

Bt Resistance – Characterization and
Strategies for GM Crops Expressing
Bacillus thuringiensis Toxins

Edited by

Mario Soberón, Yulin Gao and Alejandra Bravo



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CABI
Nosworthy Way
Wallingford
Oxfordshire OX10 8DE
UK

CABI
38 Chauncy Street
Suite 1002
Boston, MA 02111
USA

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: info@cabi.org
Website: www.cabi.org

T: +1 800 552 3083 (toll free)
E-mail: cabi-nao@cabi.org

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7 Roles of Insect Midgut Cadherin in Bt Intoxication and Resistance

Jeffrey A. Fabrick^{1*} and Yidong Wu²

¹USDA Agricultural Research Service, US Arid Land Agricultural Research Center, Maricopa, Arizona, USA; ²Department of Entomology, Nanjing Agricultural University, Nanjing, People's Republic of China

Summary

Genetically engineered crops producing *Bacillus thuringiensis* (Bt) proteins for insect control target major insect pests. Bt crops have improved yields and their use reduces the risks associated with the application of conventional insecticides. However, the evolution of resistance to Bt toxins by target pests threatens the long-term success of such transgenic crops. Insects resistant to Bt Cry toxins have been selected in the laboratory and field-evolved resistance has been reported for economically important insects in several regions of the world. Although the mechanisms of resistance have not been reported for all cases, the most common mechanism involves changes in larval midgut target sites that probably reduce binding to Bt toxins. The binding of Cry toxins to midgut cadherin represents an important step in Bt intoxication for many insects and mutations in the cadherin gene can result in resistance to Bt toxins. Here, we highlight the roles that insect midgut cadherins play in Bt Cry intoxication and review cases where changes in cadherin are involved with resistance to Cry toxins. Furthermore, we emphasize the importance of understanding the underlying molecular basis of Bt intoxication and resistance to Bt, and the implications of fundamental knowledge for resistance management strategies.

7.1 Introduction

The cadherins constitute a large family of cell surface transmembrane proteins, conserved among metazoan organisms, that play fundamental roles in development, morphogenesis, cell sorting and migration, cell signalling and the maintenance of structural integrity (Hulpiau and van Roy, 2011). Specific functions associated with cadherins include embryonic cell layer separation and the formation of tissue boundaries, synapse formation, neuron growth and connectivity, the establishment of cell polarity, mechanotransduction, cell adhesion, cell signalling and physical homeostasis (Halbleib and Nelson, 2006; Brasch *et al.*, 2012).

Given their breadth of function, it follows that cadherins are produced in various shapes and sizes. Here, we provide an overview of vertebrate and invertebrate cadherins. We specifically highlight the roles that midgut cadherin receptors for *Bacillus thuringiensis* (Bt) toxins, i.e. BtR cadherins (or BtRs), play in Bt Cry intoxication. Furthermore, we review cases where changes in BtR cadherins are associated with resistance to Cry toxins and how a comprehensive understanding of Bt intoxication and resistance has implications for designing resistance management strategies to Bt crops and biopesticides.

* Corresponding author. E-mail address: jeff.fabrick@ars.usda.gov

7.2 The Cadherin Superfamily

Cadherins are defined by the presence of an extracellular region consisting of cadherin repeat (CR) domains, a transmembrane domain and an intracellular cytoplasmic (IC) domain. The extracellular region includes a variable number of CRs, which contain the conserved motifs that include alanine (Ala), arginine (Arg), asparagine (Asn), aspartate (Asp), glutamate (Glu), phenylalanine (Phe) and proline (Pro), as well as variable amino acids (X): Asp-Arg-Glu; Asp-X-Asn-Asp-Asn-Ala-Pro-X-Phe; and Asp-X-Asp (Takeichi, 1990). Each of these CRs consists of about 110 amino acids and forms a unique immunoglobulin-like β -sandwich fold (Fig. 7.1a). The interface between these CR domains harbours calcium-binding sites that are important for the adhesive properties of cadherin, whose name arises from the contraction of 'calcium-dependent adherent protein'. The calcium-binding sites rigidify the ectodomain structure, which is important for dimerization and protection from proteolysis (Takeichi, 1991) (Fig. 7.1b).

The cadherin superfamily includes hundreds of members across species ranging from unicellular organisms to vertebrates. Vertebrate cadherins are classified into several families, including the classical cadherins, desmosomal cadherins, truncated cadherins, protein kinase cadherins, protocadherins, FAT-like cadherins, the seven-pass transmembrane/Flamingo cadherins and the *calsyntenins* (Takeichi, 2007) (Fig. 7.2a).

Classical cadherins are single-span (pass) transmembrane proteins composed of five extracellular CR domains and a conserved cytoplasmic domain. They confer calcium-dependent cell-cell adhesion through adhesive dimerization both with cadherins within the same plasma membrane (*cis* dimers) and with those from opposing cells (*trans* dimers) (Fig. 7.1b) (Brasch *et al.*, 2012). These extracellular interactions mediate homophilic cell adhesion through intracellular interactions of the cadherin cytoplasmic domain with proteins and

cytoskeletal components, such as actin filaments (Fig. 7.1c) (Brasch *et al.*, 2012).

While vertebrate classical cadherin function is well studied, members from other cadherin families perform numerous essential functions, including cell adhesion (desmosomal cadherins, truncated cadherins, protocadherins, FAT-like cadherins), tissue morphogenesis (protocadherins, FAT-like cadherins), cell signalling (protocadherins, protein kinase (PK) cadherins and *calsyntenins*), cell polarization (FAT-like cadherins, seven-pass transmembrane cadherins) and others (Morishita and Yagi, 2007). Hence, cadherins are likely to have originated to mediate mechanical cell-cell adhesion in simple organisms and their activities subsequently diversified, with parallel increases in their numbers and structural variations, to morphogenetic processes required in more complex organisms (Morishita and Yagi, 2007).

Despite their importance for multicellularity, cadherins are lacking from non-metazoan multicellular organisms (e.g. fungi and plants) (Abedin and King, 2008). Cadherins are found in the unicellular choanoflagellates, which are the earliest predecessors of metazoans (Abedin and King, 2008). Whereas mammalian genomes have over 100 genes belonging to the cadherin superfamily, but basal metazoan organisms generally have fewer cadherin genes. For example, a lancelet, a sea anemone and a placozoan have 30, 16 and eight cadherin genes, respectively (Hulpiau and van Roy, 2011). The *Caenorhabditis elegans* genome encodes 13 cadherins, including representatives of the major cadherin families that are conserved between insects and vertebrates: the classic, FAT-like, Flamingo and *calsyntenin* families (Pettitt, 2005). Within insects, a total of 17 cadherin genes are found in *Drosophila melanogaster* (Hill *et al.*, 2001), 38 genes in *Anopheles gambiae* (Moita *et al.*, 2005), 19 in *Tribolium castaneum* (B.S. Oppert, Kansas, 2014, personal communication) and 13 in *Bombyx mori* (Duan *et al.*, 2010).

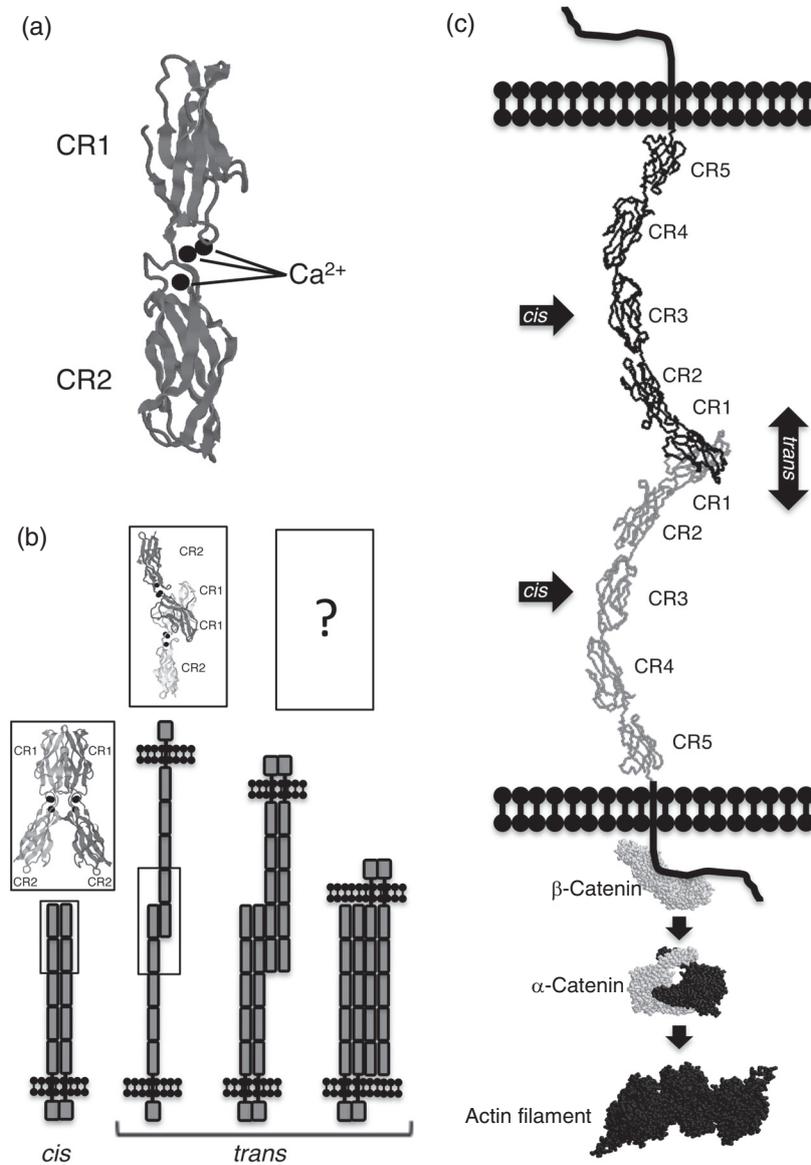


Fig. 7.1. Structure of the cadherin repeat domain and the architecture of classical cadherins forming adhesive dimers. (a) Cadherin repeats (CRs) assume an immunoglobulin-like β -sandwich fold; these occur in tandem and are separated by a linker region that harbours three calcium-binding sites (*Mus musculus* E-cadherin; Protein Data Bank (PDB) accession number (ID), 2QVF). (b) Vertebrate classical cadherins form *cis* and *trans* dimers. A *cis* dimer consists of two cadherin molecules laterally associated within the same plasma membrane (example shown is *M. musculus* E-cadherin; PDB ID, 1EDH), whereas cadherins that associate from opposing cells are *trans* dimers (example shown is *Gallus gallus* VE-cadherin; PDB ID, 3PPE). Both *cis* and *trans* interactions mediate homophilic cell adhesion. Although structures are not yet available (indicated by question mark), the extent of lateral overlap between the extracellular regions may increase the magnitude of the intercellular contact and simultaneously decrease the intercellular distance. (c) Cadherins bridge the intermembrane space between cells and the cytoplasmic domain of classical cadherins interacts with the cytoskeleton through specific adapter proteins, such as the catenins (example shown for *M. musculus* N-cadherin; PDB ID: 3Q2W).

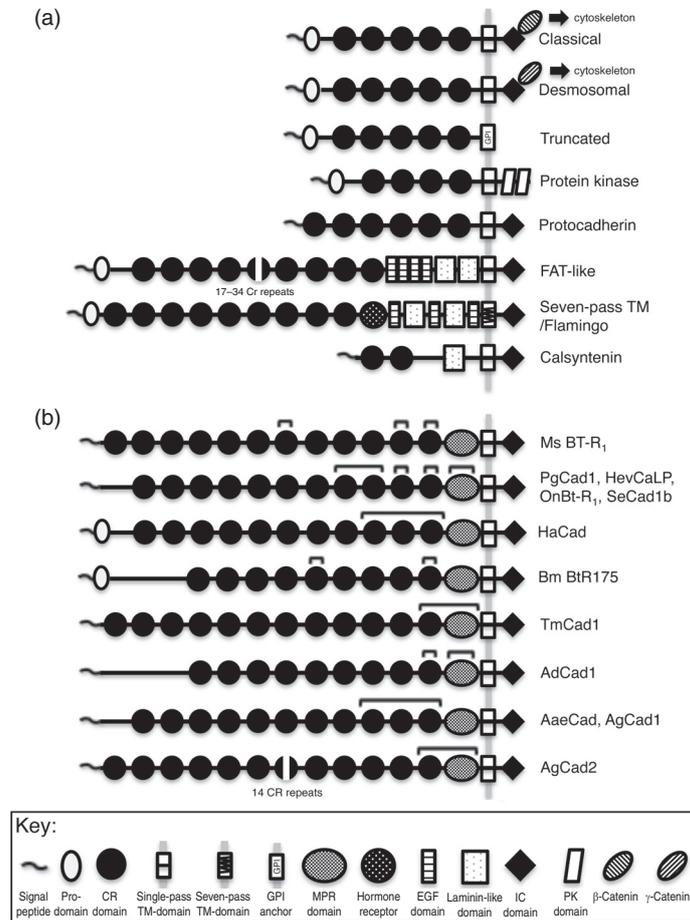


Fig. 7.2. Schematic domain organization of vertebrate cadherin families and BtR cadherins (BtRs) that function as *Bacillus thuringiensis* (Bt) Cry toxin receptors. (a) Cadherins are characterized by the presence of two or more extracellular cadherin repeat (CR) domains. Classical and desmosomal cadherins have five CR domains, but have distinct intracellular cytoplasmic (IC) domains that link through intermediate proteins to the intracellular cytoskeleton. Truncated cadherins resemble classical cadherins, but lack a transmembrane domain and attach to cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Protein kinase cadherins have four CR domains and an intracellular protein kinase (PK) domain involved in signal transduction. Protocadherins are the largest subgroup of cadherins and can have 5–27 CRs. FAT-like cadherins have a large extracellular region composed of 17–34 tandem CRs, epidermal growth factor (EGF) motifs, and laminin domains. Seven-pass transmembrane (TM) cadherins are anchored in the membrane by a seven-pass transmembrane domain and have nine CRs, EGF, laminin-like and Flamingo hormone receptor-like domains. Calsyntenins have two CRs, a single-pass TM domain and a cytoplasmic domain. (b) BtRs are receptors of Cry Bt toxins from Lepidoptera (*Manduca sexta* BT-R₁, Ms BT-R₁; *Pectinophora gossypiella* cadherin 1, PgCad1); *Heliothis virescens* cadherin, HevCaLP; *Ostrinia nubilalis* Bt-R₁, OnBt-R₁; *Spodoptera exigua* cadherin 1, SeCad1b; *Helicoverpa armigera* cadherin, HaCad; *Bombyx mori* BtR175, Bm BtR175), Coleoptera (*Tenebrio molitor* cadherin 1, TmCad1; *Alphitobius diaperinus* cadherin 1, AdCad1) and Diptera (*Aedes aegypti* cadherin, AaeCad; *Anopheles gambiae* cadherin 1, AgCad1 or BT-R₂; *An. gambiae* cadherin 2, AgCad2). BtRs all have similar predicted domain organization, including 9–14 CRs, a membrane proximal region (MPR), a single TM domain and a cytoplasmic domain. One or more BtRs are listed together because they share predicted domain structures. Brackets indicate experimentally determined toxin binding regions (TBRs) and are shown only for the first named BtR (even though experimental evidence for indicated TBRs may not be available or in agreement between all co-listed BtRs). The wide vertical grey lines represent the cell membrane.

7.3 Insect BtR Cadherins

The Lepidoptera, Coleoptera and Diptera possess phylogenetically unique cadherins, otherwise known as 'cadherin-like protein', 'Cad', 'CADR' or '12-cadherin domain' that were co-opted by the bacterium *B. thuringiensis* as a receptor of Bt endotoxins (see below). As already noted, here we refer to these insect midgut cadherins as 'BtRs'. BtRs do not align well with established vertebrate cadherin families. Although the *Manduca sexta* BT-R₁ was previously classified as an atypical cadherin from the protocadherin group (Midboe *et al.*, 2003), the sequence conservation between BtRs and protocadherins is extremely low. Furthermore, protocadherins represent the largest family of vertebrate cadherins (Morishita and Yagi, 2007). Consequently, the initial characterization by Vadlamudi *et al.* (1995) and Candas *et al.* (2002) of BT-R₁ as a 'new type of insect cadherin' remains accurate, with the BtRs representing a novel family of cadherins that evolved independently from other known cadherins.

7.3.1 Inherent BtR function

While the roles of BtRs as functional Bt receptors have been extensively studied (see below), the fundamental roles of these proteins in insect physiology are not known. Because of their shared sequence similarities with metazoan cadherins with described functions, it has been inferred that the BtRs have similar three-dimensional structures and therefore share roles in cell signalling, cell adhesion and the maintenance of cell integrity (Dorsch *et al.*, 2002; Midboe *et al.*, 2003). The *M. sexta* BT-R₁ has two cell-adhesion recognition sequences (HAV) and two cell-attachment sequences (RGD and LDV), from which it was inferred that it is a heterophilic surface adhesion protein with the capacity to bind other cadherins and/or other cell-adhesion protein partners (Dorsch *et al.*, 2002). However, no direct evidence for a role in cell adhesion has been demonstrated. Williams *et al.* (2011) provide indirect evidence of BtR involvement in the

maintenance of cell-cell integrity. They showed that pink bollworm (*Pectinophora gossypiella*) larvae harbouring mutations in the midgut cadherin PgCad1 exhibited greater permeation by the phytochemical gossypol than larvae lacking such mutations. Thus, the altered cadherin protein may facilitate increased uptake of the plant defence compound, perhaps through compromised cell integrity.

Expression of BtRs in *B. mori*, *M. sexta*, *An. gambiae* and *Aedes aegypti* larvae is primarily within the midgut epithelial microvilli, and correlates with Bt Cry toxin binding sites. The *B. mori* cadherin BtR175 is most abundant on columnar cell microvilli in the posterior midgut, but not in the lateral membranes where cell-cell contacts are prevalent (Hara *et al.*, 2003). BT-R₁ is localized at the base of microvilli throughout the entire midgut and at the apex of microvilli in the middle and posterior regions of the midgut (Chen *et al.*, 2005). Both *An. gambiae* cadherin 1 (AgCad1) and *Ae. aegypti* cadherin (AaeCad) are located primarily within the microvilli apices in the posterior midgut as well as in the *Ae. aegypti* apical gastric caecae, which co-localizes with Cry toxin binding (Hua *et al.*, 2008; Chen *et al.*, 2009). The expression of BtRs at the apical ends of microvilli suggests that these proteins may play a role beyond cell-cell contact, and resembles that of the *Drosophila* Cad99C, which specifically localizes to the apices of the microvilli apices of ovarian follicle cells (D'Alterio *et al.*, 2005). In this case, whereas the overexpression of Cad99C leads to a dramatic increase of microvillus length and the overproduction of microvilli bundles, the loss of Cad99C results in short, abnormal microvilli (D'Alterio *et al.*, 2005). Even though a number of insect species harbour cadherin mutations, and RNA interference (RNAi) has been used to reduce the production of cadherin protein, experiments have not demonstrated that changes in cadherins correlate with shortened/abnormal microvilli or altered junctions between cells.

Although BtRs are produced within midgut epithelium, only limited expression profiling and functional analyses have been

performed. Hence, BtRs may play unknown, pleiotropic roles throughout development and in different tissues. For example, the pink bollworm cadherin PgCad1 may have an additional cryptic function in reproduction, as mRNA is present in testes, and mutations in PgCad1 affect apyrene sperm transfer (Carrière *et al.*, 2009). What is more, Yang *et al.* (2009) showed that knock-down of *Plutella xylostella* cadherin by RNAi affects fecundity, egg hatch, pupal weight and adult eclosion, indicating cryptic functional roles of BtRs. Comprehensive expression profiling and functional analysis are needed to determine the inherent biochemical and physiological roles played by BtRs in insects.

7.3.2 Molecular and biochemical characteristics of BtRs

The genetic structures of lepidopteran BtR genes are highly conserved. Bel and Escrìche (2006) showed that the genomic structure for midgut cadherins from *Ostrinia nubilalis*, *Helicoverpa armigera* and *B. mori* consists of 35 exons joined by 34 introns. The pink bollworm cadherin gene (*PgCad1*) shares a similar exon/intron structural pattern, with 34 exons and 33 introns (Fabrick *et al.*, 2011), though the 5' untranslated region has not been assessed for the putative intron 1 identified in other lepidopterans (Bel and Escrìche, 2006). These genes are large, encompassing 19.6, 20.0 and 41.8 kb for cadherins from *O. nubilalis*, *H. armigera* and *B. mori*, respectively (Bel and Escrìche, 2006). *PgCad1* and the *M. sexta* cadherin (*BT-R₁*) are similarly large, as ascertained by Southern blot analysis (Franklin *et al.*, 1997; Fabrick *et al.*, 2011). While there is thought to be a single functional copy of the midgut cadherin gene in Lepidoptera, Franklin *et al.* (1997) implicated the presence of a second and related pseudogene in *M. sexta*. Furthermore, two *An. gambiae* cadherin genes, *AgCad1* (also named *BT-R₃*) and *AgCad2*, encode different BtR cadherin proteins that function as receptors for Cry4Ba and Cry11Ba, respectively (Hua *et al.*, 2008, 2013; Ibrahim *et al.*, 2013).

Transcriptional analysis of lepidopteran cadherin indicates that expression is primarily localized within the larval midgut epithelium (Midboe *et al.*, 2003; Carrière *et al.*, 2009). Cadherin transcripts and/or proteins are most abundant in the posterior of the midgut, where columnar epithelial cells are abundant and have well-developed microvilli that extend into the gut lumen (Hara *et al.*, 2003; Midboe *et al.*, 2003; Chen *et al.*, 2005, 2009; Aimanova *et al.*, 2006; Hua *et al.*, 2008).

BtRs are transmembrane proteins of 175–250 kDa composed of four domains: (i) an extracellular domain consisting of repetitive CRs, (ii) a membrane proximal region (MPR), (iii) a single transmembrane domain, and (iv) an IC domain (Fig. 7.2b). Several BtRs have been cloned and characterized, but all are predicted to have similar domain structures and vary primarily in the number of extracellular CR domains (Fig. 7.2b). *M. sexta* BT-R₁ was the first BtR to be cloned and characterized (Vadlamudi *et al.*, 1995; Dorsch *et al.*, 2002; Hua *et al.*, 2004b). BtRs that function as Bt Cry receptors were subsequently identified from three insect orders, including: HevCaLP from *Heliothis virescens* (Gahan *et al.*, 2001; Xie *et al.*, 2005); OnBt-R₁ from *O. nubilalis* (Flannagan *et al.*, 2005); HaCad from *H. armigera* (Wang *et al.*, 2005; Zhang *et al.*, 2012b); BtR175 from *B. mori* (Nagamatsu *et al.*, 1998a; Atsumi *et al.*, 2008); *PgCad1* from *P. gossypiella* (Morin *et al.*, 2003; Fabrick and Tabashnik, 2007); SeCad1b from *Spodoptera exigua* (Park and Kim, 2013; Ren *et al.*, 2013; Chen *et al.*, 2014); TmCad1 from *Tenebrio molitor* (Fabrick *et al.*, 2009); AdCad1 from *Alphitobius diaperinus* (Hua *et al.*, 2014); AgCad1 (also known as BT-R₃) and AgCad2 from *An. gambiae* (Hua *et al.*, 2013; Ibrahim *et al.*, 2013); and AaeCad from *Ae. aegypti* (Chen *et al.*, 2009).

Cadherin binding sites for Cry toxins map primarily to the CR domains adjacent to the membrane-proximal regions of the protein (Nagamatsu *et al.*, 1999; Gómez *et al.*, 2001, 2002a, 2003; Dorsch *et al.*, 2002; Hua *et al.*, 2004a, 2008; Wang *et al.*, 2005; Xie *et al.*, 2005; Fabrick and Tabashnik, 2007; Fabrick *et al.*, 2009; Ibrahim *et al.*,

2013) (Fig. 7.2b). Cry1A toxin binding to BT-R₁ is reported in three specific toxin binding regions (TBRs) or epitopes, including TBR1 in CR7, TBR2 in CR11 and TBR3 in CR12 (Dorsch *et al.*, 2002; Gómez *et al.*, 2002a; Hua *et al.*, 2004a) (Fig. 7.2b). At least two TBRs that bind Cry1Aa are found in BtR175, including one in CR5 (that has a homologous sequence to TBR1 of BT-R₁) and in CR9 (Nagamatsu *et al.*, 1999; Gómez *et al.*, 2001). Recombinant peptides from PgCad1 corresponding to CR8-CR9, CR10, CR11 and MPR bound to both activated Cry1Ac and Cry1Ac protoxin, indicating multiple TBRs in this BtR cadherin (Fabrick and Tabashnik, 2007). Among the coleopteran BtR cadherins, TBRs are found within the final CR domain and the MPR (Fabrick *et al.*, 2009; Hua *et al.*, 2014). AgCad, AaeCad and HaCad all have Cry toxin binding regions localized within their final three CR domains (CR9-CR11) (Wang *et al.*, 2005; Chen *et al.*, 2009; Ibrahim *et al.*, 2013). Hence, the critical TBR required for the binding and toxicity of Cry toxins is predominantly found in the final CR domain (Fig. 7.2b). The proximity to the membrane surface suggests that BtRs play a role in concentrating toxin at the membrane surface, which promotes oligomerization and pore formation (see Section 7.3.3).

Chen *et al.* (2007) discovered that a recombinant cadherin fragment corresponding to the CR12-MPR from BT-R₁ increased Cry1 toxicity when fed to several species of lepidopterous larvae. The TBR3 in CR12 was shown to be critical for toxin binding and toxin synergy (Chen *et al.*, 2007). Because the peptide alone was inactive against larvae and because it must be mixed with the Cry1 toxin before delivery, they hypothesized that the observed synergy resulted from an interaction of the toxin with the BtR fragment that either increased interaction with receptors or accelerated oligomerization and insertion into the membrane (Chen *et al.*, 2007). It has since been demonstrated that the peptide fragment

increases toxin oligomerization of Cry toxins, which may increase the formation of pre-pore oligomers and enhance cytotoxicity through pore formation (Gómez *et al.*, 2002b; Fabrick *et al.*, 2009; Liu *et al.*, 2009; Pacheco *et al.*, 2009b; Peng *et al.*, 2010).

Cry toxin potentiation by BtR peptides has been observed in lepidopterans, dipterans and coleopterans (Chen *et al.*, 2007; Hua *et al.*, 2008, 2013, 2014; Liu *et al.*, 2009; Pacheco *et al.*, 2009b; Park *et al.*, 2009; Peng *et al.*, 2010; Gao *et al.*, 2011). Some cadherin fragments can synergize Cry toxins in species other than those from which the BtR was isolated (Chen *et al.*, 2007; Park *et al.*, 2009; Gao *et al.*, 2011). In fact, the fragment corresponding to CR12-MPR from the *T. molitor* cadherin 1 (TmCad1) elicits enhances mortality and/or reduces the time to kill larvae from several unrelated beetle species (Gao *et al.*, 2011) and in a lepidopteran (Oppert *et al.*, 2008/2013). These results suggest similarities in the Cry toxin mode of action are shared among different insect orders.

Post-translational processing of BtRs is probably important for cadherin protein folding and function. BtRs have membrane signal peptides that are important for membrane localization on the cell surface (Nagamatsu *et al.*, 1998b; Wang *et al.*, 2005), though proteolytic removal of the signal peptide may not occur in all cases (Pigott and Ellar, 2007). Some BtRs may be produced as proproteins that require proteolytic processing for maturation (Pigott and Ellar, 2007). Candas *et al.* (2002) showed that calcium directly influences the structural integrity of BT-R₁, as bound calcium protects the ectodomain from proteolytic cleavage. This suggests that calcium-dependent protection and proteolytic processing may regulate the functional properties of the cadherin ectodomain. Furthermore, the glycosylation of BtRs may be important for their inherent function, and most likely contributes to minor discrepancies between calculated and observed molecular weights (Vadlamudi *et al.*, 1993; Nagamatsu *et al.*, 1998a).

7.3.3 Role of BtRs in Cry Bt toxin mode of action

Insecticidal crystalline (Cry) proteins from *B. thuringiensis* are widely used as biopesticides or produced in genetically engineered crops to control some major insect pests. Commercial Cry toxin-based products have been available for >50 years, despite limited knowledge of their modes of action. Recently, progress in characterizing Cry intoxication has implicated at least two mechanisms by which insect midgut cells are specifically killed. These include the sequential/pore formation model and the signal transduction model. Both models require the action of BtRs (see also Chapter 6, Bravo *et al.*).

The pore formation model

Although Cry toxins are used to target lepidopteran, dipteran and coleopteran pests, the pore formation mode of action is best characterized for Cry1 toxin in lepidopteran larvae (Gómez *et al.*, 2014) (Fig. 7.3). For Cry1 intoxication, the protein must be ingested either as an insoluble Cry protoxin contained as protein inclusion body within the Bt bacterium or as a soluble recombinant protein produced by a transgenic Bt plant. As the protoxin passes through the insect alimentary canal, it is solubilized within the alkaline environment and proteolytically activated by endogenous endopeptidases present in the midgut (Choma *et al.*, 1990). Activated monomeric Cry toxin binds with relatively low affinity to glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN) or alkaline phosphatase (ALP) proteins in the midgut epithelium (Gómez *et al.*, 2006; Pacheco *et al.*, 2009a). These binding interactions concentrate the activated toxin on the membrane where it binds with high affinity to the BtR receptor. This interaction with cadherin facilitates proteolytic removal of helix α -1 of domain I from the toxin and promotes the formation of toxin oligomeric structures, known as pre-pore oligomers (Gómez *et al.*, 2002b; Atsumi *et al.*, 2008). Pre-pore oligomers bind with high affinity to the GPI-

anchored receptors (APN and ALP), which are thought to aid the insertion of the pre-pore structure into the membrane, thereby causing pore formation and cell lysis (Pardo-López *et al.*, 2006). A recent extension to the pore formation model proposes the ABC transporter protein ABCC2 aids in irreversible insertion of the pre-pore oligomer into the membrane through coordinated opening and closing of the ABC transporter channel (Gahan *et al.*, 2010).

The signal transduction model

In the signal transduction model, the toxicity of Cry proteins is caused by activation of an intracellular pathway that leads to cell death (Zhang *et al.*, 2006, 2008; Ibrahim *et al.*, 2013) (Fig. 7.3). In this model, the steps leading up to cadherin binding are identical to those of the pore formation model, except that the Cry1A toxin monomer binds to the cadherin receptor and activates an intracellular Mg^{2+} -dependent signal transduction pathway (Fig. 7.3). The univalent toxin binding to cadherin stimulates heterotrimeric G protein and adenylyl cyclase (also known as adenylate cyclase) to increase intracellular cAMP. cAMP activates a protein kinase A that is thought to propagate a secondary messenger system that results in changes to the cytoskeleton and ion fluxing, ultimately leading to cell death (Zhang *et al.*, 2005, 2006).

Due to the technical limitations of working with intact insect midgut epithelium, neither model has been definitively validated within actual insect midgut tissue. The signal transduction pathway is based exclusively on experiments performed in non-midgut insect cell cultures and the induction of cell death signalling by Cry toxins and has not been verified *in vivo*. Further, while more empirical data has been amassed in support of the pore formation mode, no direct evidence has been provided for intact living cells or insects. So it is possible that neither model occurs *in vivo* and that variations or new undiscovered modes of action are functioning. Alternatively, both models may

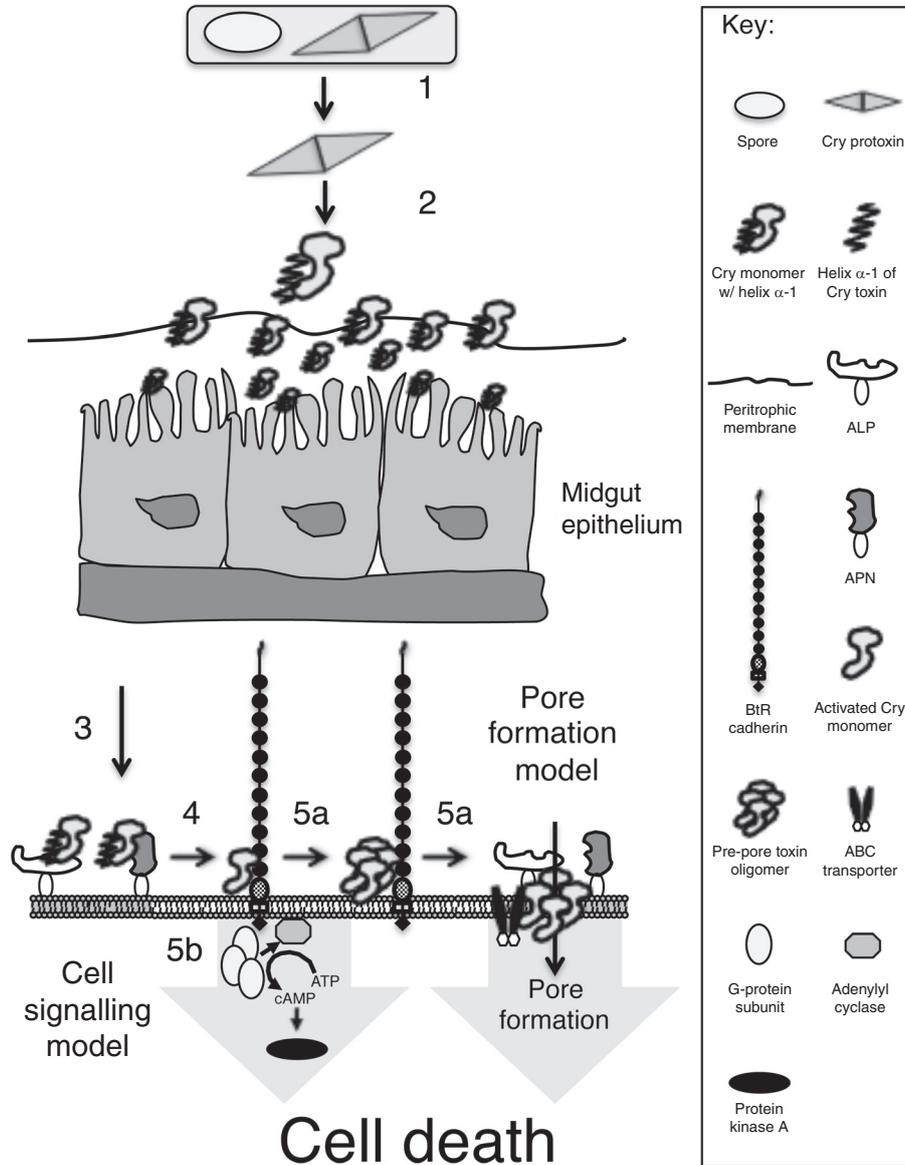


Fig. 7.3. The role of lepidopteran BtRs (*Bacillus thuringiensis* Cry toxin cadherin receptors) in the mode of action of Cry1 toxins. The five steps for Bt intoxication (1–5) include the following: (1) Ingestion of Bt Cry protoxin either solubilized from the Bt bacterium or from a transgenic Bt plant; (2) proteolytic activation of the protoxin by midgut endopeptidases; (3) binding of monomeric Cry1 toxin to GPI (glycosylphosphatidylinositol)-anchored aminopeptidase N (APN) and/or alkaline phosphatase (ALP); (4) binding of monomeric Cry toxin to BtR and proteolytic removal of the domain I α -helix; and (5), either (5a) oligomerization of Cry monomers and binding of the oligomer to GPI-anchored APN or ALP, which leads to insertion of the pre-pore toxin oligomer via ABC transporter into the cell membrane to form pores and ultimately leads to cell death (sequential/pore formation model), or (5b) binding of monomeric Cry toxin to cadherin, which activates an intracellular signal transduction pathway that leads to cell death (cell signalling model) (5b).

function simultaneously and some form of both mechanisms may be responsible for Cry intoxication. Hence, deciphering the mechanisms by which Bt toxins function is of utmost importance in order to implement better 'biorationale' (biologically rational) pest control tools, as well as to delay the evolution of resistance to the currently available Bt technologies.

7.4 BtRs and resistance to Bt toxins

7.4.1 BtR mutations

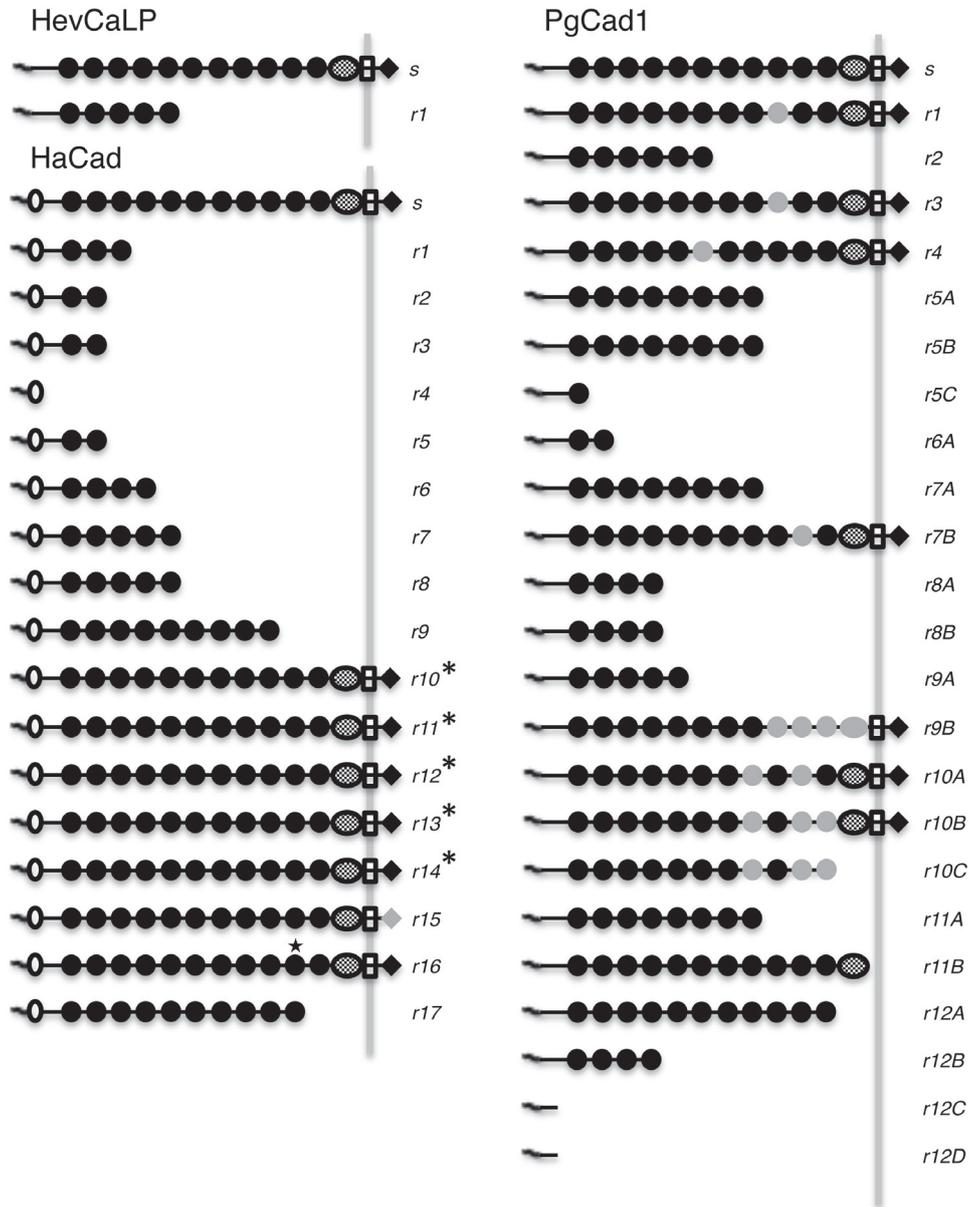
Although several mechanisms of resistance to Bt toxins are known, the most common type involves mutations that reduce the binding of Bt toxins to larval midgut proteins (Caccia *et al.*, 2010; Jurat-Fuentes *et al.*, 2011). Cadherin mutations that confer resistance to Bt toxins have been isolated from *H. virescens* (Gahan *et al.* 2001), *P. gossypiella* (Morin *et al.*, 2003; Fabrick and Tabashnik, 2012; Fabrick *et al.*, 2014) and *H. armigera* (Xu *et al.*, 2005; Yang *et al.*, 2007;

Zhao *et al.*, 2010; Zhang *et al.*, 2011, 2012a,b; Nair *et al.*, 2013) (Fig. 7.4).

Genetic mapping indicated that a BtR mutation from the YHD2 strain of *H. virescens* was genetically linked with Bt resistance (Gahan *et al.*, 2001). A tight linkage was observed between resistance to Cry1Ac in the *H. virescens* YHD2 strain and the resistance (*r*) allele *r1* of *HevCaLP* (Gahan *et al.*, 2001). The insertion of an LTR (long terminal repeat)-type retrotransposon disrupts *HevCaLP* and causes the introduction of a premature stop codon in the cadherin coding sequence. The truncated *HevCaLP r1* protein probably lacks regions important for toxin binding and membrane localization (Fig. 7.4).

Seventeen cadherin resistance alleles (*r1*–*r17*) are known from laboratory and field-collected *H. armigera* in China and India (Fig. 7.4). The *r1* mutation of *HaCad* (synonymous with *Ha_BtR*) was identified from the laboratory-selected GYBT strain (Xu *et al.*, 2005), and later from three resistant strains isolated from the field-selected Anyang population in Henan Province of northern

Fig. 7.4. Predicted BtR (*Bacillus thuringiensis* toxin cadherin receptor) proteins from *Heliothis virescens*, *Helicoverpa armigera* and *Pectinophora gossypiella* cadherin alleles. A single resistance (*r1*) allele from *H. virescens* in the USA was the first allele producing a mutant cadherin protein (*HevCaLP*) that was mapped and genetically linked with resistance to Cry1Ac Bt toxin (Gahan *et al.*, 2001). A total of 17 resistance alleles encoding mutated/truncated *H. armigera* cadherin (*HaCad*) proteins have been isolated from *H. armigera* in northern China and western India (Xu *et al.*, 2005, Yang *et al.*, 2007, Zhao *et al.*, 2010, Zhang *et al.*, 2012a,b, Nair *et al.*, 2013). The *HaCad r1*–*r15* alleles produce cadherin protein isoforms obtained from *H. armigera* in China. Products from *HaCad r10* to *r14* (indicated by *) differ from wild type *HaCad* protein only by amino acid substitutions, as the alleles have no premature stop codons, deletions or insertions. *HaCad r16* and *r17*, previously named by Nair *et al.* (2013), are here renamed from *r9* and *r10*, respectively. The *HaCad s* and *r16* protein sequences differ by three amino acid substitutions (R1296T, arginine to threonine at position 1296; R1308K, arginine to lysine at 1308; A1313S, alanine to serine at 1313) and *r16* has an insertion of a single codon encoding N1341 (asparagine at 1341, indicated by ★). Twelve cadherin resistance alleles (*r1*–*r12*) encoding mutant *P. gossypiella* cadherin (*PgCad*) proteins were isolated in the USA and western India (Morin *et al.*, 2003; Tabashnik *et al.*, 2004; 2005a; Carrière *et al.*, 2006; Fabrick and Tabashnik, 2012; Fabrick *et al.*, 2014). Four of these cadherin alleles (*r1*–*r4*) are genetically linked with resistance to Cry1Ac in laboratory-selected strains from Arizona and another eight (*r5*–*r12*) are associated with resistance to Cry1Ac Bt cotton in fields from western India. Nineteen different isoforms (*r5A*, *r5B*, etc.) of mutant *PgCad1* alleles *r5*–*r12* are shown. Predicted proteins are shown for cDNA of the susceptible (*s*) allele and resistant (*r*) mutant alleles. The amino-terminal membrane signal sequence, putative prodomain, cadherin repeats domain (CR), membrane proximal region (MPR), transmembrane (TM) region, and cytoplasmic domain (IC) are shown. Truncated structures indicate proteins predicted from cDNA with premature stop codons. Grey indicates missing regions of proteins caused by deletions. The wide vertical grey lines represent the cell membrane.



China (Zhang *et al.*, 2012a). The *r1* allele features a deletion in the *HaCad* gene corresponding to exons 8–24, which causes a frame shift and the introduction of a premature stop codon in the coding sequence (Xu *et al.*, 2005; Yang *et al.*, 2007). The coding sequences of alleles *r2*, *r3* and *r5–r8* are disrupted by the insertion of putative transposable DNA elements, and all encode a truncated *HaCad* protein (Yang *et al.*, 2007; Zhao *et al.*, 2010) (Fig. 7.4). Whereas *HaCad r9* is produced by incorrect mRNA splicing between exons 24 and 25 (Zhang *et al.*, 2012a), alleles *r4* and *r10–r14* have mutations causing amino acid substitutions (Zhao *et al.*, 2010, 2012a). Nair *et al.* (2013) identified two *r* alleles from *H. armigera* collected from western India. To avoid repetitive nomenclature, here we rename alleles *r9* and *r10* of Nair *et al.* (2013) to *r16* and *r17*, respectively. The coding sequence of the *r16* allele differs from that of the wild type susceptible (*s*) allele by the insertion of a single codon producing an additional amino acid residue at position 1340 in CR10 (Nair *et al.*, 2013). The *r17* mRNA encodes a truncated *HaCad* protein missing 413 amino acid residues beyond CR10 (Nair *et al.*, 2013). The *r15* allele isolated from field-selected *H. armigera* is unique because it is a non-recessive mutation resulting from the loss of 55 bp in the intracellular domain of *HaCad* (Zhang *et al.*, 2012b). All other cadherin mutations from *H. armigera* are recessive and affect extracellular regions. The *r15* mutation indicates that the cytoplasmic domain is important for Cry intoxication and therefore supports contributions by both the pore formation and signal transduction mechanisms in the efficacy of Bt toxins (Jurat-Fuentes and Adang, 2006; Zhang *et al.*, 2012b).

A total of 12 *PgCad1* resistance alleles were isolated in *P. gossypiella* from the USA and India (Morin *et al.*, 2003; Fabrick and Tabashnik, 2012; Fabrick *et al.*, 2014) (Fig. 7.4). The four US mutations (*r1–r4*) are genetically linked with resistance to Bt cotton producing Cry1Ac in laboratory-selected strains from Arizona (Morin *et al.*, 2003; Tabashnik *et al.*, 2004, 2005a; Carrière

et al., 2006; Fabrick and Tabashnik, 2012). While the *r2* allele has a deletion of 202 bp that introduces a premature stop codon, the others (*r1*, *r3* and *r4*) have only a single deletion of 24, 126 and 15 bp, respectively (Morin *et al.*, 2003; Fabrick and Tabashnik, 2012). The *r2* deletion results in a protein truncated following the CR6 domain; the protein products from the *r1*, *r3* and *r4* alleles lack 8, 42, and five amino acids, respectively. The 126 bp deletion from the *r3* allele results from the insertion of a non-LTR chicken-repeat retrotransposon (*CR1-1_Pg*) that causes splicing out of exon 21 from mRNA (Fabrick *et al.*, 2011). Recently, eight *PgCad1* resistance alleles (*r5–r12*) were isolated from *P. gossypiella* collected from Bt cotton fields in India (Fabrick *et al.*, 2014). From these eight alleles, a total of 19 transcript isoforms each containing a premature stop codon, a deletion of at least 99 bp, or both, were identified. Seven of the eight disrupted alleles involved alternative splicing of mRNA, which represents a novel genetic mechanism by which pests generate genetic diversity and accelerate the evolution of resistance to Bt crops (Fabrick *et al.*, 2014).

7.4.2 Reduced transcription/expression

Cadherin-based resistance to Cry1A toxin is associated with altered gene expression (due to spontaneous mutations and those introduced by the transposition of mobile DNA) and changes to mRNA (caused by alternative splicing and downregulation of transcription). Downregulation of BtRs is implicated in at least two cases of resistance. First, Bonin *et al.* (2009) found polymorphisms in *AaeCad* of *Ae. aegypti* that are consistent with positive selection for Bt resistance. Furthermore, cadherin gene expression was lower in a resistant strain of *Ae. aegypti* than in a susceptible strain (Bonin *et al.*, 2009). In the sugarcane borer, *Diatraea saccharalis*, Yang *et al.* (2011) showed that cadherin transcript levels were lower in a Cry1Ab-resistant strain than in a Cry1Ab-susceptible strain. Experiments

using RNAi to knock down BtR expression have demonstrated a corresponding decrease in susceptibility to Cry intoxication in several insects (Soberón *et al.*, 2007; Fabrick *et al.*, 2009; Yang *et al.*, 2011; Hua *et al.*, 2013; Park and Kim, 2013; Ren *et al.*, 2013; Chen *et al.*, 2014). These results support the role of BtRs as functional receptors of Cry toxins and implicate reduced expression as a mechanism of resistance.

7.4.3 DNA screening of BtR mutations

The monitoring of pest populations for field-evolved resistance to Bt crops is an important component of management strategies to delay the onset of resistance. Laboratory bioassays of target pests collected from Bt crops and surrounding non-Bt hosts are the primary tools used to detect field-evolved resistance (Tabashnik *et al.*, 2009).

A complementary approach to laboratory bioassays is DNA-based molecular monitoring for known resistance marker genes. PCR-based DNA screening for BtR resistance alleles was implemented for three major insect pests in Bt cotton, including *H. virescens* (Gahan *et al.*, 2007) and *P. gossypiella* (Morin *et al.*, 2004) in the USA and *H. armigera* in China (Zhang *et al.*, 2013). The screening of more than 7000 field-collected *H. virescens* failed to detect a single *r1* HevCaLP allele (Gahan *et al.*, 2007). Similarly, in the southwest USA, beginning in 2001, the screening of approximately 10,000 *P. gossypiella* for *r1*, *r2* or *r3* cadherin alleles indicated no resistance alleles in the field (Tabashnik *et al.*, 2005b, 2006, 2010; B.E. Tabashnik, Arizona, 2014, personal communication;). With *P. gossypiella*, PCR screening results were consistent with a low resistance allele frequency in the field as determined from diet bioassays, indicating that resistance to Bt cotton did not increase (Tabashnik *et al.*, 2005b, 2006, 2010). In *H. armigera* from northern China, DNA-based screening to detect the *r15* cadherin allele identified three field-collected specimens (out of 876) that were heterozygous at the *HaCad* locus (Zhang *et al.*, 2013). However,

the recent discovery of diverse cadherin mutations associated with resistance to Cry1Ac in field-selected populations of *P. gossypiella* in India (Fabrick *et al.*, 2014) and of *H. armigera* in China (Xu *et al.*, 2005; Yang *et al.*, 2006, 2007; Zhao *et al.*, 2010; Zhang *et al.*, 2012a) implies that the monitoring of resistance in some populations by screening cadherin DNA for specific resistance alleles may be ineffective.

The molecular and biochemical mechanisms of resistance to Bt toxins and the crops that produce them can be highly variable. Comprehensive examination of these mechanisms requires pest-specific investigation of biology, genetics, ecology, biochemistry and physiology from specific isolation sites. This requires enormous investment of time and capital resources, which is often impractical or unattainable, although technological and economic advances in functional genomics and next-generation sequencing offers promise for genome- or transcriptome-wide genetic screening of samples and more comprehensive detection and evaluation of pesticide resistance traits. Nevertheless, the significance of such technical advances in the screening of pest resistance to Bt crops remains uncertain.

7.5 Conclusion

Bt crops are planted on millions of hectares throughout the world. As a result, their benefits are realized by an ever-increasing populace. However, field-evolved resistance to Bt crops continues to threaten these long-term benefits (Tabashnik *et al.*, 2013). Thus, as reliance on Bt crops increases, the need to preserve existing technologies and/or find new complementary approaches is also intensified. In order to improve current strategies to delay resistance, we must better understand how Bt crops work and how resistance evolves. We are only now making strides towards understanding the intricacies of the mode of action of Bt and of Bt resistance at the molecular and biochemical level. For example, BtRs appear to

play a central role in Bt's mode of action and Bt resistance in several insects. Without such fundamental knowledge, advances such as the development of modified or 'Mod' toxins (reviewed in Chapter 14, Soberón *et al.*) and the identification of cadherin resistance alleles would not have been possible.

Fundamental questions remain on the mode of action of Bt, the effective implementation and preservation of Bt technologies and the evolution of pest resistance. Some questions lie within the apparent differences in mechanisms between insect species and can only be answered by in-depth knowledge of each specific case. These include the following: What roles do cadherin, ABCC, APN, and ALP play and do they play more prevalent roles in some insects than others? Will cadherin-based resistance or changes to steps in the Bt mode of action become prevalent in Bt fields? Can we implement strategies to delay the evolution of resistance? How will our understanding of the basis of resistance ultimately have an impact on efforts to design resistance management strategies?

In developed countries, the technology and infrastructure exist to design, build and implement transgenic strategies for pest control, but a major challenge is to find tools with unique modes of action that complement or replace the current generation of Bt crops. There are certainly untapped resources of novel plant protectant proteins (such as Bt toxins) yet to be found, but society also benefits from action to extend the life of safe and effective pest management tools, such as Bt crops, and to delay the evolution of resistance.

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