RNAi TO UNDERSTAND AND MANAGE Bt TOXIN RESISTANCE

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ABSTRACT

Bacillus thuringiensis is the most successfully used biological control agent for both agriculturally and medically important insects in the orders of Lepidoptera, Diptera and Coleoptera. Prolonged application of Bt formulations and planting of Bt transgenic plants has been created the threat of evolution of Bt resistance in field insect populations. The mechanism of Bt-resistance has been an important focus of study since the discovery of Bt-resistance in the insects. Various mechanisms have been suggested in laboratory selected Bt-resistant insects like reduced solubilization, altered midgut proteinases, excessive degradation by the midgut proteinases (Ferre and Van Rie, 2002) and alteration of midgut trypsin activities has been observed in Bt-resistant strains of Plodia interpunctella and Ostrinia nubilalis (Oppert et al., 1997).

KEY WORDS: Bacillus thuringiensis, cry toxin, resistance, RNAi

INTRODUCTION

Bacillus thuringiensis a biopesticide, commonly known as Bt, is a naturally occurring, gram-positive, spore-forming soil bacterium. Bt has been known to be reservoir of several insecticidal proteins, such as d-endotoxins, cytolytic proteins and vegetative insecticidal proteins, etc. Among these, d-endotoxins have been more efficiently utilized for protection of a variety of crops from various insect-pests. To the date, Bt resistant populations of agriculturally important insect pests like Diamond Back Moth, *Plutella xylostella*, and the cabbage looper, *Trichoplusia ni*, have been identified in the fields or commercial vegetable greenhouses, where sprayable Bt formulations were applied. In the case of *Plutella xylostella*, Bt products became widely used in large scale commercial crucifer vegetable production when resistance too many other insecticides had rendered them useless. The evolution of Bt-resistant insect populations has prompted an urgent need to understand the mechanisms and the genetic basis for Bt resistance in various insect species of agricultural importance in order to provide the fundamental knowledge needed for the development of strategies for Bt resistance management. RNA interference (RNAi), initially coined by (Fire *et al.*, 1998), is the sequence-specific gene silencing induced by double-stranded RNA (Wall and Shi, 2003). Crops can be modified by engineering novel RNAi pathways that create small RNA molecules to alter gene expression in crops or plant pests. RNAi can generate new crop quality traits or provide protection against insects, nematodes and pathogens without

introducing new proteins into food and feed products. New methods are needed to identify RNAi crops and measure the environmental persistence of small RNAs. Recently, RNAi is used as a tool to understand and manage Bt toxin resistance. Disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked to high levels of resistance to the Bt toxin Cry1Ac in the cotton pest *Heliothis virescens* (Gahan *et al.*, 2001). The susceptibility to the Bt toxin Cry1Ab was reduced by cadherin gene silencing with RNA interference in *Manduca sexta*, confirming cadherin's role in Bt toxicity. Native Cry1A toxins required cadherin to form oligomers, but modified Cry1A toxins lacking helix α-1 don't require cadherin. The modified toxin killed cadherin-silenced *M. sexta* and Bt-resistant *Pectinophora gossypiella* that had cadherin deletion mutations (Soberon *et al*., 2007). Silencing of all three aminopeptidases N (APNs) in *Diatraea saccharalis* in vivo by RNAi resulted in a decrease in Cry1Ab susceptibility. There was reduction in expression of the three APNs is functionally associated with the Cry1Ab resistance in *D. saccharalis* (Yang *et al.*, 2010). *C. elegans* resists Cry5B is by loss of function mutations in any one of four gylcosyltransferase genes that glycosylate glycolipids specific to arthropods (Barrows *et al.*, 2007). Lack of bre-5 in the intestine led to resistance to the Bt toxin Cry5B. Wild-type but not bre-5 mutant animals were found to uptake toxin into their gut cells, consistent with bre-5 mutants lacking toxin-binding sites on their apical gut. bre-5 mutants displayed resistance to Cry14A, a Bt toxin lethal to both nematodes and insects; this indicated that resistance by loss of carbohydrate modification is relevant to multiple Bt toxins (Griffts *et al.*, 2001). Mutation of an APN in Cry1Ac-resistant H. armigera also have been reported (Herrero *et al.*, 2005 and Zhang *et al*., 2009).

Mechanism of RNAi

RNAi is mediated by 21-23 nucleotides small interfering RNAs (siRNAs) which are produced from long double-stranded RNAs by RNAse II-like enzyme dicer (Fig. 1). The resulting siRNAs are incorporated into a RNA-induced silencing complex (RISC) that targets and cleaves mRNA complementary to the siRNAs. This active RISC then targets homologous transcript by base pairing interactions and cleaves RNA of 12 nucleotides from 3' terminus of siRNA.

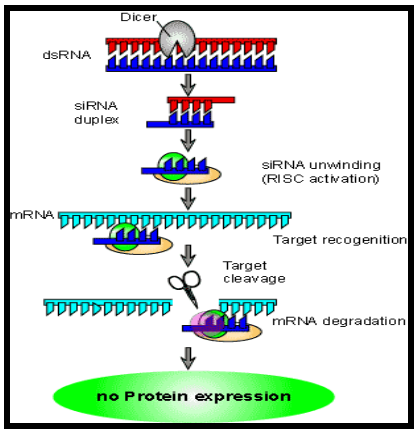

Figure 1: SiRNA Mediated RNA interference

Mode of action

Bt transgenic for environment friendly insect pest management

The crystalline Cry proteins are inactive until they get solubilised by proteases in the insect's midgut at high pH (>9.5), releasing proteins called delta-endotoxins. The toxicity is derived from the N-terminal half of the protein, which is composed of seven antiparallel alpha-helices. When insects ingest the crystalline inclusions, they are solubilised under alkaline conditions of insect midgut releasing the delta-endotoxins, which leads to proteolytic activation, exhibit a highly specific insecticidal activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvillae of susceptible insects. Alpha-helices penetrate the membrane to form an ion channel in the apical brush border membrane (Fig. 1). The formation of toxin-induced pores in the membrane allows rapid flux of ions. This disruption of gut integrity leads to death of the insect due to starvation or septicaemia.

Two different hypotheses for the mode of action of these toxins have been proposed, the first one pore formation and the second one is signal transduction (Bravo *et al*., 2007) (Fig. 3). The first steps in both models are similar: the toxin crystals are ingested by the larvae and solubilized in the gut into protoxins. These are cleaved by midgut proteases to give rise to a 60-kDa 3D Cry toxin that includes a helix α-1 at the N-terminal end. The activated toxin is able to bind to a cadherin receptor that is located in the microvilli of the midgut cells.

A. Pore formation

The pore-formation model proposes that the interaction with cadherin facilitates further proteolytic cleavage, resulting in the oligomerization of the toxin (Bravo *et al.*, 2004). The toxin oligomer then binds to secondary receptors, which are proteins, anchored to the membrane, by a glycosylphosphatidylinositol (GPI)-anchor, such as aminopeptidase N in *Manduca sexta* or alkaline phosphatase in *Heliothis virescens* (Jurat-Fuentes and Adang, 2006, Knight *et al.*, 1994 and Bravo *et al.*, 2007). In a final step, the toxin oligomer inserts into lipid raft membranes, where it forms pores and subsequently causes cells to burst, resulting in the death of the larva.

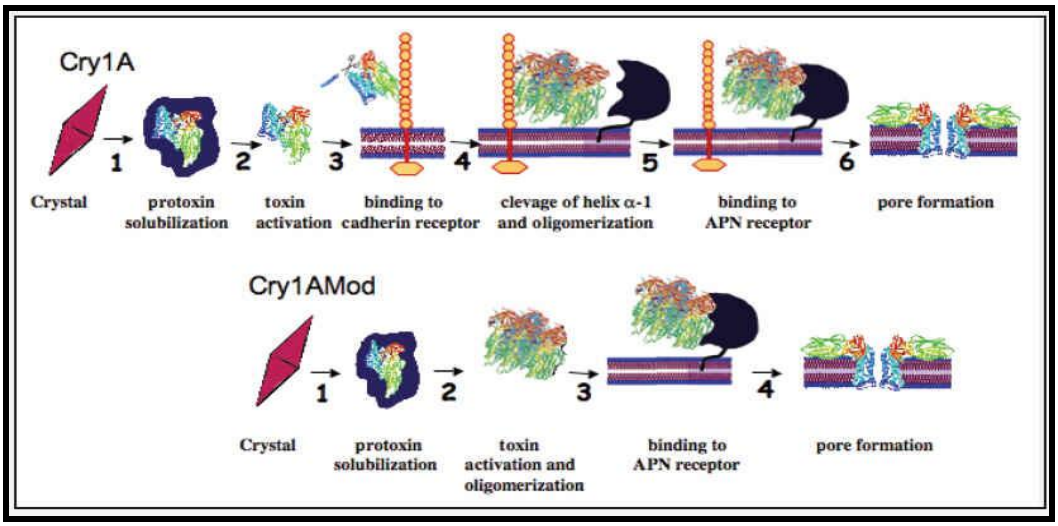

Figure 2: Mode of Action of Cry 1A toxin compared to CryMod1A toxin (Soberon *et al.*, 2007)

B. Signal Transduction

In signal transduction model, the binding of Cry1A to cadherin is assumed to trigger a cascade pathway involving the stimulation of a G protein and adenylate cyclase to increase cAMP, resulting in the activation of protein kinase A, which in turn leads to oncotic cell death (Zhang *et al*., 2006).

Recently, the midgut membrane-bound alkaline phosphatase from *H. virescens* has been identified as a receptor for Bt toxin Cry1Ac.The midgut APNs, the alkaline phosphatase is also GPI-anchored to the midgut brush border membranes and the terminal GalNAc on the alkaline phosphatase is the binding site for the toxin. Most important, the decreased level of the alkaline phosphatase in the midgut directly correlated with resistance in *H. virescens* to Bt.

Midgut cadherins are known as Bt toxin receptor proteins in the insects. Mutations of the cadherin gene in two insect species have been identified to be linked with resistance to the Bt toxin Cry1Ac. In a laboratory-selected Bt resistant *H. virescens* strain, the resistance was found to be associated with the disruption of the cadherin gene by insertion of a retrotransposon. More recently, cadherin mutations have also been identified in Bt resistant *P. gossypiella* strains. In these resistant strains, three mutant alleles (deletion mutants) were identified to be linked with the resistance to Cry1Ac

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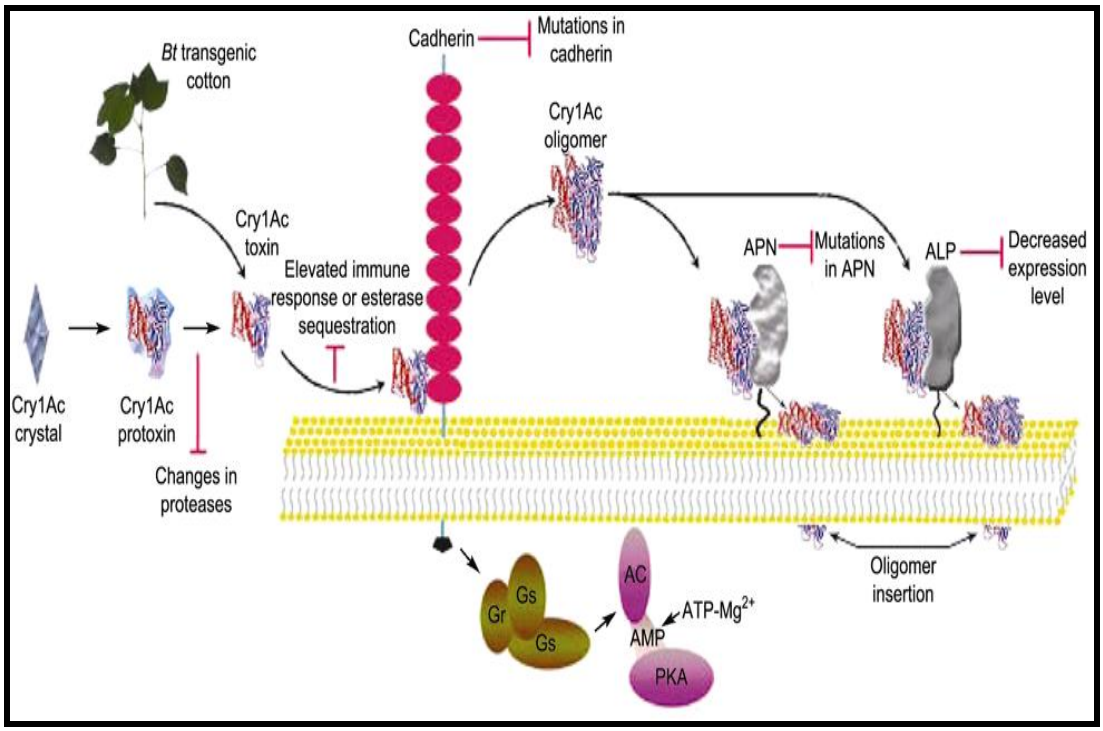

Figure.3: Models showing the mode of action of Cry toxins and resulting mechanisms of insect resistance. Two different mechanisms have been proposed: pore-formation model (top) and signal transduction model (bottom) (Liu *et al.*, 2010)

Mechanism of Bt toxin resistance

Prolonged application of Bt formulations and planting of Bt transgenic plants have created the threat of evolution of Bt resistance in field insect populations. Various mechanisms have been suggested in laboratory selected Bt-resistant insects.

1. Solubilization of Cry protein crystals in the midgut is a factor which determines the toxicity in insects. Therefore, reduced solubilisation could be a potential mechanism for Bt-resistance in insects.
2. Midgut digestive proteinases are critically involved in both activation and inactivation (degradation) of Bt toxins in the midgut. Excessive degradation of Bt toxin by the midgut proteinases could contribute to low toxicity of Bt toxins in insensitive or Bt-resistant insect hosts.
3. Insufficient activation of Bt toxins by midgut serine proteinases can also be a mechanism for Bt resistance. The alteration of midgut trypsin activities has been observed in Bt-resistant strains of *P. interpunctella* and *O. Nubilalis.*(Li *et al.*, 2004)
4. Peritrophic membrane (PM) plays a role in the toxicity of Bt toxins in insects and can be an important factor for the toxicity of Bt. It has been shown that disruption of the PM with a PM protein- specific metalloprotease could increase the toxicity of Bt in insects. Recently, trapping of Bt toxin Cry1Ac in the PM was discovered in a Cry1Ac-resistant strain of *Bombyx mori*.

RNAi is a tool to understand Bt toxin resistance

Many strategies were proposed for the construction and deployment of Bt plants to reduce the likelihood of resistance development. These included regulating the expression level of the Bt protein, using one or more Bt genes in the same plant, having the plant express the Bt protein only at specific times or in specific tissues, and using non-Bt plants as a refuge for susceptible alleles in the insect population. Because of the lack of field-developed resistance to the commercially available Bt plants (corn and cotton), however, it has been difficult to test some of the theoretical models used to evaluate these various management option. So RNAi is the new approach to understand Bt toxin resistance. This is the first functional demonstration of insect aminopeptidase-N of *H. armigera* being a receptor of Cry1Ac protein of *B. thuringiensis* (Swaminathan *et al.*, 2007).

By silencing the particular receptor protein, it's easy to understand Bt toxin resistance. Insecticidal crystal proteins of *Bacillus thuringiensis* bind to receptors in the midgut of susceptible insects leading to pore formation and death of the insect. The identity of the receptor is not clearly established. Recently a direct interaction between a cloned and heterologously expressed aminopeptidase (slapn) from *Spodoptera litura* and the Cry1C protein was demonstrated by immunofluorescence and *in vitro* ligand blot interaction (Rajagopal *et al.*, 2002). Here showed that administration of slapn double stranded RNA to *S. litura* larvae reduces its expression. As a consequence of the reduced expression, a corresponding decrease in the sensitivity of these larvae to Cry1C toxin was observed. The gene silencing was retained during the insect's moulting and development and transmitted to the subsequent generation albeit with a reduced effect. These results directly implicate larval midgut aminopeptidase N as receptor for *Bacillus thuringiensis* insecticidal proteins. APN and cadherin are involved in Cry1Ac intoxication of *H. Armigera* (Liu *et al.*, 2010).

In *Spodoptera frugiperda*, SfT6 has been identified in a subtracted cDNA library of *Spodoptera frugiperda* larval midgut

transcripts as a serine-protease gene down regulated within 24 h of intoxication with *Bacillus thuringiensis* Cry1Ca1 protein. In the present study, the specific role of SfT6 during Cry1Ca1 intoxication was investigated by RT-PCR and in vivo RNA interference. Quantitative real-time RT-PCR analysis showed SfT6 mRNA levels in the midgut tissue were significantly reduced after injecting or feeding 4th-instar larvae with specific long-size dsRNA. Gut juice-mediated in vitro protoxin activation and susceptibility for Cry1Ca1 were investigated in Sft6-knockdown larvae and compared with control treated with nonspecific dsRNA. Results demonstrated that SfT6 plays a determinant role in Cry1Ca1 toxicity against *S. frugiperda* since a decreased expression caused a reduced protoxin activation by larval gut juice and reduced susceptibility of insects to toxin in bioassays. It is proposed that SfT6 down-regulation occurring at the early stages of Cry1Ca1 intoxication is part of a complex and multifaceted defensive mechanism triggered in the insect gut to withstand *B. thuringiensis* pathogenesis (Rodriguez-Cabrera *et al.*, 2010).

Aminopeptidase N (APN) proteins located at the midgut epithelium of some lepidopteran species have been implicated as receptors for insecticidal proteins from *Bacillus thuringiensis*. cDNAs of three APN isoforms, DsAPN1, DsAPN2, and DsAPN3, from Cry1Ab-susceptible (Cry1Ab-SS) and -resistant (Cry1Ab- RR) strains of the sugarcane borer, *Diatraea saccharalis* (Lepidoptera: Crambidae), were identified and sequenced using reverse transcriptase polymerase chain reaction (RT-PCR) and 50 rapid amplification of cDNA end (50 RACE). The characteristic APN sequence features were derived from deduced amino acid sequences of the cloned cDNAs. cDNA sequences of the three APN genes were identical between the Cry1Ab-SS and -RR strains. However, total APN proteolytic activity and gene expression of the three APNs from Cry1Ab-RR larvae were significantly lower than those of the Cry1Ab-SS strain. RNA interference (RNAi) was employed using an oral droplet feeding technique for the three APNs of the Cry1Ab-SS strain. Down-regulating expressions of the three APN genes by RNAi were corresponding to the reductions in the specific APN activity. In addition, silencing of all three APNs in *D. saccharalis* in vivo by RNAi resulted in a decrease in Cry1Ab susceptibility.

Disruption of a cadherin-superfamily gene by retrotransposon-mediated insertion was linked to high levels of resistance to the Bt toxin Cry1Ac in the cotton pest *Heliothis virescens*. The cadherin protein Bt-R1a is a receptor for *Bacillus thuringiensis* Cry1A toxins in *Manduca sexta*. Cry1Ab toxin is reported to bind specific epitopes located in extracellular cadherin repeat (CR) 7 and CR11 on Bt-R1a, CR12 is the essential Cry1Ab binding component on Bt-R1a that mediates Cry1Ab induced cytotoxicity (Gang *et al*., 2004).

CONCLUSION

Due to evolution, a one-size-fits all the solution to solve insect caused crop damage may never be achieved. Pests will be always find a new way to resist natural and genetically engineered toxic effects. RNAi technology is a safe alternative to the chemical pesticides because it is specifically target the genes in plant pests. In particular, figuring out which insect genes confer resistance will aid in making target dsRNAs. RNAi use in plants has the potential to be used with Bt crops and other pesticides in order to decrease damage and increase crop yield.

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