Chapter 8
Expression of *Bacillus thuringiensis* Toxins in Insect Cells

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Abstract Baculoviruses and *Bacillus thuringiensis* (Bt) are biological control agents used for the control of agricultural insect pests and also insect vectors of human diseases. The expression of Cry proteins in insect cells using recombinant baculoviruses has been shown to be an alternative means of production of these proteins for functional and/or structural studies. The combination of the insecticidal activity of Bt and baculovirus lethal infection also has the potential to improve viral pathogenicity toward their insect hosts. The easy manipulation of baculovirus genomes and the increased number of full baculovirus genome sequences available could facilitate the expression of Cry proteins and, besides improving their pathogenicity, also retard the development of resistant insects to both Cry proteins and virus replication. In this chapter, the construction of recombinant baculoviruses containing different *cry* genes (*cry1, cry2, cry4, cry10*, and *cry11*) and the expression of the corresponding Cry proteins in insect cells and insect larvae are described.

Keywords Baculovirus • *Bacillus thuringiensis* • Cry protein • Insect cells

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8.1 Baculovirus

Baculoviruses are large dsDNA insect viruses which have been highly studied due to their insecticidal activity toward agricultural insect pests (Ribeiro and Crook 1998; Moscardi 1999) and also due to their use as expression vectors for heterologous proteins in insect cells and insects (Miller 1997). These viruses belong to the Baculoviridae family of insect viruses and are divided into four genera (alpha-, beta-, gamma-, and deltabaculoviruses) depending on the similarity of various conserved proteins (Jehle et al. 2006; Rohrmann 2013). These viruses are also known as nucleopolyhedroviruses (NPVs) and granuloviruses (GV), and their main characteristics are the production of large amounts of occlusion bodies (OBs) in insect cells at late postinfection times. The nucleopolyhedroviruses (alpha-, gamma-, and deltabaculoviruses) produce OBs in the nuclei of infected cells, and granuloviruses (betabaculoviruses) produce smaller OBs inside the cell's nucleus and cytoplasm (Rohrmann 2013).

The most studied baculovirus to date is the *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) which is the type species of NPVs and was isolated from the alfalfa looper, *Autographa californica* (Rohrmann 2013). Baculoviruses are named by the initials of the first insect where they were isolated. However, these viruses can infect more than one host (Rohrmann 2013).

During the replication of AcMNPV in insect cells, two viral phenotypes are produced: the extracellular (ECV) or budded (BV) virus is formed in the initial phase of infection and is responsible for the spread of infection inside the insect host and the occlusion-derived virus (ODV) is responsible for the spread of infection among insects and is formed at late postinfection times in the nuclei of infected cells. ODVs are surrounded by an occlusion body made mainly of a single polypeptide called polyhedrin, forming the OB (also known as polyhedra) (Smith and Summers 1978).

The regulation of AcMNPV gene expression in susceptible insect cells is mainly divided into genes expressed before viral DNA replication (early phase) and those expressed after the onset of viral DNA replication (late phase). These can be subdivided into immediate early phase, where viral genes are expressed in the first few hours after infection, and in very late phase, where genes are expressed after 18 h of infection (Maruniak 1986). The immediate early phase is characterized by the expression of viral transcriptional factors and genes responsible for preparing the cell for viral DNA replication (Friesen 1997). During the late phase, high amounts of proteins responsible for the production of BV particles are produced (Lu and Miller 1997). In the very late phase of infection, proteins involved in the production of OBs are highly expressed. For instance, polyhedrin, a 30 kDa protein, as cited above, is the main protein of the OBs and is highly produced in this phase of infection (Rohrmann 1986; Jarvis 1997). Due to its high expression, most baculovirus expression vectors were developed to use the polyhedrin promoter in order to express recombinant protein in insect cells (O'Reilly et al. 1992).

OBs are responsible for the transmission of ODVs from insect to insect. When a susceptible insect ingests OBs, the polyhedrin protein is solubilized in the alkaline

environment of the midgut, and the ODV particles are released (Funk et al. 1997). The released ODVs will cross the perithrophic membrane and infect the columnar cells of the midgut (Horton and Burand 1993; Haas-Stapleton et al. 2004). After entering the midgut, the virus can replicate in the midgut cell producing new BV particles or be transported to the basal side of the cell and directly infect tracheal cells or hemocytes spreading the infection to other tissues inside the insect body where the virus will replicate, and in a few days, the infected insect dies full of OBs that are released in the environment (Rohrmann 2013).

8.2 Baculovirus Expression Vectors (BEVs)

Expression of heterologous proteins in insect cells or insects using recombinant baculoviruses has several advantages compared to other expression systems. For instance, insect cells are eukaryotic cells with most of the posttranslational machinery for the correct processing of complex eukaryotic proteins, such as glycosylation (O'Reilly et al. 1992; Jarvis 1997, 2003). Strong promoters like the polyhedrin gene promoter ensure high expression level of most proteins (Datal et al. 2005). This technology is based on plasmid transfer vectors for the transfer of foreign genes to the genome of the baculovirus by homologous recombination or site-specific transposition (Rohrmann 2013), and several vectors are available commercially (Ribeiro et al. 2015).

8.3 *Bacillus thuringiensis*

Biological alternatives to chemical control of insect pests have received more attention in the last few years due to widespread presence of resistant insects, the lack of development of new molecules, and the increase in regulatory restrictions for their use worldwide (https://www.epa.gov/science-and-technology/pesticides-science). There is a high diversity of microorganisms with entomopathogenic activity, and they represent a valuable resource for the development of new biological products (Perlak et al. 1990; Lacey et al. 2015).

Among these microorganisms, the bacterium *Bacillus thuringiensis* (Bt), has been shown to be an excellent choice since it is used efficiently for the control of different insect pests worldwide for decades (Bravo et al. 2011). Bt is known to produce crystalline inclusion bodies composed of insecticidal proteins called δ-endotoxins (Cry and Cyt proteins) and secreted toxins called Vip and Sip (Donovan et al. 2006). These inclusions vary in size and composition and confer distinct entomopathogenic spectra on different strains of this bacterium. When ingested by susceptible insects, the δ-endotoxin crystalline inclusions are, like the occlusion bodies of baculoviruses, solubilized in the alkaline environment of the midgut and proteolytically processed by midgut proteases. The processed proteins attach to membrane

receptors at the midgut columnar cells and induce cell lysis and eventually insect death (Schnepf et al. 1998; Bravo et al. 2007).

Hundreds of δ-endotoxin (*cry* and *cyt*) genes have been sequenced to date, and Cry proteins are classified in 74 groups organized in different subgroups; besides three groups of Cyt proteins based in their amino acid identity and due to the development of new sequencing technologies, the number of novel *cry* genes is still growing (Crickmore et al. 1998, 2016).

Cry proteins have shown toxicity to different insects and have the potential to be used for the control of agricultural insect pests and insect vectors of human and animal disease. However, some Bt strains produce Cry proteins without known insecticidal activity but with toxic activity toward human cancer cells. These proteins were named parasporins, and so far, six classes of parasporin have been identified and are classified as parasporin-1 (PS1), parasporin-2 (PS2), parasporin-3 (PS3), parasporin-4 (PS4), parasporin-5 (PS5), and parasporin-6 (PS6) (Okumura et al. 2016). According to the classification of Cry proteins, parasporins are classified as Cry31A (PS1), Cry41A (PS3), Cry45A (PS4), Cry46A (PS2), Cry63A (PS6), and Cry64A (PS5) (Ohba et al. 2009; Okumura et al. 2016).

Besides Cry proteins, Bt produces and secretes other proteins with insecticidal activity during vegetative growth toxic to coleopteran, hemipteran, and lepidopteran insects that are called vegetative insecticidal proteins (VIP) which do not form crystalline inclusion bodies (Chakroun et al. 2016). Both *vip* and *cry* genes are located on large Bt plasmids (Estruch et al. 1996). Since VIP proteins are produced in the initial Bt growth phase, the mixture of spore, crystals, and supernatant of the culture can be used for the formulation of a bioinsecticide product (Monnerat and Bravo 2000). VIP proteins have shown toxic activity toward coleopteran (Vip1 e Vip2) and lepidopteran (Vip3) insects (Warren et al. 1998). No insecticidal toxic activity has yet been shown for the recently identified Vip4 proteins (Palma et al. 2014).

8.4 Baculovirus and *Bacillus thuringiensis*

8.4.1 Cry1 Proteins

Cry1 proteins are expressed as protoxins during Bt sporulation with a molecular mass of around 130 kDa, forming bipyramidal-shaped crystals. These protoxins are proteolytically processed at their N- and C-terminal ends within the midgut of a susceptible insect to a toxic fragment around half of the size of the protoxin (around 60–65 kDa).

The full-length Cry1Ab and Cry1Ac proteins were the first Cry proteins expressed in insect cells using recombinant baculoviruses (Merryweather et al. 1990; Martens et al. 1990; Ribeiro and Crook 1993, 1998). These proteins were shown to be toxic to lepidopteran larvae and were detected in the cytoplasm of infected cells, but only the Cry1Ab proteins were shown to form inclusion bodies in the cytoplasm of infected cells. However, no improvement of the recombinant

virus insecticidal activity was shown. Truncated versions of the Cry1Ab protein containing the active portion of the protein were also successfully expressed in insect cells using recombinant baculoviruses and shown to be biologically active (Ribeiro and Crook 1993; Martens et al. 1995). However, again no improvement of the viral insecticidal activity was shown. Other Cry1 proteins were also expressed in insect cells using recombinant baculoviruses. For instance, a truncated version of the Cry1C protein (N-terminal 804 amino acids and around 65 kDa) was expressed in insect cells and shown to be toxic to *Spodoptera frugiperda* and *Anticarsia gemmatalis* larvae. This protein also produced cuboid-shaped crystals in the cytoplasm of infected cells. However, the effect on viral pathogenicity was not determined (Aguiar et al. 2006). The full-length Cry1I protein also formed cuboidal-shaped crystals when expressed in insect cells using recombinant baculoviruses (Martins et al. 2008) and shown to be toxic to an important coleopteran pest, the cotton boll weevil, *Anthonomus grandis* (Coleoptera: Curculionidae).

8.4.2 Cry2 Proteins

Cry2 proteins form cuboid-shaped crystals in Bt and have a molecular mass between 65 and 71 kDa (Höfte and Whiteley 1989). Cry2Aa and Cry2Ac have known toxic activity to lepidopteran and dipteran insects, and Cry2Ab is known to be toxic only to lepidopteran insects (Höfte and Whiteley 1989; Widner and Whiteley 1989; Dankocsik et al. 1990). Although Cry2Aa and Cry2Ab share high amino acid identity, a few amino acid differences determine their difference in toxicity (Widner and Whiteley 1989). Another aspect of these proteins is the presence of accessory proteins which are necessary for the high level of expression and crystallization of these proteins in Bt (Crickmore and Ellar 1992). Since these proteins bind to different receptors than Cry1 proteins in susceptible lepidopteran insects, they are used to construct transgenic crops expressing two or more Bt toxins that kill the same insect pest in order to delay evolution of pest resistance (Hernandez-Rodriguez et al. 2009; Gouffon et al. 2011; Carrière et al. 2015).

Cry2Aa and Cry2Ab genes from a Brazilian *B. thuringiensis* subsp. *kurstaki* S447 (Btk) strain were separately inserted into the genome of a baculovirus, and the recombinant viruses were used to infect insect cells and larvae (Lima et al. 2008). Both proteins were expressed and shown to have a molecular mass of around 65 kDa in SDS-PAGE of insect cell extracts. No crystals were detected in cultured *S. frugiperda* cells (Sf21) infected by the recombinant viruses. However, cuboidal crystals similar to the ones produced by Bt were seen in larval extracts from *S. frugiperda* infected with the recombinant viruses. These crystals were significantly larger than the ones produced by Bt. This was also seen in other Cry proteins expressed in insect cells (Ribeiro and Crook 1993; Aguiar et al. 2006). The size of the recombinant protein might be dependent on the space for the crystal to grow inside the bacterial or insect cell and/or the presence of host proteins. Since the production of Cry2 crystals in Bt is dependent on the expression of accessory proteins that might

be acting as chaperones, the production of Cry2 crystals in insect larvae cells might be due to the presence of host insect cellular chaperons that are not present in the Sf21 cell line. Recombinant Cry2Aa and Cry2Ab produced in insect cells were tested against *Anticarsia gemmatalis* and *S. frugiperda* and showed similar LC_{50} when compared to the crystals produced by *B. thuringiensis* subsp. *kurstaki* HD-1 (Lima et al. 2008).

8.4.3 Bti *Cry Proteins*

Bacillus thuringiensis subsp. *israelensis* (Bti) is widely used as biological insecticide to control mosquito vectors of human diseases, such as *Culex* spp., *Aedes* spp., and *Anopheles* spp. (Höfte and Whiteley 1989; Schnepf et al. 1998; Beltrão and Silva-Filha 2007; Harwood et al. 2015). At least five Cry proteins can be found in parasporal inclusions in sporulating Bti cells. These proteinaceous crystals are composed of distinguishable classes of δ-endotoxins: Cry11Aa, Cry10Aa, Cry4Aa, Cry4Ba, Cyt1Aa, and Cyt2Ba. The genes responsible for the expression of the crystal-forming proteins are located on a 128 kDa plasmid, pBtoxis (Berry et al. 2002). Genes involved in the expression of auxiliary proteins, P19 and P20, which can function as chaperones and participate in crystal formation, are also present in this megaplasmid (Manasherob et al. 2001). Cry and Cyt proteins have been found to act synergistically in the midgut of mosquito larvae and on cultured mosquito cell lines (Thomas and Ellar 1983; Poncet et al. 1995; Corrêa et al. 2012a, b). Cyt proteins are toxic to mosquito larvae, although the toxicity achieved is usually lower than that observed for Cry proteins (Chang et al. 1993). It has been demonstrated, for example, that Cyt1Aa may act as a primary receptor for Cry11Aa, facilitating the formation of oligomers of this protein (Pérez et al. 2005). Experiments conducted with Cyt1Aa and Cry4Ba also showed interaction between these two proteins (Cantón et al. 2010). Cyt2Ba protein also showed some level of interaction with Cry11Aa and Cry4Aa (Corrêa et al. 2012a, b).

As part of efforts to obtain isolated mosquitocidal Cry toxins for toxicity studies, the *cry4Aa* and *cry4Ba* genes obtained from two Brazilian Bti strains (S1806 and S1989) were separately inserted into the baculovirus AcMNPV genome by homologous recombination or site-specific transposition (Corrêa et al. 2013). Overall, Cry4Aa and Cry4Ba are closely related toxins which exhibit a high level of amino acid sequence identity. However, their proteolytically activated N-terminal portions show only around 55% of identity (Chungiatupornchai et al. 1988). While Cry4Aa is highly toxic to *Culex* and *Aedes*, its activity against *Anopheles* is low. On the other hand, Cry4Ba shows strong toxicity to *Aedes* and *Anopheles*, but *Culex* larvae are less susceptible to this toxin.

The recombinant baculoviruses produced Cry4Aa or Cry4Ba in insect cells (Corrêa et al. 2013). However, cytoplasmic Cry4Aa crystals from the recombinant

virus constructed using the site-specific transposition method seemed to be two- to threefold larger than those produced by homologous recombination. A plausible explanation for this difference in size may reside in the fact that the first recombinant virus lacked the polyhedrin gene. Thus, the synthesis of Cry4Aa crystals may be enhanced as the protein expression machinery of infected cells is not busy producing large amounts of polyhedrin. Morphological differences were observed between Cry4Aa and Cry4Ba crystals expressed in insect or in Bt cells. Cuboidal- or bipyramidal-shaped crystals were formed in insect cells, while spherical crystals are expressed in Bt cells (Schnepf et al. 1998). This structural difference can be related to interactions of heterologous Cry proteins with cellular proteins. Bioassays with Cry4Aa and Cry4Ba expressed in *S. frugiperda* larvae were performed with second instar *A. aegypti* larvae and showed that these proteins were toxic to this insect. Cry4Aa toxins produced by recombinant viruses constructed by either homologous recombination or transposition were equally toxic to *A. aegypti* with no significant difference in LC_{50} (Corrêa et al. 2013).

Cry10Aa is also a toxin produced by *B. thuringiensis* subsp. *israelensis* as a protoxin of around 80 kDa, which is proteolytically cleaved by insect gut proteases to a toxic fragment of around 60 kDa. Among the δ-endotoxins produced by Bti, Cry10Aa seem to be expressed in low levels and show no relevant toxic activity toward different mosquito species (Hughes et al. 2005; Hernández-Soto et al. 2009). The mosquitocidal activity of Bti was shown to be due primarily to the Cry4, Cry11, and Cyt proteins (Martins et al. 2007).

In order to test the toxicity of Cry10Aa against other insect species, Cry10Aa from a Brazilian *B. thuringiensis israelensis* strain (S1804) was expressed in insect cells using a recombinant baculovirus (Aguiar et al. 2012) and tested against *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), an important cotton pest. In contrast to the spherical crystals produced by the S1804 strain, the expressed Cry10A protein was produced as a cuboidal-shaped crystal in insect cells and shown to be highly toxic (LC_{50} of 7.12 μg/mL) to neonate larvae of *A. grandis* when compared to Cry1I (LC_{50} 21.5 μg/mL) or Cry1B (305.32 μg/mL) (Martins et al. 2010). These results indicate the potential of this protein to be used for the construction of transgenic plants for the control of this important insect pest.

Cry11A is another Bti mosquitocidal δ-endotoxin that has been expressed in insect cells by a recombinant baculovirus. Cry11A is the most toxic Bti toxin to *A. aegypti*. It has a molecular mass of 65 kDa, and since it is a "truncated" protein lacking the C-terminal portion found in 130 kDa Cry proteins, Cry11A crystals are formed in Bti cells with the help of two auxiliary proteins, P19 and P20 (Agaisse and Lereclus 1994; Yamagiwa et al. 2002). The *cry11A* gene, obtained from the Brazilian Bti strain S1989, was used to generate a recombinant baculovirus by site-specific transposition (Lima 2009). Crystals of Cry11A were able to form in *S. frugiperda* larvae in the absence of P19 and P20 and shown to be toxic to second instar *A. aegypti* larvae with an estimated LC_{50} of 53.3 ng/ml (Lima 2009).

8.4.4 *Polyhedrin/Cry Fusion*

Chang et al. (2003) constructed a recombinant baculovirus containing a fused gene comprised of the polyhedrin gene, an 1836 bp DNA fragment coding for the N-terminal toxic region of the Cry1Ac protein of Bt subsp. *kurstaki* HD-73 and the green fluorescent protein. This recombinant virus also contained an extra copy of the polyhedrin gene. The fused protein was shown to be expressed and incorporated into the OBs. These OBs were then shown to be highly toxic toward *Plutella xylostella* larvae. This recombinant virus showed a dramatic improvement on the virus pathogenicity since it was able to kill infected larvae by replication of the virus and also by the Cry1Ac toxicity, which should, in theory, reduce the chance of development of resistant insects to both Cry1Ac toxin and the baculovirus.

8.5 Concluding Remarks

The expression of Cry proteins in insect cells using recombinant baculoviruses has been shown to be an alternative to the production of these proteins for functional or structural studies (Table 8.1). The combination of the toxic activity of these proteins and the infectivity of these viruses to different insect species is still not widely used. The genomes of more than 73 baculovirus species have been sequenced to date (Ardisson-Araújo et al. 2016), but only the AcMNPV genome has been genetically

Table 8.1 List of *cry* genes inserted into the genome of a baculovirus and their target insects that showed susceptibility to the expressed toxin

Genes *cry*	Target insect	Reference
cry1Aa	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ab	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ac	*P. xylostella*	Chang et al. (2003)
cry1Ac	*H. virescens*	Ribeiro and Crook (1993, 1998)
cry1Ca	*S. frugiperda and A. gemmatalis*	Aguiar et al. (2006)
cry1Ia	*Anthonomus grandis*	Martins et al. (2008)
cry2Aa	*S. frugiperda and A. gemmatalis*	Lima et al. (2008)
cry2Ab	*S. frugiperda and A. gemmatalis*	Lima et al. (2008)
cry10Aa	*Anthonomus grandis*	Aguiar et al. (2012)
cry4Aa	*A. aegypti and C. quinquefasciatus*	Corrêa et al. (2013)
cry4Ba	*A. aegypti and C. quinquefasciatus*	Corrêa et al. (2013)
cry1Ab	*Pieris brassicae*	Martens et al. (1990)
cry1Ac	*Trichoplusia ni*	Merryweather et al. (1990)
cry1Ab	*Spodoptera exigua*	Martens et al. (1995)
cry11A	*A. aegypti*	Pang et al. (1992), Lima (2009)

manipulated to harbor *cry* genes. Therefore, other baculoviruses might be used for the expression of Cry proteins in order to improve their pathogenicity toward different insect species and also retarding the development of resistant insects to both Cry proteins and virus replication.

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