In this system, lectin-mediated disruption of gut physiology complemented Cry-induced epithelial damage, enhancing overall efficacy while reducing reliance on a single mechanism of action (Najeebullah et al., 2025). Beyond protein-based defenses, metabolic enhancement strategies have also been explored to improve Bt efficacy. In cotton, two Bt Cry genes (*Cry1Fa* and *Cry32Aa*) were co-expressed with two pectin methyl esterase (*pme*) genes, which increase methanol production from the plant cell wall. Bioassays demonstrated that plants co-expressing *cry* and *pme* genes caused 100% mortality of *H. armigera*, 70% mortality of *P. gossypiella*, and 95% mortality of *Earias fabia*, substantially exceeding mortality levels observed in plants expressing Bt Cry genes alone (Razzaq et al., 2021). These results demonstrate that coupling Bt toxins with traits that modify plant metabolic by-products, such as methanol, can markedly enhance insecticidal efficacy against multiple pests, diversify modes of action, and improve the durability and effectiveness of transgenic insect-resistant crops.

2.7. RNA interference (RNAi)

Following the discovery of double-stranded RNA (dsRNA)-mediated gene silencing (Fire et al., 1998), RNA interference (RNAi) has progressed from a molecular biology tool to a practical insect pest management strategy. Its feasibility was first demonstrated in transgenic maize expressing dsRNA that silenced a vacuolar ATPase gene and suppressed feeding in *Diabrotica virgifera virgifera* (Baum et al., 2007). RNAi reached commercial deployment in 2017 with U.S. approval of SmartStax® PRO maize, the first crop expressing dsRNA targeting the *D. v. virgifera* Snf7 (Sucrose non-fermenting 7) gene (Head et al., 2017).

RNA interference is a sequence-specific, post-transcriptional gene-silencing mechanism triggered by dsRNA that targets complementary messenger RNA for degradation. In the cytoplasm, dsRNA is processed by Dicer endonucleases into approximately 21-23 nucleotide small interfering RNAs (siRNAs), one strand of which is incorporated into the RNA-induced silencing complex (RISC). RISC contains an Argonaute protein with RNase-H like activity that mediates sequence-specific cleavage of target mRNA (Fig. 4) (Singh et al., 2016). Functionally, siRNAs typically exhibit perfect complementarity and induce mRNA degradation, whereas microRNAs (miRNAs) often show partial complementarity and regulate gene expression through translational repression or, when fully complementary, mRNA cleavage via interactions with the 3' untranslated region (Gu & Kay, 2010). The effectiveness of RNAi-based transgenic plants depends on the selection of essential target genes, sufficient accumulation and delivery of intact dsRNA (including via in planta expression), and insect physiological factors, all of which influence dsRNA uptake and gene silencing efficiency (Price & Gatehouse, 2008; Li et al., 2022). Following ingestion, plant-derived dsRNA enters insect cells through clathrin-mediated endocytosis (CME) and/or SID-mediated transport. Unlike plants, insects lack RNA-dependent RNA polymerases and therefore cannot amplify the RNAi signal, making silencing efficiency highly dependent on the amount of dsRNA internalized (Li et al., 2022). Additionally, species-specific gut environments, particularly the presence of dsRNases in some lepidopterans, can rapidly degrade dsRNA and reduce RNAi effectiveness (Guan et al., 2018; Singh et al., 2017; Fan et al., 2021).

Effective and environmentally safe RNAi-based pest control requires targeting genes that are essential to the target insect but absent or nonessential in non-target organisms (Ni et al., 2017). Castellanos et al. (2022) evaluated non-target risks of RNAi-based pesticides using the egg parasitoid *Telenomus podisi* exposed to dsRNA targeting *Euschistus heros*. Despite 17-21 nt sequence similarity between dsRNA constructs and parasitoid genes, no alterations in gene expression were detected. In this study, sequence similarity alone did not result in detectable off-target effects.

Multiple classes of insect genes have been successfully targeted, including those involved in basal metabolism, detoxification, digestion, suppression of plant defenses, and symbiont-mediated functions (Chung et al., 2021). Additionally, RNAi constructs can be designed to silence a single gene or gene fragment, multiple regions within a gene, multiple alleles, or even multiple genes across one or more species (Guo et al., 2015). Several representative examples illustrate the breadth of RNAi targets and outcomes. Silencing of a cuticular protein gene in *Myzus persicae* feeding on *Arabidopsis thaliana* reduced aphid performance (Bhatia & Bhattacharya, 2018). Oral delivery of dsRNA targeting the salivary effector Bt56 in *Bemisia tabaci* disrupted host defense suppression and reduced survival and fecundity (Xu et al., 2019). Knockdown of the glutathione S-transferase gene *GSTs5* (involved in detoxifying glucosinolates expressed in Brassicaceae) in *Bemisia tabaci* via *Arabidopsis thaliana* transgenic plants expressing dsRNA, increased susceptibility to glucosinolates and delayed development (Eakteiman et al., 2018). RNAi-mediated disruption of symbiosis-related genes (*AmiD* and *LdcA1*) in *Acyrtosiphon pisum* reduced *Buchnera aphidicola* abundance and aphid growth (Chung et al., 2018, 2021). Fatty acyl-CoA reductases (FARs) represent another promising class of RNAi targets. FARs are involved in fatty alcohol biosynthesis and play essential roles in insect pheromone production and reproduction, including bombykol synthesis in *Bombyx mori* (Moto et al., 2003; Lin et al., 2018). RNAi-mediated knockdown of the FAR

gene in *Adelphocoris suturalis* suppressed ovarian development and female fertility (Luo et al., 2017). Similarly, in *Chilo suppressalis*, transgenic rice expressing dsRNA targeting FAR (*dsFAR*) significantly increased larval mortality, caused epidermal abnormalities and conferred strong resistance to *C. suppressalis* infestation (Sun et al., 2022). RNAi has also been deployed through artificial microRNAs (amiRNAs) in transgenic crops, as demonstrated by Faisal et al. (2021) against *M. persicae* in transgenic tomato cultivars. Similarly, artificial microRNAs targeting *Sex-lethal*, acetylcholinesterase, and *Orcokinin* genes in *Bemisia tabaci* conferred enhanced resistance in transgenic *Nicotiana tabacum*, resulting in sustained reductions in whitefly abundance across generations (Zubair et al., 2020). Collectively, these studies demonstrate that targeting multiple, functionally unrelated pathways can diversify selection pressures and reduce the likelihood of rapid resistance evolution. Ni et al. (2017) developed the first Bt-RNAi cotton pyramids targeting juvenile hormone biosynthesis and transport genes (*JHAMT* and *JHBP*) in *H. armigera*. These plants killed both Bt-susceptible and Bt-resistant strains without cross-resistance, and Bt and RNAi acted independently yet synergistically. Simulation modeling based on northern China production systems predicted that Bt + RNAi pyramids could substantially delay resistance evolution, particularly when supported by large natural refuges (~50%) (Ni et al., 2017). Because the durability of Bt pyramids can be compromised by cross-resistance and antagonistic interactions among toxins (Carrière et al., 2015, 2016), RNAi has emerged as a promising complementary strategy. Combining RNAi with Bt traits can reduce the likelihood of resistance evolution by introducing mechanistically distinct selection pressures (Saakre et al., 2024).

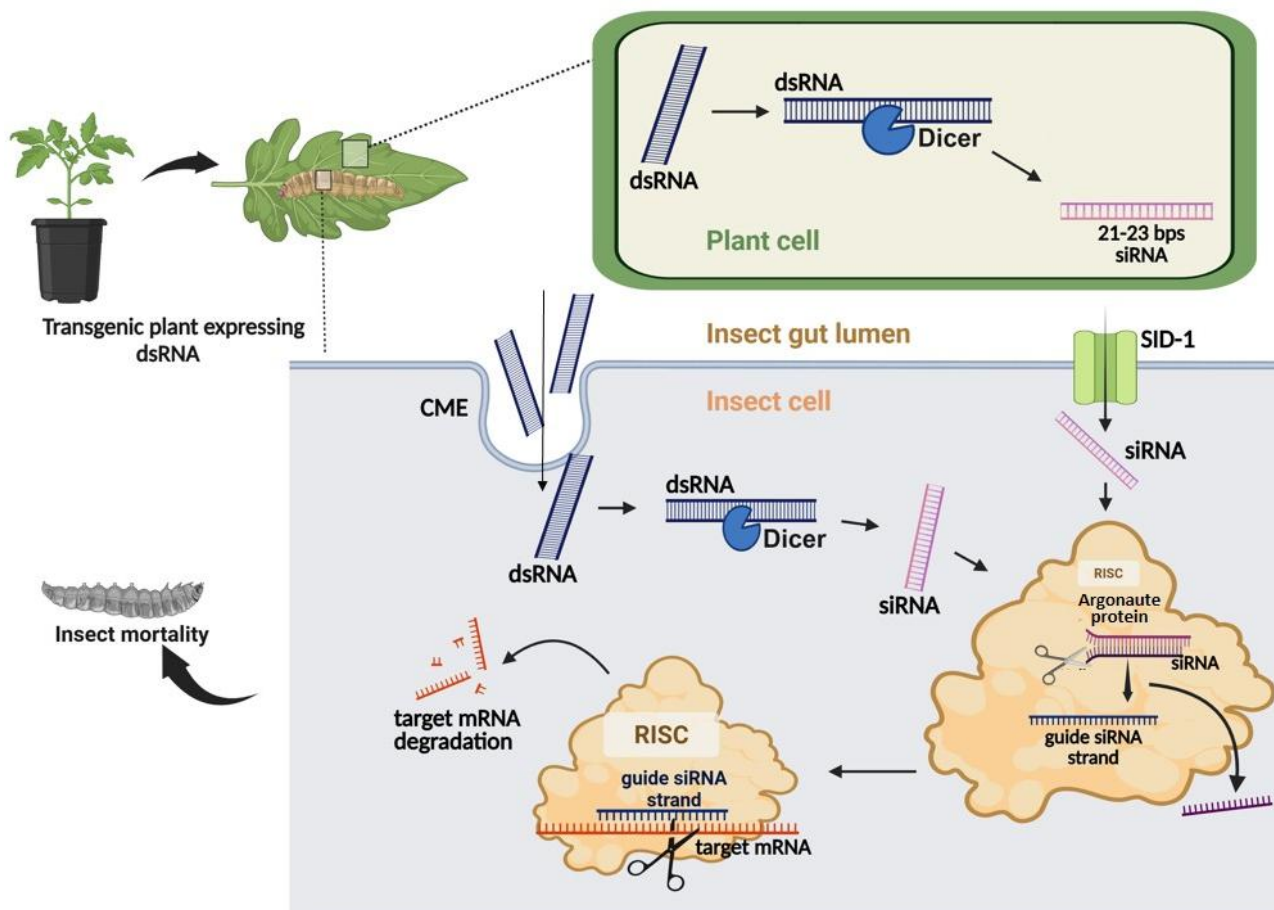


Fig. 4. Mechanism of action of RNA interference (RNAi): Transgenic plants produce double-stranded RNA (dsRNA), which enters the insect digestive system during feeding. Within plant cells, Dicer enzymes process dsRNA into small interfering RNAs (siRNAs) of 21-23 base pairs. In the insect gut, dsRNA is internalized by midgut epithelial cells mainly through clathrin-mediated endocytosis (CME), whereas siRNA uptake may involve Systemic RNA Interference Defective 1 (SID-1)-mediated transport. Within insect cells, Dicer further cleaves dsRNA into siRNAs, which are subsequently incorporated into the RNA-induced silencing complex (RISC), containing the Argonaute protein. The siRNA duplex is unwound, allowing the guide strand to remain associated with RISC. This complex recognizes and cleaves complementary target messenger RNA (mRNA), thereby suppressing gene expression and potentially leading to insect mortality (Figure created using BioRender).

2.8. CRISPR/Cas9

CRISPR/Cas9 is a flexible tool for genome editing composed of the Cas9 endonuclease and single-guide RNAs (sgRNAs), with multiplex genome editing simultaneously inducing double-stranded breaks at multiple genomic

loci, representing one of its most powerful applications (Cheng et al., 2025). Because many insect pests, particularly polyphagous species, rely on plant-derived cues such as volatiles, gustatory signals, visual traits, and oviposition cues for host recognition and selection (Mookiah et al., 2021), targeted genome editing of these plant traits provides a promising alternative or complementary strategy for pest management (Kumari et al., 2022).

At the molecular level, Cas9 functions as a sequence-specific endonuclease, while sgRNAs guide Cas9 to defined genomic loci, where it introduces double-strand breaks adjacent to protospacer-adjacent motif (PAM) sequences (Courtier-Orgogozo et al., 2017). These breaks are repaired either through error-prone non-homologous end joining (NHEJ), resulting in insertions or deletions that disrupt gene function, or through homology-directed repair (HDR), which enables precise gene knockouts, knock-ins, or sequence modifications when donor templates are supplied (Yin et al., 2017). This flexibility allows both loss- and gain-of-function manipulation of plant genes involved in defense and insect interactions.

Genome editing of plant metabolic and defense pathways has demonstrated clear potential for enhancing resistance to insect pests. For example, suppression of serotonin biosynthesis in rice through mutation of the cytochrome P450 gene *CYP71A*, which catalyzes the conversion of tryptamine to serotonin, resulted in increased salicylic acid accumulation and enhanced resistance to planthoppers and stem borers (Lu et al., 2018). Such modifications highlight how altering secondary metabolism can indirectly strengthen plant defenses against multiple insect guilds.

In addition to metabolic pathways, CRISPR/Cas9-mediated editing of plant defense and immunity genes offers a direct route to insect resistance. For example, transgenic *Arabidopsis thaliana* plants engineered to express high levels of E β f synthase produce the aphid alarm pheromone, the sesquiterpene (E)- β -farnesene, upon aphid infestation; this compound deters feeding and attracts natural enemies such as the parasitoid *Diaeretiella rapae*, thereby suppressing aphid populations (Beale et al., 2006). More directly, loss-of-function editing of the soybean UDP-glycosyltransferase gene *GmUGT* significantly enhanced resistance to *H. armigera* and *S. litura*, demonstrating the feasibility of CRISPR/Cas9-based manipulation of immunity-related genes for broad-spectrum insect resistance (Zhang et al., 2022). In many countries, gene-edited crops face fewer regulatory barriers to commercialization than conventional GM crops (Waltz, 2018).

2.9. Resistance monitoring

The U.S. Environmental Protection Agency (EPA) identifies Bt resistance monitoring as one of the seven core components required in insect resistance management (IRM) plans for Bt crops. A central requirement for effective IRM is a cost-efficient monitoring system capable of estimating initial Bt resistance allele frequencies at very low levels in field populations and detecting early shifts in these frequencies, thereby enabling timely, proactive interventions before field-level control failures occur (Huang, 2006). Conventional approaches for monitoring Bt resistance include dose-response and diagnostic-dose bioassays, F₁ iso-female line screens, F₂ screens, and sentinel plots (Huang, 2006). Dose-response and diagnostic bioassays are primarily used to confirm resistance or diagnose field control failures but are insufficiently sensitive to detect resistance at very low allele frequencies (e.g., $<10^{-3}$). In contrast, F₁ and F₂ screening methods are highly sensitive for early detection of rare resistance alleles (Andow & Ives, 2002). The F₂ screen evaluates resistance in F₂ progeny derived from iso-line families established from wild-mated females or paired wild collections (Andow et al., 1998), whereas the F₁ screen assesses offspring from crosses between field-collected insects and laboratory strains with known resistance (Gould et al., 1997). Despite their sensitivity, these methods are labor-intensive, costly, and operationally complex, limiting their large-scale deployment.

Molecular approaches offer greater sensitivity and flexibility than phenotypic assays by enabling direct detection of resistance alleles in both heterozygous and homozygous individuals, independent of dominance or recessive traits (Morin et al., 2003; Huang, 2006). Elucidation of the molecular basis of *Bacillus thuringiensis* resistance has facilitated the development of DNA-based diagnostics to monitor resistance allele frequencies and support proactive resistance-management strategies (Yang et al., 2007). However, diverse genetic mechanisms and multiple resistance mutations can constrain broad application of DNA-based tools (Wu, 2014). Despite these limitations, DNA-based assays have been developed for resistance alleles characterized in field populations, such as mutated cadherin alleles in *H. armigera* (Yang et al., 2007). Whole-genome sequencing further enables simultaneous detection of known and novel resistance mutations, exemplified by identification of a 12-bp insertion in exon 15 of *ABCC2* associated with Cry1F resistance in *S. frugiperda* (Guan et al., 2021). Targeted sequencing represents a cost-effective alternative to whole-genome sequencing by focusing coverage on candidate resistance genes, thereby increasing sensitivity while reducing sequencing costs, although its utility depends on prior knowledge of resistance loci and high-quality genome annotation (Tandy et al., 2023). Using highly multiplexed PCR-based targeted sequencing, resistance alleles in *SfABCC2* have been detected and tracked in *S. frugiperda* populations across multiple geographic regions, enabling identification of both known and novel candidate resistance mutations and their spatial spread (Schlum et al., 2021; Tandy et al., 2023).

3. Conclusion and future prospects

The success of Bt crops in suppressing major insect pests has been substantial, yet decades of deployment have shown that resistance is not merely a theoretical concern but an expected evolutionary risk under sustained selection pressure. The high-dose/refuge strategy delayed the onset of resistance by preserving susceptible alleles within pest populations, and it continues to provide a measure of protection where refuge compliance is maintained. This strategy was not designed to function indefinitely as a standalone measure, however, and its

underlying assumptions: true high-dose toxin expression, recessive inheritance, and adequate refuge planting, are not uniformly met under field conditions. Departures from these assumptions can substantially reduce the effectiveness of HDR and have been associated with field-evolved resistance in several crop-pest systems.

Gene pyramiding, modified and novel Bt toxins, non-Bt insecticidal proteins, RNA interference, and CRISPR/Cas9-based genome editing each address particular limitations of the high-dose/refuge model, but none constitutes a self-sufficient solution. Pyramids can lose much of their resistance-management advantage when their component toxins exhibit substantial cross-resistance. Protease inhibitors and lectins provide mechanistically distinct plant-derived defenses, but their effectiveness and contribution to resistance management vary among crop-pest combinations and require further validation under field conditions. RNAi has progressed beyond laboratory proof-of-concept to commercial deployment, although its broader application remains constrained by species-specific differences in dsRNA stability, uptake and silencing efficiency. Many CRISPR-based insect-resistance applications remain at the experimental stage and their contribution to field-scale IRM remains to be established. These evidences point to a consistent conclusion: durable resistance management depends on combining mechanistically distinct tactics rather than relying on any single approach, while maintaining appropriate refuge and surveillance strategies.

Resistance monitoring must become sufficiently rapid and inexpensive to be integrated into routine agricultural practice rather than confined to periodic research surveys. DNA-based and genomic diagnostic tools have advanced considerably in this direction, although cost and accessibility remain limiting factors, particularly in smallholder farming systems, which account for a substantial share of global Bt crop cultivation. A further consideration is the disparity between the performance of these strategies under controlled experimental conditions and their behaviour on commercial farms, where mixed pest complexes, inconsistent refuge adoption, and economic constraints on growers all shape outcomes. Therefore, greater emphasis should now be placed on multi-year, field-scale evaluation that complements, rather than replaces, laboratory studies.

These considerations do not diminish the contribution that Bt technology has made to pest management; rather, they indicate that the next phase of insect resistance management will require a shift from safeguarding individual traits toward managing resistance as a continuously evolving biological process. Achieving this will depend on sustained collaboration among plant breeders, molecular biologists, entomologists, and growers, supported by monitoring systems capable of detecting resistance before it compromises field efficacy. The extent to which such coordination is achieved, more than the introduction of any single new toxin or technology, is likely to determine the long-term viability of Bt crops in global pest management.

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Author Contributions

All authors contributed to the study conception and manuscript preparation. A. Balaji performed the literature search. The first draft of the manuscript was written by A. Balaji and N. Balakrishnan. A. Balaji created the figures. N. Balakrishnan, N. Chitra, A. Subramanian and R. Raghu critically reviewed and edited the manuscript. All authors commented on previous versions of the manuscript.

Data availability

No datasets were generated or analysed during the current study.

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