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FIELD-EVOLVED RESISTANCE TO BT TOXINS IN INSECT PESTS: A MOLECULAR INSIGHTS

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ABSTRACT

Genetically modified (GM) crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt) have revolutionized pest management in agriculture, significantly reducing synthetic insecticide use and enhancing crop productivity since their commercial introduction in 1996. However, the sustained selection pressure exerted by these crops has inevitably led to the evolution of field-evolved resistance in several key insect pests, threatening the long-term efficacy and sustainability of Bt technology. This comprehensive review synthesizes the current understanding of the complex molecular mechanisms underlying Bt resistance, predominantly focusing on altered toxin binding due to mutations or reduced expression of membrane receptors, but also exploring impaired toxin activation, enhanced detoxification, and altered signal transduction pathways. It critically examines the essential role of surveillance diagnostics, including both traditional bioassays (e.g., diagnostic dose assays for resistance allele frequency determination) and advanced molecular tools (e.g., PCR-based assays, sequencing for specific mutations like cadherin and aminopeptidase N mutations, and gene expression analysis for receptor downregulation), in the early detection and monitoring of resistance evolution. The review extensively details integrated mitigation strategies crucial for delaying resistance, emphasizing the cornerstone refuge strategy (e.g., structured refuges, refuge-in-a-bag, high-dose/refuge principle), the power of gene pyramiding (stacking multiple Bt toxins with different modes of action), and the importance of integrating Bt crops into broader Integrated Pest Management (IPM) frameworks (e.g., crop rotation, biological control). Case studies, such as the varied success and challenges with *Helicoverpa armigera* resistance to Cry1Ac in China (where resistance was rapid due to poor refuge compliance and rapid gene flow, e.g., Cry1Ac resistance allele frequency > 0.5 in some regions by 2005) versus the sustained susceptibility of *Pectinophora gossypiella* to Cry1Ac in the US (where high-dose/refuge strategy maintained susceptibility, with a frequency of resistance alleles to Cry1Ac remaining below 0.005), highlight the critical importance of effective resistance management plans. Despite significant progress in understanding resistance mechanisms and developing management tactics, challenges remain in predicting resistance evolution, ensuring farmer compliance with refuge requirements and managing resistance in multi-gene Bt crops and against new toxin types. Future directions emphasize the need for novel Bt toxins, next-generation technologies like RNA interference, and adaptive regulatory frameworks to ensure the long-term sustainability of this vital pest control technology.

Key words : Bt crops, Insect resistance, *Bacillus thuringiensis*, Molecular mechanisms, Surveillance, Diagnostics, Refuge strategy, Gene pyramiding, IPM, Resistance management.

Introduction

Global agriculture faces the perennial challenge of insect pest management, which significantly impacts crop productivity and food security. Historically, this challenge has been addressed primarily through the widespread application of synthetic chemical insecticides. While effective, the overuse of these chemicals has led to severe

environmental consequences, including biodiversity loss, water contamination, non-target organism toxicity and the rapid evolution of insecticide resistance in pest populations (Georghiou and Lagunes-Tejeda, 1991). The search for more sustainable and environmentally benign pest control alternatives became a paramount objective.

The advent of genetically modified (GM) crops expressing insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt) marked a revolutionary shift in pest management. First commercialized in 1996 with Bt cotton and Bt maize, these crops incorporate genes encoding specific Bt toxins (primarily Cry and Vip proteins) directly into the plant genome, allowing the plant itself to produce the insecticidal agent (Shelton *et al.*, 2002). This innovation offered several compelling advantages:

Reduced Insecticide use : Bt crops dramatically reduced the need for synthetic broad-spectrum insecticides, thereby minimizing environmental pollution and benefiting non-target organisms, including natural enemies of pests (Romeis *et al.*, 2006).

Targeted Specificity : Bt toxins are highly specific, generally toxic only to particular insect orders (e.g., Lepidoptera, Coleoptera, Diptera), thus posing minimal risk to humans, livestock and most beneficial insects.

Enhanced Efficacy and Yield Protection : Bt crops provide season-long protection against key lepidopteran and coleopteran pests, leading to more consistent and often higher yields, especially under heavy pest pressure (Qaim and Zilberman, 2010).

Farmer Benefits : Farmers benefited from reduced input costs (fewer insecticide sprays), less exposure to chemicals and improved economic returns (Brookes and Barfoot, 2021).

The global adoption of Bt crops has been remarkable. By 2022, GM crops, predominantly Bt cotton and Bt maize, were cultivated on millions of hectares worldwide, with significant acreage in the United States, Brazil, Argentina, India and China (ISAAA, 2022). Their widespread success in controlling major pests like cotton bollworms (*Helicoverpa armigera*, *Pectinophora gossypiella*) and corn borers (*Ostrinia nubilalis*, *Diatraea grandiosella*) is well-documented.

However, the widespread and continuous selection pressure exerted by Bt crops created an inevitable evolutionary challenge: the evolution of resistance in target insect pests. Just as insects develop resistance to chemical insecticides, they possess the genetic potential to evolve resistance to Bt toxins (Tabashnik *et al.*, 2008). The high selection pressure, coupled with the potential for rapid reproduction and high genetic variability in insect populations, means that if not managed proactively, resistance can evolve swiftly, rendering Bt technology ineffective. This phenomenon, known as field-evolved resistance, represents a critical threat to the long-term sustainability of Bt crops and the substantial benefits they provide.

Understanding the molecular mechanisms by which insects circumvent Bt toxins, developing robust surveillance and diagnostic tools to detect resistance early, and implementing effective integrated mitigation strategies are paramount for prolonging the utility of this invaluable technology. This comprehensive review aims to synthesize the current knowledge on these critical aspects. We will delve into the molecular intricacies of Bt toxin mode of action and the diverse mechanisms of resistance, explore the methodologies used for monitoring resistance evolution, and critically evaluate the effectiveness of integrated resistance management strategies, illustrated by key case studies of success and failure in the field. Finally, we will discuss the challenges ahead and outline future directions to ensure the continued efficacy of Bt crops in a dynamic agricultural landscape.

Bt Toxins and their Mode of Action

Understanding the mode of action of *Bacillus thuringiensis* (Bt) toxins is fundamental to comprehending how insects evolve resistance. Bt produces a diverse array of insecticidal proteins, primarily classified into two major families: Cry (crystalline) proteins and Vip (vegetative insecticidal proteins) proteins. While both are lethal to susceptible insects, they differ significantly in their structure, secretion, and mechanism of action, which is crucial for resistance management.

Cry proteins

Cry proteins (Crystal proteins) are the most common and well-studied Bt toxins, produced during the sporulation phase of the bacterium as parasporal crystalline inclusions. These proteins are protoxins, meaning they require a series of specific events to become active and exert their insecticidal effect.

Ingestion and Solubilization

When a susceptible insect larva ingests plant tissue expressing a Bt Cry protein, the alkaline environment (pH 8-12) of the insect's midgut lumen, along with specific reducing conditions, causes the insoluble crystalline protoxin to solubilize (Bravo *et al.*, 2007). This is the first critical step; if the protoxin does not solubilize, it cannot proceed to the next steps.

Proteolytic activation

Upon solubilization, the protoxin is cleaved by specific proteases (e.g., trypsin, chymotrypsin-like enzymes) present in the insect midgut. This proteolytic processing removes N- and C-terminal sequences, releasing a smaller, biologically active toxin (the core toxin) (Oppert *et al*., 2005). The active toxin typically retains a characteristic three-domain structure (Domain I, II and III).

Domain I: Composed of seven α-helices, believed to be involved in membrane insertion and pore formation.

Domain II: Composed of three anti-parallel β-sheets, often involved in receptor binding specificity.

Domain III: Also composed of three anti-parallel β-sheets, involved in receptor binding and stabilization of the toxin structure.

Binding to Midgut Receptors

This step is highly specific and crucial for toxicity. The active Bt toxin binds to specific, high-affinity receptor proteins located on the apical brush border membrane of the midgut epithelial cells of susceptible insects (Bravo *et al.*, 2011). This binding event is often considered the primary determinant of insecticidal specificity.

Key Receptors : Several types of midgut proteins have been identified as putative or confirmed receptors for Cry toxins:

Cadherin (CAD) proteins : These are large, single-pass transmembrane proteins that have been extensively implicated as primary receptors for many Cry1A and Cry2A toxins. Binding to cadherin is thought to facilitate subsequent steps in toxin activation and membrane insertion (Vadlamudi *et al*., 1995 and Ma *et al*., 2005).

Aminopeptidase N (APN) proteins : These are glycosylphosphatidylinositol (GPI)-anchored proteins found on the midgut brush border membrane. Different APN isoforms can bind to various Cry toxins (Cry1Ac, Cry1Ab, Cry1F, Cry1C) and contribute to their toxicity (Knight *et al*., 1994).

Alkaline Phosphatase (ALP) proteins : Also GPI-anchored proteins, ALPs have been identified as receptors for some Cry toxins, particularly Cry1Ac (Ohlemeyer *et al*., 2011).

Glycolipids (e.g., glycosphingolipids, GSLs) : These can also act as receptors or co-receptors, forming part of the multi-protein receptor complex (Bravo *et al*., 2007).

Sequential Binding Model : The prevailing model suggests a sequential binding process for many Cry1A toxins, where initial binding to cadherin facilitates further proteolytic processing by secondary proteases (e.g., APN-associated proteases), leading to a modified toxin that then binds to secondary receptors like APN or ALP (Bravo *et al*., 2004). This multi-step binding enhances specificity and efficacy.

Membrane Insertion and Pore Formation

After binding to receptors, the toxin undergoes conformational changes, inserts into the midgut epithelial cell membrane and oligomerizes (forms pores) (Bravo *et al*., 2007). These pores disrupt the integrity of the cell membrane, leading to ion imbalance, loss of cell homeostasis and ultimately, osmotic lysis of the midgut cells.

Septicemia and Insect Death

The disruption of the midgut barrier allows gut contents, including symbiotic and pathogenic bacteria from the insect's midgut, to enter the hemocoel (insect body cavity), leading to septicemia and the eventual death of the insect due to starvation and systemic infection (Broderick *et al*., 2006).

Vip Proteins

Vip (Vegetative insecticidal proteins) are secreted by Bt during the vegetative growth phase, unlike Cry proteins which are produced during sporulation. Vip proteins are not crystalline and are typically secreted as soluble proteins (Estruch *et al*., 1996).

Mode of action : While less extensively studied than Cry proteins, Vip proteins are also protoxins that require proteolytic activation in the insect midgut. However, they generally bind to different receptors on the midgut epithelial cells than Cry proteins, and their mechanism of membrane insertion and pore formation may also differ. This distinct mode of action and receptor binding makes Vip proteins valuable tools for resistance management by providing a different target for pests to overcome (Chakrabarty *et al*., 2014).

Specificity : Vip proteins (e.g., Vip3A) typically have a broader spectrum of activity against lepidopteran pests than individual Cry toxins, but they retain insect specificity.

Importance in Resistance Management : The non-overlap in binding sites and mode of action between Vip and Cry proteins makes Vip proteins excellent candidates for gene pyramiding (stacking) strategies, as resistance to one type of toxin is unlikely to confer cross-resistance to the other.

Understanding these detailed modes of action is critical for developing effective resistance management

strategies, particularly by targeting different biochemical pathways or receptor binding sites to mitigate the evolution of resistance.

Evolution of Resistance to Bt Toxins

The evolution of insect resistance to Bt toxins in crops is a classic example of natural selection in action. When Bt crops are widely deployed, they exert strong selective pressure on target pest populations. Susceptible insects are killed, while rare, naturally occurring resistant individuals survive and reproduce, passing their resistance genes to the next generation. Over time, the frequency of these resistance alleles increases in the population, leading to the emergence of resistant populations that can cause significant damage to Bt crops.

Principles of Resistance evolution

Pre-existing Genetic variation : Resistance does not "appear" in response to Bt exposure. Rather, it arises from rare, naturally occurring resistance alleles already present in pest populations at very low frequencies (Gassmann *et al*., 2014). These alleles are typically recessive and may confer a fitness cost in the absence of selection pressure.

Selection Pressure : Bt crops provide continuous, high-level exposure to the toxin, acting as a powerful selective agent. Insects feeding on Bt crops either die or suffer severe fitness penalties, creating a strong advantage for individuals carrying resistance alleles.

Inheritance : For most cases of field-evolved resistance to Bt toxins, resistance is inherited as a single, recessive, or partially recessive gene (Tabashnik *et al*., 2003). This pattern of inheritance is crucial for the success of the "high-dose/refuge" strategy, as heterozygotes (carrying one resistance allele and one susceptible allele) would be killed by the high dose of toxin.

Gene Flow and Assortative Mating : The movement of resistant individuals (gene flow) and mating patterns (e.g., assortative mating where resistant individuals preferentially mate) within and between populations can influence the spread of resistance alleles.

Fitness Costs : Resistance mechanisms often impose a fitness cost on the insect in the absence of the selective agent (Bt toxin). This means resistant individuals may grow slower, have lower fecundity, or be less competitive than susceptible individuals in a non-Bt environment (Gassmann *et al*., 2009). Fitness costs are important because they can help to dilute resistance alleles if selection pressure is removed or reduced.

Factors Influencing Resistance Evolution Rate

The rate at which resistance evolves in a pest population is influenced by several biological, ecological, and operational factors.

Biological Factors of the Pest

Initial Frequency of Resistance Alleles : The rarer the resistance alleles are in the natural population, the slower resistance will evolve (Gould, 1998).

Inheritance of Resistance : Recessive resistance evolves slower than dominant resistance because heterozygous individuals are susceptible and do not contribute to resistance allele frequency increase on Bt crops.

Number of Genes conferring Resistance : If resistance is controlled by a single gene (monogenic), it can evolve faster than if it's controlled by multiple genes (polygenic), as fewer mutations are required. Most observed field resistance to single-Bt toxin crops has been monogenic and recessive.

Fitness Costs of Resistance : If resistant insects have significant fitness costs in the absence of Bt, their numbers will decline in refuge areas, slowing the overall rate of resistance evolution.

Life History Traits : Insects with short generation times, high fecundity, and high mobility tend to evolve resistance more rapidly due to faster population turnover and greater gene flow.

Ecological factors

Host Range and Alternative Hosts : Pests with broad host ranges (polyphagous) may feed on non-Bt plants, potentially diluting resistance alleles. However, if they have alternative non-Bt hosts that are also widely grown, they might not face full selection pressure on Bt crops.

Gene Flow : Migration of susceptible individuals from non-Bt areas (refuges, alternative crops, wild hosts) into Bt crop fields can dilute resistance alleles and delay resistance. Conversely, migration of resistant individuals from areas with high selection pressure can accelerate resistance spread.

Environmental factors : Environmental conditions (e.g., temperature, rainfall) can affect pest population dynamics and the efficacy of Bt toxins, indirectly influencing selection pressure.

Operational Factors of Bt Crop Deployment (Resistance Management Strategies)

Dose of Toxin : The "high-dose" component of the high-dose/refuge strategy is crucial. A high dose of Bt toxin in the plant ensures that homozygous susceptible (SS) and heterozygous (RS) individuals are killed, leaving

only rare homozygous resistant (RR) individuals to survive (Gould, 1998). If the dose is not sufficiently high (e.g., a "low dose"), heterozygous individuals can survive, accelerating resistance.

Refuge Size and Structure : The "refuge" is a critical component. A refuge is an area planted with non-Bt versions of the crop, providing a source of susceptible insects to mate with rare resistant individuals emerging from Bt fields. The size, spatial arrangement, and proximity of refuges to Bt fields significantly impact their effectiveness (Tabashnik *et al.*, 2008).

Structured refuges : Separate blocks or strips of non-Bt crop.

Unstructured Refuges : Non-Bt plants mixed within the Bt crop (e.g., refuge-in-a-bag or RIB).

Gene Pyramiding/Stacking : Deploying crops that express multiple Bt toxins targeting the same pest species but with different modes of action (i.e., different binding sites) reduces the likelihood of resistance. An insect would need to simultaneously evolve resistance to multiple distinct toxins, a much rarer event (Roush, 1998).

Integrated Pest Management (IPM) : The broader adoption of IPM practices (e.g., crop rotation, biological control, cultural practices, judicious use of conventional insecticides) alongside Bt crops can reduce overall pest pressure and potentially dilute selection for Bt resistance.

Monitoring and Compliance: Effective monitoring programs to detect early signs of resistance are crucial. Farmer compliance with refuge requirements and other management guidelines is paramount for the long-term sustainability of Bt technology (Carrière *et al.*, 2016).

Understanding and proactively managing these factors is essential for delaying the evolution and spread of Bt resistance, thus prolonging the efficacy of this valuable pest control technology. The success of Bt crops globally hinges on rigorous implementation of resistance management strategies.

Molecular Mechanisms of Bt Resistance

The evolution of insect resistance to Bt toxins is a complex phenomenon at the molecular level, primarily driven by alterations in the interaction between the toxin and its specific receptors in the insect midgut. While altered toxin binding is the most common mechanism, other pathways have also been identified or hypothesized. Understanding these mechanisms is crucial for developing effective diagnostic tools and designing new toxins or management strategies to circumvent resistance.

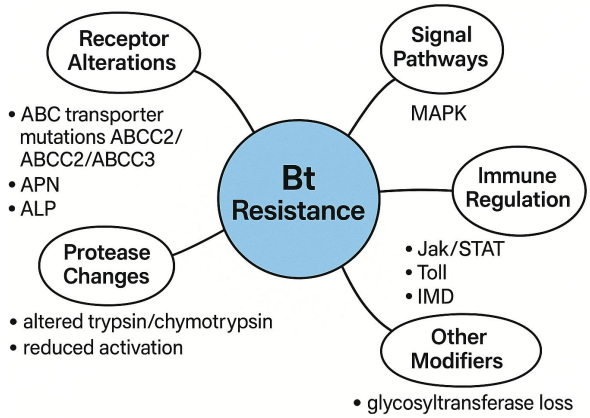

Fig. 1 : Molecular Mechanisms of Bt Resistance.

Altered Bt Toxin Binding

This is by far the most prevalent and well-characterized mechanism of resistance to Cry toxins. It involves modifications in the midgut epithelial cells that prevent or reduce the binding of the active Bt toxin to its specific receptor proteins, thereby inhibiting subsequent steps of toxicity (pore formation and cell lysis).

Mutations in Receptor Genes

Genetic mutations (e.g., deletions, insertions, point mutations) in the genes encoding key Bt toxin receptors are a primary cause of altered binding. These mutations can lead to:

Truncated or non-functional Receptors : A common mechanism is the introduction of premature stop codons or frameshift mutations, leading to the synthesis of truncated, non-functional receptor proteins that cannot bind the toxin effectively (Baxter *et al.*, 2011).

Altered Receptor Structure : Point mutations or small insertions/deletions can subtly alter the three-dimensional structure of the receptor's binding domain, reducing or eliminating the toxin's affinity for it without necessarily rendering the protein completely non-functional for its native biological role (Ahmad *et al.*, 2011).

Key Receptor Genes

Cadherin (CAD) gene : Mutations in cadherin genes have been strongly linked to high levels of resistance to Cry1Ac and Cry2Ab in several lepidopteran pests, including *Pectinophora gossypiella* (Pink Bollworm), *Helicoverpa armigera* (Cotton Bollworm) and *Heliothis virescens* (Tobacco Budworm) (Tabashnik *et al.*, 2013). For instance, in *P. gossypiella*, a 12-bp deletion in the cadherin gene (r1 allele) results in a truncated protein that fails to bind Cry1Ac, conferring high resistance.

Aminopeptidase N (APN) gene : Mutations or reduced expression of APN proteins have been implicated

in resistance to various Cry toxins (e.g., Cry1Ab, Cry1Ac, Cry1Fa) in insects like *Plutella xylostella* (Diamondback Moth) and *Spodoptera frugiperda* (Fall Armyworm) (Bautista *et al.*, 2009; Wang *et al.*, 2017). These mutations can affect glycosylation sites or specific binding regions.

Alkaline Phosphatase (ALP) gene : Downregulation or mutations in ALP genes have also been associated with resistance in some cases, though less frequently than CAD or APN (Ohlemeyer *et al.*, 2011).

Reduced Expression of Receptor Genes (Downregulation)

Instead of direct mutations, some insects evolve resistance by reducing the expression levels of receptor genes. This means fewer receptor proteins are produced on the midgut membrane, leading to fewer binding sites for the Bt toxin (Gahan *et al.*, 2001).

Mechanism : This can be due to mutations in regulatory regions of the gene (e.g., promoter), epigenetic modifications, or altered mRNA stability.

Example : In some Cry1Ac resistant strains of *Helicoverpa armigera*, reduced expression of cadherin and APN has been observed without clear structural mutations in the coding regions, suggesting a regulatory mechanism (Rajagopal *et al.*, 2010).

Masking of Binding Sites

This mechanism involves the production of other molecules in the midgut lumen or on the brush border membrane that bind to the Bt toxin, preventing it from interacting with its intended receptors (Jurat-Fuentes *et al.*, 2002).

Example : Specific carbohydrates or mucins in the gut could potentially sequester the toxin. While conceptually plausible, strong experimental evidence for this as a primary resistance mechanism in field-evolved cases is less common compared to altered receptor binding.

Impaired Toxin Activation (Proteolytic Processing)

As Bt protoxins require proteolytic cleavage to become active toxins, modifications in the insect's midgut proteases can theoretically lead to resistance.

Altered Protease activity : If the insect's midgut proteases are modified in terms of their quantity, specificity, or activity, they might fail to properly cleave the protoxin into its active form (Oppert *et al.*, 2005).

Example : Some laboratory-selected strains of *Spodoptera exigua* showed reduced trypsin-like activity or altered protease profiles, leading to inefficient processing of Cry1Ac (Li *et al.*, 2004). However, this mechanism is less commonly observed in field-evolved resistance, possibly because it might also impact the insect's normal digestive processes, incurring a fitness cost.

Enhanced Detoxification

This mechanism involves the insect breaking down or sequestering the active Bt toxin before it can reach its target receptors or exert its toxic effect.

Increased Detoxification Enzymes : Overexpression or altered activity of enzymes such as esterases, cytochrome P450 monooxygenases (P450s), or glutathione S-transferases (GSTs), commonly associated with chemical insecticide resistance, could potentially detoxify Bt toxins (Tang *et al.*, 2015).

Example : While enhanced detoxification is a major mechanism for chemical insecticide resistance, compelling evidence for it as a primary or major mechanism for field-evolved Bt resistance is limited. Some studies have suggested its potential involvement, particularly in some laboratory-selected strains or in combination with other mechanisms.

Altered Signal Transduction

After toxin binding and pore formation, a cascade of intracellular events typically leads to cell lysis. If the insect can alter these downstream signaling pathways, it might survive toxin exposure.

Changes in Intracellular Pathways : This could involve modifications in ion channels, cellular repair mechanisms, or stress response pathways that enable the cell to tolerate the membrane disruption caused by the toxin (Soberón *et al.*, 2009).

Example : This mechanism is largely hypothetical and difficult to study. Current research indicates that cell death initiated by Bt toxins is quite rapid and overwhelming, making it challenging for insects to evolve robust resistance through altered downstream signaling alone.

Genetic Basis of Resistance

The genetic basis of resistance is fundamental for resistance management.

Monogenic vs. Polygenic : Most documented cases of high-level field-evolved resistance to a single Bt toxin (e.g., Cry1Ac) have been linked to a single, major gene mutation with recessive or partially recessive inheritance (Tabashnik *et al.*, 2013). This fits the predictions of the high-dose/refuge strategy. However,

low-level or complex resistance, especially to pyramided toxins or new toxin types, might involve multiple genes (polygenic inheritance).

Recessive Inheritance : The recessive nature of most high-level resistance alleles (meaning only homozygous resistant individuals survive a high dose of toxin) is critical for the success of the refuge strategy. This ensures that susceptible individuals emerging from the refuge can mate with resistant individuals from the Bt crop, producing susceptible heterozygote offspring, thereby diluting the resistance alleles in the population.

In summary, altered toxin binding due to mutations in receptor genes (especially cadherin and APN) or reduced expression of these receptors is the most significant and well-established molecular mechanism of field-evolved Bt resistance. Understanding these mechanisms at a fine molecular scale is crucial for developing robust surveillance diagnostics and informing the design of next-generation Bt toxins and resistance management strategies.

Surveillance and Diagnostics for Bt Resistance

Effective surveillance and robust diagnostic tools are indispensable components of any successful Insect Resistance Management (IRM) program for Bt crops. Early detection of resistance is paramount to enable timely implementation of mitigation strategies, thereby protecting the long-term efficacy of Bt technology. Surveillance involves systematically monitoring pest populations for signs of evolving resistance, while diagnostics provide the tools to confirm and characterize resistance.

Importance of Surveillance

Early Warning System : Surveillance acts as an early warning system, detecting incipient resistance before it causes significant crop damage (Storer *et al*., 2010). This allows managers to intervene proactively rather than reactively.

Informing Management Decisions : Data from surveillance informs decisions on whether to adjust refuge strategies, deploy new Bt traits, or implement additional IPM tactics.

Assessing IRM effectiveness : Continuous monitoring helps assess the effectiveness of deployed IRM strategies (e.g., refuge compliance, gene pyramiding efficacy) and identify areas where improvements are needed.

Understanding Resistance Dynamics : Long-term surveillance data provides crucial insights into the spatial and temporal patterns of resistance evolution, informing research on resistance mechanisms and population genetics.

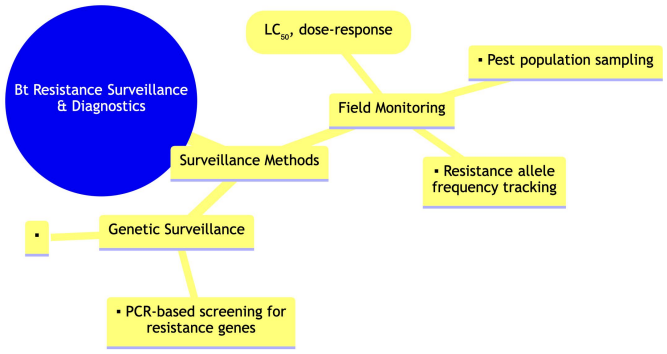

Fig. 2 : Surveillance and Diagnostics for Bt Resistance.

Bioassays (Phenotypic Diagnostics)

Bioassays are the gold standard for directly measuring the phenotypic response of insect populations to Bt toxins. They involve exposing insects to varying concentrations of toxins and observing mortality or growth inhibition.

Diagnostic Dose (or Discriminating Concentration) assays

Principle : These assays use a single, high concentration of Bt toxin (the diagnostic dose) that is expected to kill 99-100% of susceptible individuals, but allows resistant individuals to survive (Andow and Miller, 2012).

Procedure : Field-collected insects (usually larvae) are exposed to Bt toxin via diet overlay, diet incorporation, or direct topical application. A control group is exposed to untreated diet. Mortality is recorded after a defined period.

Interpretation : Survival at the diagnostic dose indicates the presence of resistant individuals. The frequency of survivors at this dose provides an estimate of the frequency of resistance alleles in the population, assuming resistance is recessive and the dose is high enough to kill heterozygotes.

Strengths

Direct Measure of Resistance : Provides a direct measure of an insect's ability to survive Bt toxin exposure in a standardized laboratory setting.

Broad applicability : Can be applied to any pest-Bt toxin combination.

Estimation of Allele frequency : When calibrated with known susceptible and resistant strains, it can estimate resistance allele frequency, crucial for IRM models.

Table 1 : Molecular Mechanisms of Bt Resistance in Insect Pests.

Mechanism Category	Specific Mechanism	Description	Example (Pest & Bt Toxin)	Impact on Bt Toxicity	Diagnostic Approach
Altered Toxin Binding (Most Common)	**Mutation in Receptor Gene**	Genetic changes (e.g., deletions, insertions, point mutations) in genes encoding midgut receptors (Cadherin, APN, ALP) leading to truncated or structurally altered proteins.	*Pectinophora gossypiella* to Cry1Ac (Cadherin deletion) *Helicoverpa armigera* to Cry1Ac (Cadherin point mutations) *Plutella xylostella* to Cry1Ab/Ac (APN mutations)	Toxin cannot bind or binds with reduced affinity to midgut cell membranes, preventing pore formation.	DNA sequencing of receptor genes; allele-specific PCR.
	Reduced Receptor Expression (Down-regulation)	Decreased production of receptor proteins on the midgut membrane, due to regulatory mutations or epigenetic changes.	*Helicoverpa armigera* to Cry1Ac (Cadherin/APN downregulation observed in some strains)	Fewer binding sites available, reducing the effective concentration of bound toxin.	Gene expression analysis (qPCR) of receptor genes; Western blot for protein levels.
	Masking of Binding Sites	Production of other molecules (e.g., carbohydrates, mucins) that bind to the toxin, physically preventing it from reaching its target receptors.	Hypothetical for most field cases; some lab evidence suggests mucins might interfere.	Toxin is sequestered or neutralized before binding to its specific receptor.	Biochemical assays for toxin-binding inhibitors.
Impaired Toxin Activation	**Altered Proteolytic Processing**	Modifications in midgut proteases that lead to inefficient or incorrect cleavage of the Bt protoxin into its active form.	*Spodoptera exigua* to Cry1Ac (Lab-selected, reduced trypsin activity)	Protoxin is not converted into the active, pore-forming toxin.	Protease activity assays; sequencing of protease genes.
Enhanced Detoxification	**Increased Detoxifying Enzymes**	Overexpression or altered activity of general detoxifying enzymes (e.g., P450s, esterases, GSTs) that could break down or modify the Bt toxin.	Limited field evidence; some suggestions in lab-selected strains.	Toxin is chemically modified or degraded before reaching its target or acting.	Enzyme activity assays; gene expression of detoxification genes.
Altered Signal Transduction	**Modifications in Downstream Pathways**	Changes in cellular pathways that allow midgut cells to tolerate the initial membrane damage caused by toxin insertion.	Largely hypothetical/poorly understood in field resistance.	Cell is able to repair or compensate for membrane damage, preventing lysis.	Cell biology experiments; proteomics/metabolomics.

Limitations

Labor and Time Intensive : Requires rearing insects, preparing diets, and meticulous observation, making it time-consuming and labor-intensive, especially for large-scale surveillance.

Sensitivity : May not detect very low frequencies of resistance alleles accurately.

Environmental Variability : Results can be influenced by environmental conditions during assays.

"Survivors" vs. Resistant Individuals : Survival might be due to factors other than true genetic resistance if the dose is not truly diagnostic or if the insect is unhealthy.

Fitness Costs : The assays don't account for fitness costs of resistance in a field setting.

Concentration-Response Assays (Dose-Response Assays)

Principle : Involves exposing insect populations to a range of toxin concentrations to determine a dose-mortality curve (LC_{50} or EC_{50}, the lethal or effective concentration that kills/affects 50% of the population).

Procedure : Similar to diagnostic dose assays but uses multiple toxin concentrations.

Interpretation : A shift in the LC50 value to a higher concentration compared to a known susceptible reference strain indicates a decrease in susceptibility or an increase in resistance (Tabashnik *et al*., 2013). The slope of the dose-response curve can also provide insights.

Strengths : Provides a quantitative measure of the magnitude of resistance.

Limitations : Even more labor and time intensive than diagnostic dose assays, making it impractical for routine, large-scale surveillance. Usually reserved for confirming resistance in field-evolved populations.

Molecular Diagnostic Tools (Genotypic Diagnostics)

Molecular tools provide rapid, high-throughput, and increasingly cost-effective methods for detecting and monitoring specific resistance alleles, especially when the underlying molecular mechanisms are known.

PCR-based Assays (e.g., Allele-Specific PCR, qPCR)

Principle : Designed to detect specific DNA mutations (e.g., single nucleotide polymorphisms - SNPs, small deletions/insertions) in target receptor genes known to confer resistance.

Procedure : Uses specific primers to amplify DNA fragments containing the mutation. Allele-specific PCR (AS-PCR) uses primers that specifically bind to either the susceptible or resistant allele. Quantitative PCR (qPCR) can measure the frequency of alleles or the expression levels of genes.

Strengths

High throughput : Can screen many individual insects rapidly.

Cost-effective : Generally less expensive than full sequencing for routine screening.

Early detection : Can detect resistance alleles at very low frequencies, even before phenotypic resistance is observed in bioassays.

Non-destructive (sometimes) : Can be performed on small tissue samples, allowing the insect to be reared further.

Limitations

Requires Known Mutations : Only works if the specific molecular resistance mutations are already identified and validated. If resistance evolves through novel mutations or different mechanisms, these assays will not detect them.

Cross-resistance : Cannot assess cross-resistance to other Bt toxins unless specifically designed.

Cannot Detect all Mechanisms : Cannot directly detect mechanisms like altered protease activity or general detoxification without specific assay development.

DNA Sequencing

Principle : Direct sequencing of candidate receptor genes (e.g., cadherin, APN, ALP) to identify novel mutations or confirm known resistance alleles.

Procedure : Genomic DNA is extracted from individual insects, and target genes are amplified by PCR and then sequenced. Next-generation sequencing (NGS) allows for sequencing of many individuals or multiple genes simultaneously.

Strengths

Discovery of Novel Mutations : Essential for identifying new resistance alleles or mechanisms when resistance is detected phenotypically but the molecular basis is unknown.

Comprehensive : Provides full sequence information, allowing for precise characterization of mutations.

Limitations

High Cost and Time : Historically expensive and time-consuming, though NGS is reducing this.

Bioinformatics Expertise : Requires significant bioinformatics resources for data analysis.

Cannot Assess Expression levels : Only provides genetic information, not information on gene expression or protein levels.

Gene Expression Analysis (e.g., Quantitative PCR - qPCR, RNA-Seq)

Principle : Measures the abundance of mRNA transcripts of specific receptor genes or other genes involved in Bt toxin processing or detoxification.

Procedure : RNA is extracted from insect midguts, converted to cDNA, and then quantified using qPCR or subjected to RNA sequencing (RNA-Seq).

Strengths

Detects Downregulation : Directly detects resistance mechanisms involving reduced gene expression (e.g., downregulation of cadherin or APN).

Identifies Novel Regulatory Mechanisms : Can potentially identify genes involved in novel resistance pathways or compensatory responses.

Limitations

Tissue Specificity : Requires specific tissues (midgut).

Environmental Variability : Gene expression can be highly dynamic and influenced by environmental factors.

Requires Reference Genes : Accurate quantification relies on stable reference genes.

Protein-based Assays (e.g., ELISA, Western Blot, Ligand Blots)

Principle : Detects changes in the quantity or quality of receptor proteins directly (e.g., Western blot), or assesses the ability of Bt toxins to bind to midgut proteins (e.g., ligand blot, ELISA) (Bravo *et al*., 2011).

Strengths : Provides direct evidence of altered protein levels or binding efficacy.

Limitations : More labor-intensive, requires specific antibodies or purified toxins, and often requires larger amounts of insect tissue. Less suitable for high-throughput screening compared to PCR-based methods.

Population Genetics approaches

Population Structure and Gene Flow : Using molecular markers (e.g., microsatellites, SNPs) to understand gene flow patterns between pest populations in Bt and non-Bt areas. High gene flow of susceptible individuals from refuges is critical for resistance management.

Resistance Allele Frequency Monitoring : Combining bioassay data with molecular diagnostic tools to track the frequency of resistance alleles over time in target pest populations. Models predict that if the frequency of resistance alleles exceeds a certain threshold (e.g., 0.01-0.05), field-evolved resistance may be imminent or already occurring at low levels (Storer *et al*., 2010).

The integration of both phenotypic (bioassay) and genotypic (molecular) diagnostic approaches is essential for a comprehensive and robust Bt resistance surveillance program. Bioassays provide the ultimate confirmation of functional resistance, while molecular tools offer high-throughput, early detection, and mechanistic insights, enabling more proactive and adaptive resistance management strategies.

Integrated Mitigation Strategies for Bt Resistance

To ensure the long-term sustainability of Bt crop technology, rigorous and integrated resistance management strategies are essential. These strategies aim to delay the evolution of resistance by reducing selection pressure and promoting the survival of susceptible individuals.

The High-Dose/Refuge Strategy

This is the cornerstone of Bt resistance management globally, particularly for Lepidopteran pests, and its success hinges on specific biological assumptions and strict implementation (Gould, 1998; Tabashnik *et al*., 2008).

High-Dose Component

Principle : The Bt crop is engineered to produce a concentration of Bt toxin high enough to kill not only homozygous susceptible (SS) insects but also heterozygous (RS) insects (those carrying one resistance allele and one susceptible allele). This means only very rare homozygous resistant (RR) individuals can survive on the Bt crop.

Importance : If the dose is not "high," heterozygous individuals (RS) can survive and reproduce, rapidly increasing the frequency of resistance alleles. This is considered "low-dose" or "intermediate-dose" selection, which significantly accelerates resistance evolution.

Implication for Resistance Genetics : Assumes that field-evolved resistance is controlled by a single, highly

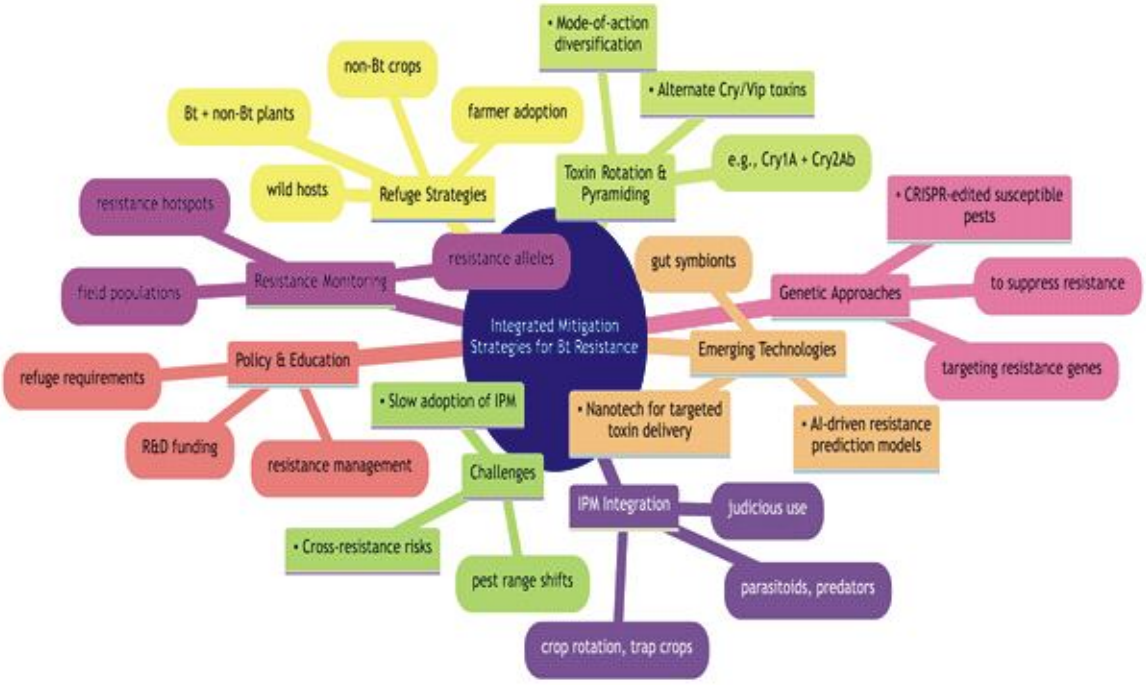

Fig. 3 : Integrated Mitigation Strategies for Bt Resistance.

recessive gene. If resistance were dominant or polygenic, the high-dose strategy would be less effective.

Refuge Component

Principle : An area of non-Bt plants (refuge) is planted alongside or within the Bt crop. This refuge serves as a source of susceptible insects that are not exposed to the Bt toxin and therefore do not develop resistance.

Purpose : The large population of susceptible insects produced in the refuge is intended to mate with the rare resistant insects emerging from the Bt crop. Since resistance is typically recessive, the offspring of a resistant (RR) x susceptible (SS) mating will be heterozygous (RS) and, crucially, killed by the high dose of toxin in the Bt crop. This dilutes the resistance alleles in the overall pest population.

Refuge Configurations

Structured Refuges : These are separate blocks or strips of non-Bt crop planted adjacent to or within a defined distance from the Bt field. The size and proximity of the refuge are critical, typically 20% or more of the total acreage for lepidopteran pests (Tabashnik *et al.*, 2008).

Unstructured Refuges (Refuge-in-a-Bag - RIB) : For some crops (e.g., maize), non-Bt seeds are physically mixed with Bt seeds in the seed bag at a specific ratio (e.g., 5% to 10% non-Bt seeds). This ensures that every Bt field contains a small percentage of non-Bt plants, providing refuge on an individual plant basis.

Advantages of RIB : Simplifies farmer compliance, as separate planting of refuges is not required. Ensures closer proximity of susceptible and resistant insects for mating.

Disadvantages of RIB : May not provide sufficient refuge for highly mobile pests, or if pest pressure on non-Bt plants is too low.

Refuge compliance : Farmer compliance with refuge requirements is absolutely critical for the success of this strategy. Poor compliance (e.g., planting too small a refuge, treating the refuge with insecticides that kill susceptible insects) is a major reason for accelerated resistance evolution in some regions.

Gene Pyramiding (Bt Toxin Stacking)

Gene pyramiding, also known as gene stacking, involves engineering Bt crops to express two or more different Bt toxins that target the same pest species but have independent modes of action (i.e., bind to different receptors or act via different biochemical pathways) (Roush, 1998).

Principle : For an insect to evolve resistance to a pyramided Bt crop, it would need to simultaneously evolve resistance to all component toxins. If the mechanisms of resistance to each toxin are independent, the probability of an insect having all the necessary resistance alleles is extremely low (the product of individual resistance allele

Table 2 : Key Strategies for delaying Bt Resistance and their Mechanisms.

Strategy	Mechanism of Action	Target Pest Resistance Phase	Advantages	Challenges
High-Dose/Refuge (HDR)	High Dose: Kills SS and RS genotypes, leaving only rare RR individuals on Bt crop. Refuge: Produces abundant susceptible (SS) moths/insects to mate with rare RR survivors from Bt field. Offspring (RS) are then killed on Bt.	Delays initial increase in R allele frequency; dilutes R alleles through mating with susceptible insects.	Proven effective for recessive resistance; environmentally friendly by reducing pesticide use.	Farmer compliance (refuge planting/management); susceptibility of R allele to high dose.
Gene Pyramiding (Stacking)	Crops express ed2 Bt toxins with different modes of action. Insect needs to develop resistance to *multiple* toxins simultaneously, which is highly improbable.	Delays onset of resistance by requiring multiple independent resistance mutations.	Significantly increases durability of Bt resistance management; broadens pest spectrum.	Higher cost of seed; potential for some cross-resistance; finding multiple toxins with independent modes of action.
Integrated Pest Management (IPM)	Crop Rotation: Breaks pest life cycles, reduces pest density. Biological Control: Natural enemies suppress pest populations (both in Bt and refuge). Cultural Practices: Reduce initial pest pressure. Judicious Chemical Use: Reduces overall selection pressure and non-target effects.	Reduces overall selection pressure for Bt resistance; enhances ecosystem resilience; diversifies pest control tactics.	Sustainable and environmentally friendly; improves overall pest control efficacy.	Requires integrated knowledge; farmer adoption varies; potential for conflicts with specific Bt strategies (e.g., insecticide use on refuge).
Novel Bt Toxins/ Next-Gen Technologies (e.g., RNAi)	Provides new modes of action/targets for pests, circumventing existing resistance mechanisms.	Offers solutions when current toxins face resistance; provides new options for stacking.	Maintains efficacy against resistant pests; broadens spectrum of control.	Time and cost of R&D; regulatory hurdles; potential for new resistance mechanisms to evolve; public acceptance of new technologies.

SS = Homozygous Susceptible; **RS** = Heterozygous Resistant; **RR** = Homozygous Resistant.

frequencies).

Mechanism : If an insect develops resistance to Toxin A (e.g., by mutating its receptor), it will still be susceptible to Toxin B (which binds to a different receptor), and thus killed by the pyramided crop.

Importance : Pyramided Bt crops significantly increase the durability of Bt technology compared to single-gene Bt crops, as the selection pressure for resistance to multiple toxins simultaneously is greatly reduced.

Deployment : Most newly developed Bt crops are pyramided traits (e.g., SmartStax maize contains multiple Cry and Vip proteins for both lepidopteran and coleopteran pests).

Refuge for Pyramided Crops : Even with pyramided crops, a refuge strategy is still necessary, though often at a reduced percentage compared to single-gene Bt crops (e.g., 5% non-Bt maize for SmartStax in some regions) (Carrière *et al*., 2016). The high dose ensures the rare double-resistant individuals are selected against, and the refuge provides susceptible mates.

Integrated Pest Management (IPM) Principles in Bt Systems

Integrating Bt crops into broader IPM frameworks can further delay resistance by reducing overall pest pressure and diversifying management tactics.

Crop Rotation : Rotating Bt crops with non-host crops or non-Bt varieties of the same crop can break pest life cycles and reduce pest population sizes,

thereby diluting selection pressure for Bt resistance (Storer *et al*., 2010).

Crop Diversification : Increasing crop diversity at the landscape level can enhance natural enemy populations and dilute pest pressure across the agroecosystem.

Biological control : Conserving and enhancing natural enemies (predators, parasitoids, entomopathogens) can help suppress pest populations on both Bt and non-Bt refuge plants, contributing to overall pest management and potentially diluting selection for resistance (Romeis *et al*., 2006). Bt crops are generally compatible with beneficial insects, unlike broad-spectrum insecticides.

Cultural Practices : Practices such as optimal planting dates, destruction of crop residues, and proper sanitation can reduce early-season pest populations, thereby reducing overall selection pressure.

Judicious Chemical control : If chemical insecticides are used in Bt crop systems, they should be applied judiciously, strategically (only when thresholds are met), and with careful consideration of their impact on natural enemies and the development of insecticide resistance (which can interact with Bt resistance). Avoiding insecticide sprays on refuges is critical.

Novel Bt Toxins and Next-Generation Technologies

Continuous discovery and development of new Bt toxins and alternative pest control technologies are crucial for staying ahead of resistance evolution.

New Cry and Vip Toxins : Identifying and characterizing novel Cry and Vip proteins with different modes of action and receptor binding sites is an ongoing effort. These new toxins can be stacked with existing ones or deployed in rotation.

Bt-Hybrid Toxins : Engineering hybrid toxins by combining domains from different Bt proteins to create novel toxins with altered binding specificities or enhanced toxicity (Bravo *et al*., 2013).

RNA Interference (RNAi) : RNAi-based pesticides or GM crops engineered to express RNAi constructs can target essential insect genes, leading to their suppression and insect mortality (Baum *et al*., 2007). This offers a novel mode of action distinct from Bt proteins, providing a valuable tool for resistance management.

Gene Drives : While highly controversial and in early research stages, gene drive technologies could potentially spread resistance-breaking genes or genes that reduce pest fertility through insect populations.

Policy and Regulatory Frameworks

Effective resistance management requires robust policy and regulatory support.

Mandatory Refuge Requirements : Regulatory agencies (e.g., EPA in the US) mandate specific refuge requirements for Bt crops, and these are enforced through various compliance measures (e.g., grower agreements, seed sales tracking).

Monitoring Programs : Support for national and regional resistance monitoring programs is critical for detecting resistance early and informing adaptive management strategies.

Adaptive Management : Regulatory frameworks should be flexible enough to allow for adaptive management strategies, where IRM plans can be modified based on new scientific data on resistance evolution or pest dynamics.

Farmer Education and Extension : Continuous education and extension efforts are necessary to ensure farmers understand the importance of IRM strategies and comply with refuge requirements.

By integrating these strategies, particularly the high-dose/refuge approach and gene pyramiding, with broader IPM principles and by investing in novel technologies and supportive policies, the agricultural community can significantly delay the evolution of resistance and extend the useful life of Bt crop technology for sustainable pest management.

Case Studies of Field-Evolved Bt Resistance

The history of Bt crop deployment provides valuable real-world examples of resistance evolution and the effectiveness, or failure, of resistance management strategies. These case studies highlight the interplay of biological, ecological and operational factors.

Success : Pink Bollworm (*Pectinophora gossypiella*) to Cry1Ac in the United States

The management of *Pectinophora gossypiella* (Pink Bollworm - PBW) resistance to Bt cotton (Bollgard I, expressing Cry1Ac) in the United States is widely regarded as a triumph of proactive resistance management (Tabashnik *et al*., 2010).

Pest and Crop : Pink Bollworm is a highly destructive pest of cotton. Bollgard I (Cry1Ac) cotton was introduced in the US in 1996.

IRM Strategy : A strict high-dose/refuge strategy was implemented, primarily in the arid southwestern US (Arizona, parts of California, Texas), where PBW populations were largely isolated from non-Bt cotton growing regions.

High Dose : Bollgard I cotton expressed Cry1Ac at a dose lethal to both SS and RS individuals.

Refuge : Farmers were mandated to plant 20% non-Bt cotton as a structured refuge (separate fields or strips). Importantly, the refuge cotton was not sprayed with insecticides active against PBW.

Biological Traits : PBW has a high initial frequency of resistance alleles to Cry1Ac (~10–10 to 10–6) and resistance is primarily monogenic and recessive.

Outcome : After over two decades of widespread cultivation (1996-2017), there was no documented field-evolved resistance of PBW to Cry1Ac in the US. Bioassay data consistently showed susceptibility, and molecular diagnostics (tracking a key cadherin mutation r1) confirmed that resistance allele frequencies remained extremely low (below 0.005) (Tabashnik *et al*., 2013). This remarkable success is attributed to several factors:

High-dose Efficacy : The Cry1Ac dose in Bollgard I was truly high against PBW.

Recessive Resistance : The recessive nature of the r1 cadherin mutation meant that heterozygous individuals (produced by RR x SS matings from the refuge) were killed on Bt cotton, effectively diluting resistance.

Effective Refuge Compliance : Farmers in the arid Southwest had high compliance with refuge planting requirements due to strong extension efforts, mandatory compliance checks and a clear understanding of the technology's benefits.

Geographical Isolation : The arid environment limited movement of PBW from other regions, facilitating contained resistance management.

Eradication : The successful suppression of PBW populations by Bt cotton, combined with other control methods, led to its eradication from commercial cotton in the US Southwest by 2017, further validating the strategy's effectiveness (Tabashnik *et al*., 2018).

Challenge : Cotton Bollworm (*Helicoverpa armigera*) to Cry1Ac in China and India

The story of *H. armigera* resistance to Cry1Ac cotton in China and India presents a contrasting picture, highlighting the difficulties of resistance management under different socio-economic and ecological contexts.

Pest and Crop : *Helicoverpa armigera* is a polyphagous and highly migratory global pest. Bt cotton (expressing Cry1Ac) was widely adopted in China from 1997 and in India from 2002.

IRM Strategy : Initially, high-dose/refuge strategies were recommended, but implementation varied significantly.

China : Small-scale farms, diverse cropping systems, and initial lack of stringent refuge compliance led to varied selection pressure. Farmers often intercropped Bt cotton with non-Bt crops (e.g., maize, groundnut) that could serve as "unstructured refuges" (Wu *et al*., 2008). However, this was often insufficient or uncontrolled.

India : Bt cotton adoption was very rapid and widespread. Initial refuge recommendations were for 20% unstructured refuge or 5% structured refuge, but farmer compliance was often low due to lack of awareness, economic pressures, and limited enforcement (Mohan and Gujar, 2010). Farmers often sprayed refuges with insecticides.

Outcome

China : Field-evolved resistance to Cry1Ac in *H. armigera* was detected within a few years of Bt cotton adoption. Studies showed significant reductions in susceptibility and increases in resistance allele frequencies (e.g., over 0.5 in some regions by 2005) (Wang *et al*., 2011). This was primarily linked to mutations in cadherin receptors. However, the polyphagous nature of *H. armigera* and its feeding on non-Bt alternative crops (e.g., maize, soybean, vegetables) in diverse agricultural landscapes might have partially mitigated resistance development by providing natural refugia and dilution (Wu *et al*, 2008).

India : Widespread and rapid evolution of resistance to Cry1Ac in *H. armigera* was observed, leading to control failures in some regions by the mid-2000s (Mohan and Gujar, 2010). This was largely attributed to very high selection pressure (due to massive adoption rates), poor refuge compliance and spraying of non-Bt refuges. The move to pyramided Bt cotton (e.g., Bollgard II, expressing Cry1Ac and Cry2Ab) was critical in restoring efficacy against *H. armigera* (Santhy *et al*., 2018).

Lessons learned : The differing outcomes highlight the critical importance of robust and mandatory refuge strategies, strong extension services, farmer education, and adapted IRM plans that consider the specific biology of the pest and the local agricultural context. The polyphagous nature of *H. armigera* provided some "natural refuge," but it was often insufficient to prevent resistance without structured management.

Challenge: Western Corn Rootworm (*Diabrotica virgifera virgifera*) to Cry3Bb1, mCry3A, and eCry3.1Ab in the US

The Western Corn Rootworm (WCR) is a major pest of maize in the US. Its evolution of resistance to several

Bt toxins targeting Coleoptera presents a significant challenge.

Pest and Crop : WCR is primarily a maize root feeder. Bt maize lines expressing Cry3Bb1, mCry3A (MON863) and eCry3.1Ab (DAS59122-7) were widely adopted in the US Corn Belt from the early 2000s.

IRM Strategy : The recommended strategy for WCR was a high-dose/refuge approach, with a 5% structured refuge or refuge-in-a-bag (RIB). However, the "high-dose" assumption for some Cry3 toxins against WCR was later questioned for specific populations, and compliance with refuges was often low, particularly for structured refuges.

Outcome : Field-evolved resistance of WCR was documented for Cry3Bb1 in Iowa in 2009, subsequently to mCry3A, and later to eCry3.1Ab (Gassmann *et al*., 2014; Wangila *et al*., 2015). This resistance was characterized by increased root damage in Bt fields.

Mechanism : While not fully elucidated, resistance to Cry3 toxins in WCR appears to be complex, potentially involving multiple genes (polygenic) and mechanisms beyond simple receptor binding (Frank *et al*., 2017). This contrasts with the simpler recessive, monogenic resistance seen in many lepidopterans.

Factors Contributing to Resistance

Lack of True High-Dose : For some WCR populations, the dose of Cry3 toxins might not have been truly high enough to kill heterozygotes, leading to faster resistance evolution.

Poor Refuge Compliance : Low farmer compliance with refuge planting was a significant factor, especially before RIB became widely available.

Monoculture : Continuous maize monoculture provided constant selection pressure.

Rapid Adaptation : WCR has shown a remarkable ability to adapt, including evolving a variant that lays eggs in soybean fields to circumvent maize rotation.

Lessons learned : Resistance in WCR highlighted that the simple high-dose/refuge model (developed for lepidopterans with recessive, monogenic resistance) might not be universally applicable, especially for coleopteran pests with potentially polygenic or complex resistance mechanisms. It underscored the need for robust dose validation, strong compliance, and diversified management tactics beyond just Bt.

Emerging Challenge: Fall Armyworm (*Spodoptera frugiperda*) to Various Cry Toxins in the Americas

The Fall Armyworm (FAW) is a highly polyphagous pest that has rapidly evolved resistance to multiple Bt toxins in its native range in the Americas.

Pest and Crop : FAW is a major pest of maize, cotton, and other crops. Various Bt maize and cotton traits expressing Cry1A.105, Cry1F, Cry2Ab2, Vip3A, and combinations thereof have been deployed.

IRM Strategy : High-dose/refuge strategies are recommended, often with RIB (Refuge-in-a-Bag).

Outcome : Rapid field-evolved resistance to Cry1F was reported in Puerto Rico by 2008 and in Brazil by 2014 (Storer *et al*., 2012 and Farias *et al*., 2014). Resistance to Cry1A.105 was also documented. More recently, there are increasing reports of resistance to pyramided traits and even to Vip3A in some areas (Omoto *et al*., 2019).

Factors Contributing to Resistance

High selection Pressure : Continuous cultivation of Bt maize and cotton over large areas.

Polyphagous Nature : FAW feeds on many non-Bt hosts (e.g., wild grasses, sorghum), which can act as uncontrolled refugia, but this can also dilute selection pressure if the "natural refuge" is extensive and diverse. However, if Bt crops become a primary host, resistance can still accelerate.

Poor Refuge Compliance : As with *H. armigera* in some regions, inconsistent refuge compliance contributes to resistance.

Rapid Generation Time : FAW has a short generation time and high fecundity, accelerating resistance evolution.

Mechanism : Resistance appears to involve multiple mechanisms, including altered binding to APN and ABC transporters and potentially complex polygenic inheritance (Wang *et al*., 2017).

Lessons Learned : FAW's rapid resistance evolution highlights the challenge of managing highly adaptable, polyphagous pests even with pyramided traits. It underscores the critical need for regional resistance management plans, effective monitoring and perhaps novel control tactics, especially as FAW rapidly spreads globally and encounters new Bt traits.

These case studies provide crucial insights into the complexities of Bt resistance evolution. They demonstrate that while a well-executed high-dose/refuge strategy can be highly effective (PBW in US), failures often arise from a combination of biological factors (pest biology, complex resistance mechanisms), ecological factors (gene flow, alternative hosts) and operational factors (poor

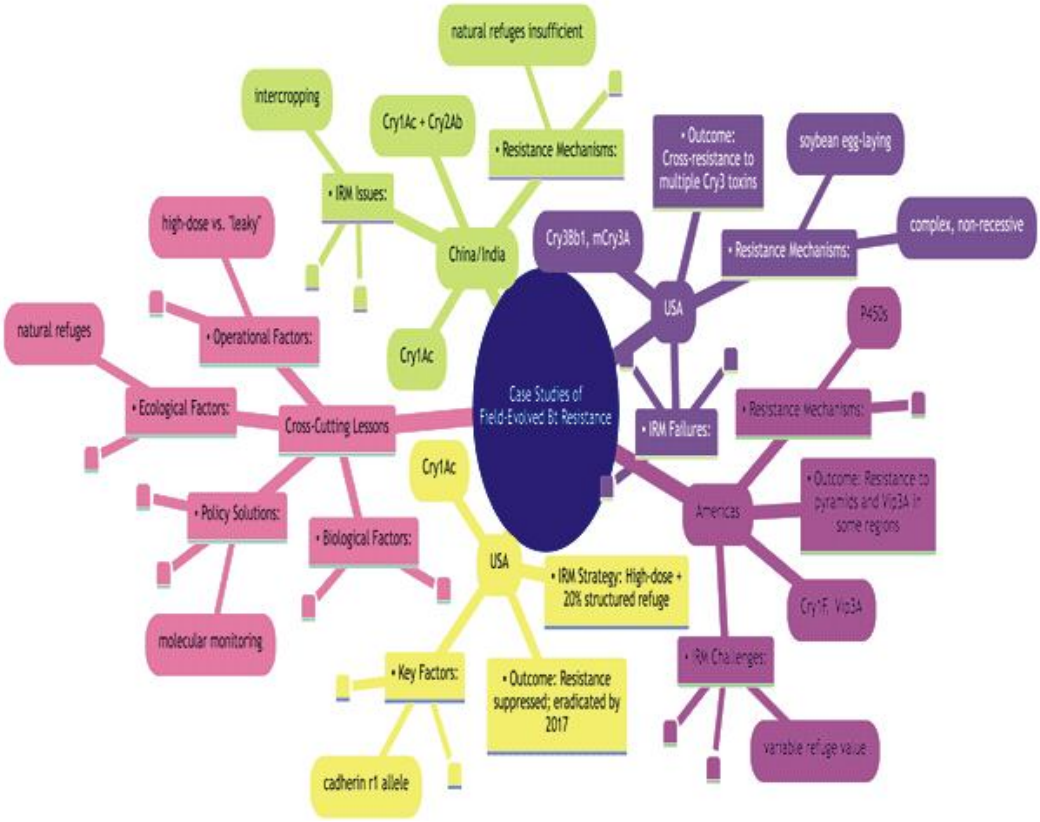

Fig. 4 : Case studies of Field-Evolved Bt Resistance.

refuge compliance, insufficient dose, lack of diversified IPM). Continued vigilance, adaptive management and investment in new technologies are essential.

Challenges and Future Perspectives

Despite the successes and advancements in understanding and managing Bt resistance, several significant challenges persist, demanding ongoing research, innovation, and adaptive strategies to ensure the long-term sustainability of this vital technology.

Challenges in Resistance Management

Managing Multiple Resistance Mechanisms and Complex Genetics

Polygenic Resistance : While most high-level resistance to single Bt toxins has been monogenic and recessive, resistance to pyramided toxins or in some coleopteran pests (e.g., Western Corn Rootworm) appears to involve multiple genes (polygenic) and more complex inheritance patterns (Gassmann *et al.*, 2014). This complicates management, as the high-dose/refuge strategy is less effective against polygenic resistance.

Diverse Molecular Mechanisms : Resistance can arise from various molecular mechanisms (altered binding, downregulation, impaired activation, detoxification). Designing diagnostic tools and new toxins that account for all potential mechanisms is challenging.

Cross-resistance : The evolution of resistance to one Bt toxin can sometimes confer cross-resistance to other toxins, even those with different Cry classifications, if they share binding sites or downstream pathways (e.g., some Cry1 toxins might share binding sites on cadherin or APN). This reduces the effectiveness of pyramiding.

Compensatory evolution : Pests might evolve compensatory mutations that restore fitness costs associated with resistance, making it harder to dilute resistance alleles in refuges.

Predicting Resistance Evolution

Uncertainty in Initial Allele Frequencies : Precisely estimating the initial frequency of resistance alleles in wild populations is extremely challenging but critical for modeling resistance evolution rates (Gassmann *et al.*, 2009).

Fitness Costs in the Field : Quantifying the fitness costs of resistance alleles under varying field conditions (absence of Bt selection) is difficult but essential for predicting how quickly resistance alleles will decline in refuges.

G x E Interactions : The efficacy of Bt toxins and the expression of resistance can be influenced by environmental factors (e.g., temperature, drought stress), adding complexity to predictions.

Modeling complexity : Integrating all relevant biological, ecological, and operational factors into predictive models, especially for large geographic areas and diverse agricultural systems, remains a formidable task.

Global Spread of Resistance and Transboundary Pests

Migration : Highly migratory pests (e.g., *Helicoverpa armigera*, *Spodoptera frugiperda*) can rapidly spread resistance alleles across vast geographical regions, transcending national borders and complicating coordinated management efforts (Early *et al*., 2018).

Asynchronous adoption : Different countries may adopt Bt crops at different rates and implement varying IRM strategies, leading to "hotspots" of resistance that can then spread to other regions.

Illicit seeds : The illegal cultivation of unapproved Bt varieties or varieties with insufficient refuge levels can undermine resistance management efforts.

Socio-Economic Factors and Farmer compliance

Complexity for Farmers : Implementing refuge strategies (especially structured refuges) can be perceived as complex, time-consuming, or economically disadvantageous by farmers.

Lack of Awareness/education : Insufficient understanding among farmers about the importance of resistance management can lead to poor compliance with refuge requirements.

Economic incentives : The short-term economic benefits of Bt crops might overshadow the long-term need for resistance management, especially if regulatory enforcement or incentives for compliance are weak.

Smallholder farmers : In regions dominated by smallholder farms, managing refuges or implementing complex rotations can be particularly challenging.

Regulatory Challenges for New Technologies

Regulatory Bottleneck : The development and approval process for new Bt toxins, pyramided traits, or novel technologies like RNAi can be lengthy and expensive, hindering their rapid deployment to address evolving resistance.

Public Perception : Public acceptance and regulatory frameworks for new genetic technologies vary widely across countries, impacting adoption.

Future Perspectives and Directions

To overcome these challenges and ensure the continued efficacy of Bt technology, future efforts must focus on integrated, adaptive and innovative approaches.

Discovery and Deployment of Novel Bt Toxins and Next-Generation Technologies

New Cry and Vip Proteins : Continued bioprospecting and engineering to identify and develop new Cry and Vip toxins with novel modes of action and no cross-resistance to existing toxins.

Bt-Derived Proteins and Hybrids : Designing synthetic Bt-derived proteins or hybrid toxins by combining domains from different Bt proteins to create novel binding specificities and enhanced potency (Bravo *et al*., 2013).

RNA Interference (RNAi) as a Standalone or Stacked Trait : Developing crops expressing dsRNA that targets essential insect genes (e.g., genes involved in molting, reproduction, or metabolism). RNAi offers a completely distinct mode of action from Bt proteins, making it an excellent tool for resistance management through stacking with Bt toxins or as a rotational strategy (Baum *et al*., 2007).

Genome Editing for Pest Control : Exploring advanced genome editing tools (e.g., CRISPR/Cas9) to engineer pests themselves (e.g., gene drive technologies to spread susceptibility alleles, or sterility) or to enhance plant defenses (though still in early research stages and raising ethical/regulatory considerations).

Enhanced Surveillance and Predictive Modeling

Real-time Monitoring Networks : Developing smart, automated surveillance networks using IoT sensors, AI-powered image recognition (for pest identification and counting), and remote sensing (for crop health monitoring) to provide real-time data on pest populations and early detection of resistance (Storer *et al*., 2010).

Advanced Predictive Models : Utilizing machine learning and AI to develop more sophisticated predictive models that integrate diverse data streams (climate data, pest population dynamics, molecular diagnostics, agricultural practices) to forecast resistance evolution and optimize management strategies on a regional scale.

Functional Genomics of Resistance : Deeper molecular characterization of novel resistance mechanisms, particularly for polygenic resistance and cross-resistance, to inform diagnostic development and new toxin design.

Strengthening Integrated Pest Management (IPM)

Diversified Cropping Systems : Promoting agricultural landscapes with higher biodiversity, diverse crop rotations, intercropping, and agroforestry systems

Fig. 5 : Challenges in Resistance Management.

that enhance natural enemy populations and reduce reliance on single control tactics (Altieri and Nicholls, 2017).

Bio-pesticides and Biological Control : Increased investment in research and deployment of microbial and botanical bio-pesticides that are compatible with Bt crops and in the conservation and augmentative release of natural enemies.

Area-wide Management : Implementing coordinated, area-wide pest management programs that transcend individual farm boundaries, especially for highly mobile pests, involving multiple stakeholders.

Adaptive Management Frameworks : Developing flexible IRM plans that can be rapidly adjusted based on real-time surveillance data and predictive models of resistance evolution.

Policy, Education and Incentives

Global Harmonization : Promoting international collaboration and harmonization of regulatory frameworks for Bt crops and new pest control technologies to facilitate their responsible and timely deployment.

Farmer Education and Support : Strengthening extension services and developing user-friendly decision-support tools to empower farmers with the knowledge and resources needed for effective IRM compliance.

Incentives for Compliance : Exploring economic incentives or policy mechanisms to encourage farmer compliance with refuge requirements and other resistance management practices.

Stewardship Programs : Continued commitment from seed companies and industry associations to robust stewardship programs that ensure proper deployment and monitoring of Bt products.

By proactively addressing these challenges and pursuing these future directions, the agricultural community can work collaboratively to safeguard the efficacy of Bt technology, ensuring its continued contribution to sustainable pest management and global food security in the face of evolving insect pests.

Conclusion

The widespread adoption of genetically modified crops expressing *Bacillus thuringiensis* (Bt) toxins has unequivocally transformed agricultural pest management, leading to significant reductions in synthetic insecticide use, improved crop yields and enhanced environmental sustainability since their introduction over two decades ago. However, the remarkable success of Bt technology has been intrinsically linked to an enduring evolutionary challenge: the inevitable evolution of resistance in target insect pests. This review underscores that field-evolved

resistance is a natural outcome of strong selection pressure and presents a critical threat to the long-term utility of Bt crops.

Our understanding of the molecular mechanisms underlying Bt resistance has advanced significantly, with altered Bt toxin binding being the predominant mechanism. This typically involves mutations or reduced expression of key midgut receptor proteins such as cadherins and aminopeptidases N (APNs), which prevent the toxin from binding and forming pores in midgut cells. For example, specific cadherin mutations have been identified as primary drivers of resistance to Cry1Ac in *Pectinophora gossypiella* and *Helicoverpa armigera*. The identification of these mechanisms has been greatly aided by sophisticated surveillance diagnostics, which combine traditional bioassays (e.g., diagnostic dose assays for resistance allele frequency determination) with cutting-edge molecular tools (e.g., PCR-based assays, sequencing for specific mutations, and gene expression analysis for receptor downregulation). These diagnostic capabilities are crucial for the early detection and monitoring of resistance evolution, providing the necessary data to inform adaptive management decisions.

The cornerstone of resistance mitigation has been the high-dose/refuge strategy, which aims to ensure that the initial frequency of resistance alleles remains low and that any resistant individuals are diluted by susceptible mates from non-Bt refuges. The exemplary success in maintaining the susceptibility of *Pectinophora gossypiella* to Cry1Ac in the US, with resistance allele frequencies remaining below 0.005 over two decades, stands as a testament to the effectiveness of this strategy when rigorously implemented. Conversely, the rapid evolution of *Helicoverpa armigera* resistance to Cry1Ac in some regions of China and India, where resistance allele frequencies climbed to over 0.5 in some areas by 2005, highlights the critical importance of farmer compliance with refuge requirements and the complexities posed by diverse agricultural landscapes and pest biology. The advent of gene pyramiding, or stacking multiple Bt toxins with different modes of action, has significantly enhanced the durability of Bt traits, as insects must simultaneously evolve resistance to multiple toxins. These strategies, coupled with broader Integrated Pest Management (IPM) principles like crop rotation, biological control, and judicious use of insecticides, form a robust framework for delaying resistance.

Despite these advancements, significant challenges persist. Predicting the rate and nature of resistance evolution remains complex, particularly for polygenic resistance or in highly migratory pests like *Spodoptera frugiperda*, which has shown rapid resistance to multiple Cry toxins. Ensuring consistent farmer compliance with refuge requirements, especially among smallholder farmers, and navigating fragmented regulatory landscapes across different regions continue to be major hurdles. Furthermore, the constant threat of new resistance mechanisms emerging or the global spread of existing resistance demands continuous vigilance.

Looking ahead, the long-term sustainability of Bt technology hinges on an adaptive and multi-pronged approach. This includes the ongoing discovery and deployment of novel Bt toxins and next-generation technologies like RNA interference, which offer new modes of action to circumvent existing resistance. Crucially, investments in real-time, high-throughput surveillance, advanced predictive modeling integrating artificial intelligence and a deeper understanding of the functional genomics of resistance are vital. Finally, strengthening global collaboration, enhancing farmer education and incentives and fostering supportive policy and regulatory frameworks are paramount to ensure that Bt crops continue to contribute effectively to sustainable pest management and global food security for generations to come.

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