Recent advancement on chemical arsenal of *Bt* toxin and its application in pest management system in agricultural field

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Abstract

Bacillus thuringiensis (*Bt*) is a Gram-positive, spore-forming, soil bacterium, which is very popular bio-control agent in agricultural and forestry. In general, *B. thuringiensis* secretes an array of insecticidal proteins including toxins produced during vegetative growth phase (such as secreted insecticidal protein, Sip; vegetative insecticidal proteins, Vip), parasporal crystalline δ -endotoxins produced during vegetative stationary phase (such as cytolytic toxin, Cyt; and crystal toxin, Cry), and β -exotoxins. Till date, a wide spectrum of Cry proteins has been reported and most of them belong to three-domain-Cry toxins, Bin-like toxin, and Etx_Mtx2-like toxins. To the best of our knowledge, neither *Bt* insecticidal toxins are exclusive to *Bt* nor all the strains of *Bt* are capable of producing insecticidal *Bt* toxins. The lacuna in their latest classification has also been discussed. In this review, the updated information regarding the insecticidal *Bt* toxins and their different mode of actions were summarized. Before applying the *Bt* toxins on agricultural field, the non-specific effects of toxins should be investigated. We also have summarized the problem of insect resistance and the strategies to combat with this problem. We strongly believe that this information will help a lot to the budding researchers in the field of modern pest control biotechnology.

Keywords *Bacillus thuringiensis* · Insecticides · Cry toxins · Cyt toxins · Mechanism of action · *Bt* resistance

Introduction

Bacillus thuringiensis or *Bt* is an important bacterium that possesses entomopathogenic activity and thus has high demand in agricultural sectors. Due to its colonization ability in the gut of insect, *Bt* is often used in agriculture (Deist et al. 2014), forestry (Zhang et al. 2016), and public health programmes as a bio-control agent (Ibrahim et al. 2010; De Schrijver et al. 2015). *Bt israelensis* (*Bti*) was the first subspecies observed to be toxic against the dipteran larvae of noxious species such as mosquitoes, black flies, and chironomid midges. The demand for *Bt*-based insecticides in agriculture sectors declined in the mid 1970s, due to availability of more effective chemical pesticides. However, in

the 1980s, progress in biotechnology has again accelerated the demand for *Bt* products. In an investigation, Schnepf and Whiteley (1981) cloned a crystal toxin gene from *Bt kurstaki* (*Btk*) and expressed it into *Escherichia coli*. Since then, much studies have been performed to improve the target spectra and to discover more infectious strains of *Bt*. According to Tabashnik et al. (2013), the commercial application of *Bt* genes in plant (expressing *Bt* toxins) has been started from mid 1990s. In 1999, different types of *Bt* products such as *Bt* corn, *Bt* cotton, and *Bt* potatoes were produced worldwide.

During spore-forming stage, *B. thuringiensis* secretes different types of δ -endotoxins [belong to the cytolytic (Cyt) or crystal (Cry) families], which vary greatly in structure, mode of action, and specificity (de Maagd et al. 2003; Deist et al. 2014; Land and Miljand 2014), whereas the other insecticidal toxins such as Vip are secreted during the vegetative phase of growth (Palma et al. 2014). While, another group of novel *Bt* proteins, termed as parasporins (Brasseur et al. 2015), have unique cytotoxic activity. The parasporins are parasporal-like protein that are non-hemolytic in nature, but have the ability to preferentially kill cancer cells (Xu et al.

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2014). We have not discussed parasporin in this review, because it is beyond the scope of insecticidal activity.

In this review, we have categorically analyzed the chemical arsenal of *Bt*, which are insecticidal in nature. We have also discussed about Cyt, vegetative insecticidal proteins (Vip), and secreted insecticidal protein (Sip) toxins along with different type of Cry toxins. The sequential-binding model or classical model of Cry toxins has already studied intensively by researchers. In this review along with the sequential-binding model, we also have summarized the signaling pathway model or the alternate action pathway of Cry toxins which trigger apoptosis in gut cells of the pest. We also have summarized the problems of insect resistance and the strategies to combat with these problems.

Insecticidal *Bt* strains and *Bt* toxins

The use of *Bt* strains has increased tremendously, and currently, it is about 90% of the world microbial pest control agent (MCPA) market. Due to beneficial effects, about 84 strains of *Bt* were recorded in the world data centre for microorganism (WDCM) (<http://www.wfcc.info/>); however, all the strains of *Bt* are not insecticidal in nature (Ibrahim et al. 2010). For example, *Bt* strain NTB-88 possess non-insecticidal parasporal inclusions and flagellar antigen H8a8b (Ibrahim et al. 2010).

There are several strains of *Bt* with different host specificity. The toxins produced by *Bt israeliensis* (*Bti*) are very specific and effective against dipteran pest, including mosquito and black fly, and thus has great demand in public health programmes (kills 95–100% of mosquito larvae within 24 h) (Land and Miljand 2014). Most of the *Bt kurstaki* (*Btk*) strains are active against lepidopteran pests, while strains of *Bt tenebrionis* (*Btt*) are active against coleopteran pests (Azizoglu et al. 2015). The commercial *Bt* strains, their target pest, and their use in different sectors (agriculture, forestry, and public health programmes) are presented in Table 1.

Bt toxins produced by other bacterial candidates

The expression of *Bt* toxins like Cry and Cyt is not limited to *Bt* strains, and several other microorganisms like *Clostridium bifermentans*, *Paenibacillus popilliae*, *P. lentimorbus*, and *B. sphaericus* have been reported as good sources of *Bt* toxin. The sphaericolysin/anthrolysin family of toxins is highly conserved across isolates from *Lysinibacillus sphaericus*, *Bt*, *Bacillus cereus*, and *Paenibacillus alvei* (Berry 2012; Castagnola and Patricia Stock 2014). Mtx2 protein and aerolysin were also identified from *Clostridium* sp. and *Aeromonas hydrophila*,

Table 1 Selection of *Bt* strains and strains originated from *Bt* used as commercial pesticides in agriculture, forestry, and public health programmes

Pest control sectors	Insecticidal <i>Bt</i> strains/ genes	Target pest
Agriculture	Natural strains	
	<i>Bt kurstaki</i> (<i>Btk</i>)	Lepidoptera
	<i>Bt san diego</i> (<i>Btsd</i>)	Coleoptera
	<i>Bt tenebrionis</i> (<i>Btt</i>)	Coleoptera
	Improved strains	
	<i>Btk</i> HD1	Lepidoptera
	<i>Btk</i> SA-11	Lepidoptera
	<i>Btk</i> SA-12	Lepidoptera
	Conjugated strains	
	EG2424 (<i>Btk</i> × <i>Btt</i>)	Lepidoptera and Coleoptera
	EG2348 (<i>Btk</i> × <i>Bta</i>)	Lepidoptera
	Recombinant strains	
	<i>E. coli</i> expressing Cry1Ac (x2), Cry3A Cry3Bb	Lepidoptera
	<i>E. coli</i> expressing Cry1Ac (x3), Cry2A Cry1C	Lepidoptera
	<i>E. coli</i> expressing Cry1Aa, Cry1Ac (x2), Cry2A Cry1F-1Ac	Lepidoptera
Forestry	Natural strains	
	<i>Bt aizawai</i> (<i>Bta</i>)	Lepidoptera
	<i>Btk</i>	Lepidoptera
	Improved strains	
	<i>Btk</i> HD1	Lepidoptera
	<i>Btk</i> SA-11	Lepidoptera
Public health	Natural strains	
	<i>Bt israeliensis</i> (<i>Bti</i>)	Diptera
	<i>Bt medellin</i> (<i>Btm</i>)	Diptera

respectively (Szczesny et al. 2011; Palma et al. 2014). On the other hand, different strains of *L. sphaericus* also produce the BinA/B toxin, Mtx1, Mtx2, Mtx3, Mtx4, sphaericolysin, Cry48, and Cry49 (Berry 2012). *Photobacterium* strains were reported to produce PirA/B and Mcf toxins (Sato et al. 2014).

Genetically modified organism with *Bt* toxins

To combat with the insect pest, the diversity of *Bt* toxin must be enriched, and this can be attained by conjugation or recombinant DNA technology [for example, EG2348 (*Btk* × *Bta*) and EG2424 (*Btk* × *Btt*)] (Carlton and Gawron-Burke 1993).

Bacteria producing *Bt* toxins are non-systemic insecticides and are, therefore, ineffective against insects that do not come

into direct contact with the crystals (which includes sap sucking and piercing insects, root dwelling pests, stem, and fruit borer, etc.). This problem has been effectively addressed by creating genetically modified plants (GMP) that express the crystal proteins. The problem of narrow insect pest specificity has been effectively solved by simultaneously expressing of multiple *Bt* toxins through gene ‘stacking’ or ‘pyramiding’ (de Maagd et al. 1999). The US Environmental Protection Agency (EPA) first approved the registration of *Bt* crops (potato, corn, and cotton) in 1995. First marked GMP with *Bt* toxin was a NewLeaf potato variety expressing Cry3A developed by Monsanto. Presently, the United States is the global leader in producing GMP with *Bt* toxins followed by Argentina and Brazil. China and India are in top among countries taking most rapid adoption of GMP expressing *Bt* toxins.

Ethical, socio-economic, and regulatory issues related to *Bt* toxins

Bt is a natural insecticide and is, therefore, its field application as bacterial suspension (or any products that use live *Bt* spores and crystal toxins) is not restricted. However, the production company has to ensure that the strain used is not producing any non-specific β -exotoxins. However, the question of stringent rule, regulation, and requirement of approval is enforced in case of GMO with *Bt* toxins (Altieri and Rosset 1999). There have been several issues raised at different platforms on the use of GMP with *Bt* toxins (example included Galgene’s FlavrSavr and Ciba Giegy’s *Bt* corn). The issue of antibiotic resistance markers in selection of GMP has been tackled with auxotrophic or food grade markers (Braun et al. 2001). GMPs need to be evaluated for toxicity rigorously in animal models before their release for human consumption. In most of the countries, GMP with *Bt* toxins are require to be clearly labeled biotech (Maghari and Ardekani 2011). It is always recommended that before applying the *Bt* toxins on agricultural field in any form, the non-specific effects of toxins should be investigated. Otherwise, it will disturb the natural insect diversity and hamper the ecosystem.

Insecticidal *Bt* toxins nomenclature

Höfte and Whiteley (1989) classified Cry proteins into four major classes according to the specificity of the toxin against different insect classes (Fig. 1). However, several workers have reported the overlapping toxicity of *Bt* toxins to different insect classes. To overcome such problem, Crickmore et al. (1998) have introduced another classification scheme. According to the scheme, the protein class named CryIC (genes involved: *cry1Ca1*, *cry1Ca2*, *cry1Ca3*, *cry1Ca4*, etc.) was recorded to have toxic effects in dipterans

and lepidopterans pest (Crickmore et al. 1998). Finally, in the year 1993, the *Bt* δ -endotoxin nomenclature committee (<http://www.btnomenclature.info/>) has divided the Cry, Cyt, and Vip toxins based on amino acid identity (Fig. 1). The nomenclature committee has assigned a distinctive name for each toxin and placed closely related toxins in group or rank. Each rank was again divided into four sub ranks. Furthermore, the nomenclature style also has been changed (Roman numerals to Arabic numerals) in the primary rank (e.g., CryIIIA became Cry3A).

The classification scheme was developed on the basis of identity in primary sequence. However, it is conceivable that despite the diversity in primary sequence, many of the non three-domain Cry toxins could share significant structural/functional homology (Xu et al. 2014). On the other hand, Etx_Mtx2 like cry toxins or Bin-like cry toxins are considerable diverge than three-domain Cry (3d-Cry) proteins in their tertiary structure. Therefore, inclusion of these two groups into this present nomenclature scheme is quite misleading. Parasporins shows significant structural similarity with 3d-Cry, but is not considered into the present nomenclature scheme. Moreover, the numbers of unclassified cry toxins into this present scheme of nomenclature are ever increasing which further emphasizes the need of re-evaluation of the *Bt* toxins nomenclature.

Cry toxins

Cry proteins or parasporal inclusion proteins produced by *Bt* and other bacteria are investigated deeply in the last few years. In general, the molecular weight of Cry proteins varies between 70 and 140 kDa (de Maagd et al. 2003; Pardo-López et al. 2013; Palma 2015). It is well established that most of the Cry toxin belongs to the three-domain family protein, though two minor groups of Cry toxins such as Mtx-like and Bin-like were also introduced (Fig. 1). However, parasporins (Cry family) are the most important and studies proteins, as it possess anticancer activity (Berlitz et al. 2013).

Three-domain Cry toxin

Among insecticidal *Bt* toxins, the family of three-domain Cry (3d-Cry) proteins belong to the main section having > 53 subgroups (Bravo et al. 2013). In general, the 3d-Cry toxin contains two subunits: 65 and 130 kDa. The 130 kDa subunit is inactive and designated as protoxin. The removal of C-terminal extension by the proteolytic activity of larval midgut proteases converts it as an active one (de Maagd et al. 1999; Bravo et al. 2013). The N-terminal regions of all 3d-Cry toxins contain approximately 20–60 amino acid residues (Pardo-López et al. 2013).

It also contains eight conserved amino acid blocks; and among these, first five blocks are highly conserved and are concentrated mainly in the centre of the domains and at the junctions between the domains (Bravo et al. 2013). Pardo-López et al. (2013) have stated that the domains I and II have evolved together, whereas the domain III showed a different structural geometry due to its swapping nature (Pardo-López et al. 2013). In general, 3d-Cry proteins (or protoxin) are activated into toxin by trypsin and are highly specific in nature (e.g., Cry2Aa for dipteran–lepidopteran; Cry1Aa for lepidopteran; Cry4Aa and Cry4Ba for dipteran; Cry3Aa, Cry3Bb and Cry8Ea for coleopteran) (Guo et al. 2012; Pardo-López et al. 2013).

Epsilon toxin_metaxin 2 (Etx_Mtx2)-like Cry toxins

Along with three-domain Cry toxins, Cry nomenclature committee also has classified some other families of unrelated toxins (Fig. 1). Etx_Mtx2 like Cry toxins such as Cry15, Cry33, Cry60, etc. share a significant structural homology, and are very similar to toxin like aerolysin. Aerolysin is well-studied toxin (having pore-forming

capability) produced by *Aeromonas hydrophila* (Szczesny et al. 2011). Palma et al. (2014) have evaluated the mode of action of Etx_Mtx2 like Cry toxins that actively form a pore in the host target cell. In the aerolysin family of proteins, the conserved beta sheet or aerolysin fold is believed to adopt a barrel conformation within the membrane (Szczesny et al. 2011).

Binary (Bin)-like Cry toxins

Majority of Bin-like Cry toxins such as Cry35 and Cry36 (Toxin_10 family) have high antagonistic activity against different agricultural insect pests (Fig. 1). The structural topology of Cry35Ab1 (Kelker et al. 2014) and BinB (Srisucharitpanit et al. 2014) is very similar to those of Etx_Mtx2 like Cry toxins, as they possess aerolysin-like fold. Cry35 contains a QxW motif, which is structurally very similar to carbohydrate-binding domains of ricin and Mtx1. In recent years, a few other Bin-like Cry toxins also have been characterized such as Cry37, Cry34, and Cry23Aa, also known as ET33 (de Maagd et al. 2003; Contreras et al. 2013; Kelker et al. 2014). In an investigation, Ekobu et al. (2010) have reported the combined effects of Cry37 (ET34) and Cry23Aa against various coleopteran insects.

Unclassified Cry toxins

In recent years, several *Bt* strains have been reported which produce insecticidal toxins; however, the *Bt* δ -endotoxin nomenclature committee was unable to place these toxins into any category, due to different structure and amino acid sequences. For example, Palma et al. (2014) have reported a 42-kDa toxin from *Bt* strain, which exhibited high similarity to BinA and BinB. Similarly, Palma et al. (2014) also have reported a sphaericolysin (possess unique N-terminal sequence and cholesterol-dependent cytolysins motif) producing *Bt* strain. Sphaericolysin is considered to be a potent insecticidal toxin, which is very effective to control the *Blattella germanica* and *Spodoptera litura* (Castagnola and Patricia Stock 2014; Palma et al. 2014). Few other unclassified *Bt* toxins have also been studied against different insect pest such as *Helicoverpa armigera* (Tabashnik et al. 2015) and *Aedes aegypti* (Deist et al. 2014). Some other toxins (such as Cry6, Cry22, Cry55, Mcf, etc.) which belong to unclassified category also have been reported (Fig. 1).

Fig. 1 Schematic overview of the evolution of nomenclature system used by the *Bt* Toxin Nomenclature Committee for δ -endotoxins (Cry and Cyt) and secretable (Vip and Sip) toxins. In this example, numbers indicate different Cry proteins changing rank 1 depending of percentage amino acid similarity. The same rule applies for ranks 2, 3, and 4 assigning a different identification digit/letter

Fig. 2 Schematic representation of insecticidal *Bt* toxins classification with example of toxins and their target pest in each class

Cyt toxins

Like Cry toxin, Cyt toxins are another group of *Bt* crystal toxins that possess an in vitro and in vivo activity against dipteran pests (Fig. 2). Cyt toxins are able to bind with non-saturated membrane lipids (Chougule et al. 2013) and thus do not require any specific receptor. In general, Cyt toxins are 25 kDa protein containing a single α - β domain wrapped by two layers of α -helix hairpins (Cantón et al. 2014). The conversion of inactive protoxin Cyt1Aa (27 kDa) to active toxin (24 kDa) is achieved by the proteolytic cleavage at the amino (N) and carboxy-terminal (C) ends (Chougule et al. 2013), which is vital for membrane attachment and piercing. The topology of Cyt2Aa reveals a three-layer α - β domain with a unique fold, which permits the α -helix layers to move away and push the β -sheet into the host membrane. Furthermore, the two components of Cyt2Aa: loop β 6- α E and part of β 7 also possess high affinity to Cry11Aa (Cadauid-Restrepo et al. 2012). The cytolysin fold has critical importance, as the binding of toxin to epithelial membrane depends on the conformational geometry of the fold (Coates et al. 2013). The Cyt1Aa toxins do not have any sequence similarity to Cry polypeptides (Bravo et al. 2013).

Vip toxins

Like other *Bt* toxin, Vip are also important insecticidal protein in integrated pest management system (Baranek et al. 2015). Based on amino acid sequence, the Vip proteins may be divided into four different families: Vip1,

Vip2, Vip3, and Vip4 (Crickmore et al. 2014) (Fig. 2). Like Cyt toxins, Vip proteins are also inactive in its native form. Thus, Vip protein also undergoes a conformational changes at conserved signal peptide sequences before or after the secretion in the membrane (Baranek et al. 2015). Vip1 and Vip2 are insecticidal binary toxins against a wide range of coleopteran and Hemipteran pests (Sattar and Maiti 2011). Furthermore, in a review, Palma et al. (2014) have stated that Vip2 exhibits structural similarity with active domain of CdtA toxin produced by *Clostridium difficile*. Whereas, Vip3 (single-chain) toxins are effective against a wide range of agricultural lepidopteran pest (Palma et al. 2014; Palma 2015).

Sip toxins

The secreted insecticidal protein (Sip) is the member of *Bt* insecticidal family, which are very effective against coleopteran larvae (Fig. 2). Like Cyt and Vip toxins, a conformational change in amino acid sequence converts the inactive Sip toxin into an active one (Palma et al. 2014). Palma et al. (2014) have reported the production of Sip protein named Sip1Aa1 from the *Bt* strain EG2158. The protein Sip1Aa1 is approximately 41 kDa long that contains 367 amino acid residues. The Sip1Aa1 toxin contains a 30 amino acid long secretion signal and is recorded to be lethal for *Leptinotarsa decemlineata*, *Diabrotica undecimpunctata howardi*, and *D. virgifera virgifera* (Palma et al. 2014).

β-Exotoxins

β -Exotoxins are thermostable, non-proteinaceous secondary metabolites secreted by a few strains of *Bacillus thuringiensis*. Unlike δ -endotoxins, the target of β -exotoxins is non-specific; and sometimes, it also affects mammals, along with insect pest (Liu et al. 2014), and thus, the use of β -exotoxins producing *bt* strains is restricted in several countries of Europe and USA. β -Exotoxins are also known as thuringiensin, which are analogues of adenine (Obeidat et al. 2012) or uracil (Levinson et al. 1990). It is well established that β -exotoxins play a negative role in RNA biosynthesis (Obeidat et al. 2012).

Fig. 3 Schematic overview of the mode of action of Vip toxin triggering apoptotic pathways

Mode of action of *Bt* toxin

In the last few years, a wide spectrum of *Bt* toxins have been introduced in agricultural and forestry. All these toxins have great ability to kill different insect pests; however, their mechanism of action is different. The mechanism of cell death caused by VIP toxins is presented in Fig. 3 (de Maagd et al. 2003; Bravo et al. 2013; Palma et al. 2014). On the other hand, the exact working mechanism of Sip toxins is not clear to date; however, Sip1Aa1, a toxin of Sip class, is reported to be a pore-forming agent (Palma et al. 2014). In this review, we have discussed the mechanism of action of Cry toxins and Cyt toxins in details, as they are considered to be the most studied *Bt* toxins till date.

Working mechanism of insecticidal Cry toxins

The working mechanism of Cry toxin is well established. At present, two models have been proposed based on the working mechanism of Cry toxins. The most studied one is the sequential-binding model or classical mode of action

of Cry toxins, whereas new arrival is the signaling pathway model or alternate mode of action of Cry toxins. In both the models, upon ingestion, Cry toxins are dissolved in the insect gut juices to yield the protoxin. The conversation of protoxin to active toxin in the insect gut is only possible due to the suitable pH condition. Then, the active toxin binds with his specific receptor present in the membrane of the gut cells. The use of Cry toxin is popular to the agricultural field due to its target specificity (insect pest) and does not hamper the other insect (non-target). The strategy of such specificity is assumed to be caused by the (1) gut pH, (2) domain II and III, and (3) the presence of the toxin-specific receptors in the cell membrane. However, the binding affinity of Cry toxin to its receptor varies greatly (due to varying number of binding sites) from one insect to others (Bravo et al. 2013; Lee 2013).

Fig. 4 Sequential-binding model (classical mode of action) of Cry toxin showing toxin activation, binding with specific receptors, formation of pores, and subsequent death of pest insect larvae

The sequential-binding model (classical mode of action)

It is a multiple step process of receptor-mediated complex pathway that leads to the formation of pore in the cell membrane (Pacheco et al. 2009; Palma et al. 2014). Upon ingestion, the crystalline inclusions of the endotoxin are dissolved in the insect gut juices to yield the protoxin (Fig. 4). The conversion of protoxin to active toxin (removal of N- and C-terminal amino acids) in the insect gut is only possible due to the suitable pH condition. The removal of N-terminal amino acid by the proteolytic action by gut enzymes varies from one Cry toxin to another (for example, 49, 58, and 25–30 amino acid residues are removed in case of Cry2A, Cry3A, and Cry1 toxins, respectively) (Silvia Denise et al. 2013) (Fig. 4). However, The C-terminal end of the protoxin plays the major role in receptor-binding phenomena. During last few years, researchers have investigated and identified different types of receptor [ABC transporters (ABCC2, ABCC3), GPI-anchored alkaline phosphatase (ALP), cadherin-like protein (CADR), and glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN)] in Cry1A toxins, which are required for toxin activation (Yudina et al. 2007; Tabashnik et al. 2015). Upon binding on membrane receptor, the hydrophobic amino acid residues accelerate the pore formation process. Lepidopteran-specific Cry1Aa specifically bind with lepidopteran ABCC transporter ABCC2 and ABCC3, whereas coleopteran ABCC transporter specifically responded to a coleopteran-specific Cry8Ca toxin (Endo et al. 2017). Ben-Dov (2014) has reported that Cry4Aa or Cry4Ba are the most important parts of pre-pore structure. Finally, it enters within the membrane of the gut epithelium cells and forms a stable membrane channel (Tabashnik et al. 2015), through which different types of cation can enter freely. The entrance of outer ions dis-balances the cell physiology and osmotic balance and ultimately led to the lysis of the cell. Furthermore, the pore allows leaking out the leak of the gut content to the haemocoel, including bacteria that causes septicemia (Ben-Dov 2014) (Fig. 4).

The signaling pathway model (alternate mode of action)

Unlike sequential-binding model, the pore formation is not an important event in signaling pathway model (Fig. 4). Palma et al. (2014) have reported that upon attachment of Cry1A toxin to cadherin receptor accelerates the events of intracellular apoptotic pathways that lead to the destruction of the cell (Palma et al. 2014). However, the interaction of toxin and receptor in insect gut depends on extracellular signals (Palma et al. 2014) (Fig. 5).

Fig. 5 Signaling pathway model (alternate mode of action) of Cry toxin showing attachment of Cry toxin to cadherin receptor accelerates the events of intracellular apoptotic pathways

Mechanism of insecticidal Cyt toxins

Like Cry toxin, the mechanism of action of Cyt toxins is also very complicated; and at present, two general mechanistic pathways have been investigated.

Pore formation model

The pore-forming ability of the Cyt toxins highly depends on the structural topology of the protein. This model was suggested for the toxins like Cyt1Aa and Cyt2Ba that contain a cytotoxin fold and exhibit hemolytic activity (Ben-Dov 2014). Cyt toxins may form pore in the membrane either alone or synergistically with Cry toxin. In the insect mid-gut, digestive juice solubilizes the Cyt protein and activates it. Upon activation, the Cyt proteins bind to the membrane receptor and exposes the specific binding sites for the Cry toxins (Deist et al. 2014). The interaction of Cry11Aa and Cyt1Aa is important for membrane insertion, which depend

on the charged amino acids residues (domain II loop $\alpha 8$ of Cry11Aa and β -4 of Cyt1Aa). Ultimately, it forms a pore and destabilizes the cell physiology and osmotic balance.

Detergent action model

This model has proposed the detergent action mechanism of Cyt toxins. Upon binding on the cell surface, it disrupts the lipid packing pattern and forms a stable channel through which the cell content comes out by all-or-nothing mechanism (Doggett et al. 2013). Like Cry toxins, the modes of action of Cyt toxin may not be mutually exclusive. Bravo et al. (2007) have reported that the pore formation process of Cyt toxins (e.g., Cyt1A) and Cry toxins is quite different. Unlike Cry toxin, Cyt toxins are directly absorbed on the surface and form the pore using detergent-like mechanism (Bravo et al. 2007). In this direction, Ben-Dov (2014) has reported that the efficacy of pore formation of Cyt toxins depends on the toxin concentration and time of exposure.

Bt toxin effect on non-target insects

Until now, several reports have been published regarding the effects of *Bt* toxins on agricultural insect pest (target pest) and their control; however, the information regarding the non-target insects or unspecific effects of *Bt* toxins (specially Cyt toxin) is scanty (Bøhn et al. 2008; Resende et al. 2016). Previously, people are not so concern about the adverse effects of *Bt* toxins on non-target insect. In the year 1999, Losey et al. have reported a huge mortality of Monarch butterfly (*Danaus plexippus*) larvae fed with pollen from Cry1Ab expressing maize plants, and from then, it becomes a regulatory test approved by USA Government. The effects of *Bt* plant (such as transgenic Cry1Ab maize and Cry3A-expressing potato foliage) on non-target insects (*Spodoptera littoralis* and *Chrysoperla carnea*) have been recorded (Hussein et al. 2006; Romeis et al. 2014; Resende et al. 2016). The toxicity of *Bt* toxins was found sublethal for *Neobellieria bullata* depending on the dose of applied toxin (Cerstiaens et al. 2001). Brandt et al. (2004) have detected the presence of Cry2Ab in the *Lygus hesperus* tissues; however, the binding of Cry2Ab alone is not sufficient for toxicity. Furthermore, Rodrigo-Simon et al. (2006) stated that the gut of *C. carnea* larvae does not contain any specific receptors for Cry1Ab and Cry1Ac, and thus, the actions of these toxins are unspecific. In a review, Deist et al. (2014) also have mentioned the toxic effects of Cry3A toxins on non-target insect pests [as, for example, pea aphid (*Acyrtosiphon pisum*)]. Interestingly field studies on the NewLeaf potato (Cry3Aa) showed that the toxin specifically

affects the Colorado potato beetle has no deleterious effect on other insects in the potato field, including the beetle's natural predators (Reed et al. 2001). After detailed experimental design, Lawo et al. (2009) have concluded that *Bt* cotton has no negative impact on beneficial insects in the cotton ecosystem.

Bt resistance

Till date, there are only few instances of resistance development in the agricultural field. In laboratory conditions, due to regular use, the agricultural pests are turned to be resistant to *Bt* toxins. Emergence of resistant pest is a big threat for agricultural sectors. In general, three major events are considered that confer resistant property to the insect pests; (1) alteration of toxin–receptor interactions, (2) alterations in toxin, and (3) alterations in toxin solubilization (Ferry et al. 2004).

The attachment of *Bt* toxins on the cell surface is a receptor-binding phenomenon and any defects in receptor topology do not allow the toxin to bind on the surface. Until now, several types of receptor defects such as defects on cadherin-like receptor, defects on GPI-anchored receptor, and defects on channel protein have been investigated (Pardo-López et al. 2013). In a study Elzak (2016) has reported a mutation in the cadherin-like gene in *Heliothis virescens*, which confers resistant to Cry1Ac-resistant line YHD2 (cross resistant to Cry1A and Cry1Fa toxins). In a similar study, Chougule and Bonning (2012) have indentified mutated CADR alleles in *Pectinophora gossypiella* that confer resistant property against a wide range of Cry toxins. Furthermore, Coates et al. (2013) have stated that Cry1C-resistant colony of *Spodoptera exigua* did not express APN1 in the gut, which clearly indicates the correlation between toxin-resistant and receptor protein production. Pardo-López et al. (2013) also have established the relation between ALP receptor expression and Cry1Ac toxin-resistant property of *H. virescens* YHD2 larvae. In this direction, Park et al. (2014) have identified several types of mutation in ABCC2 transporter protein, which confer resistant property against *Bt* toxin. Recently Chakroun et al. (2016) also have reported Vip3Aa-resistant pest *Helicoverpa armigera* in Australia, which cause a huge damage in agricultural production.

Strategies for combating against Bt resistance problems

Regular and constitutive application of *Bt* 3d-Cry proteins contributed in insect resistance to these toxins. Agricultural methods like refuge strategy may only partially address the problem by delaying the appearance of resistance in insect

pests (*P. gossypiella*) as discussed by Tabashnik et al. (2010). Therefore, precise strategies are being formulated to combat with this problem in real time. Here, we have discussed and summarized different technical strategies that are being applied in agricultural field.

Discovery of novel *Bt* toxins

Bt toxin plays an important role in agriculture and forestry, and thus, searching and characterization of novel *Bt* toxin are a never-ending process. Several molecular, biological, and biotechnological techniques such as DNA microarray, HPLC, and southern hybridization have been introduced to purify and characterize new *Bt* toxin (Kelker et al. 2014). Furthermore, next-generation sequencing (NGS) technologies are also available to discover unrecognized insecticidal-toxin genes (Palma et al. 2014). The combination of novel *Bt* toxins produced by other bacteria and already known 3d-Cry toxins produced by *Bt* might be useful in controlling insect pest (Bravo et al. 2011).

Combination among Cry proteins

The information regarding the 3d-Cry toxins genes, expressed proteins, and their receptors is already available. Therefore, one of the available technologies is ‘stacking’ or ‘pyramiding’ different *Bt* toxins together to tackle multiple pest attack in the agriculture field. Till date, the best example cited may be SmartStax® (Monsanto, MI and Dow AgroSciences, IN). SmartStax® is a brand of genetically modified seed made through several combinations of Cry proteins which are very effective against resistant pest. These combinations of toxins not only kill the insect pest but also slowdown the resistance evolution process. Long-term use of *Bt* toxin creates a selective pressure for emerging *Bt* toxin-resistant pest; however, synergistic effect of two or more toxins might be an alternative way to combat with such issues. In this direction, researchers have reported the synergistic effects of Cry4Aa and Cry4Ba, Cry4Aa, and Cry11Aa, or the three Cry’s, against larvae of *Culex*, *Aedes*, and *Anopheles*, whereas Cry4Ba and Cry11Aa exhibited synergistic effect against *Ae. aegypti* (Ibrahim et al. 2010; Elleuch et al. 2015).

Synergies between Cry and Cyt proteins

Several investigations have reported the low toxicity of Cyt1Aa and of Cyt2Aa produced by *Bt kyushuensis*; however, their combined insecticidal effect is very high (Ben-Dov 2014; Deist et al. 2014). Similar synergistic effect of *Bti* Cry toxins–Cyt1Aa and Cyt toxins–Cry toxins against several insect pests, including *Culex quinquefasciatus*, also have been studies (Sena et al. 2009; Hayakawa et al. 2016).

However, better result against *C. quinquefasciatus* was observed in case of recombinant acrySTALLIFEROUS *Bti* strain that produce a mixture of *Bt* toxins (Ben-Dov 2014).

Synergies between different *Bt* toxins

The combination of VIPs and immunosuppressive proteins (animals or plants origin) may tackle the resistance problem (Price and Gatehouse 2008). Based on combinational effect of *Bt* toxin, researchers have developed alternative killing mechanism such as chitin degrading enzyme (digest chitin membrane) and Trypsin Modulating Oostatic Factor (responsible for larval starvation) (Ajamhassani et al. 2011; Han et al. 2015).

Bt toxin protein engineering

Protein engineering has opened the new window of controlling mechanism against resistant pest through several strategies such as loop substitution, domain swapping, and shuffling strategies (Deist et al. 2014). Cry1AMod toxins could effectively reduce the resistance levels in the six major crop pest species (Franklin et al. 2009; Elzak 2016). Cry1Ab.105 introduced by Monsanto (Monsanto, MI and Dow AgroSciences, IN) for their product SmartStax® is a brilliant example of *Bt* toxin protein engineering. It is very effective way to control insect pest, as well as to check the insect pest resistance problem.

Conclusion and future perspective

In agriculture, forestry, and public health sectors, insecticidal *Bt* toxins are valuable tools for pest control management. Currently, different chemical pesticides are available in the market; however, the use of such chemicals is not good for health, and, thus, have been restricted in several countries. It is now well established that the mutual interactions among the *Bt* toxins (Cry4Aa, Cry4Ba, and Cry11Aa and Cyt1Aa) confer the resistant property of *Bti*. This knowledge turns to be fundamental for the development of strategies to combat resistance problems faced in the agricultural sectors. Before applying the *Bt* toxins on agricultural field, the non-specific effects of toxins should be investigated. The specific larvicidal properties of *Bt* toxins are attributed to the complex interactions between and among Cry, Cyt, Vip, and Sip proteins and their specific receptors. In virtue of the discovery of novel insecticidal *Bt* proteins and emerging technologies like protein engineering, transgenic plants are expected to serve mankind for a longer time period than previously expected. In conclusion, the discovery of new insecticidal *Bt* toxins, development of chimeric toxins, and their expression in different combinations will definitely open a

new window for the researcher in future. It is evident that neither *Bt* insecticidal toxins are exclusive to *Bt* nor all the strains of *Bt* are capable of producing insecticidal *Bt* toxins. Therefore, the scope of research on insecticidal *Bt* toxins is not limited to *Bt* only.

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Compliance with ethical standards

Conflict of interest We declared that none of the authors have any conflict of interest.

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