The Dissertation Committee for Seongjoon Kang Certifies that this is the approved version of the following dissertation:

Toward mosquito control with *Chlamydomonas*: Expression of Cry genes from *Bacillus thuringiensis israelensis* in the chloroplast of *Chlamydomonas reinhardtii*

Committee:

David L. Herrin, Supervisor

Edward C. Theriot

G. Barrie Kitto

John W. La Claire II

Stanley J. Roux

Toward mosquito control with *Chlamydomonas*: Expression of Cry genes from *Bacillus thuringiensis israelensis* in the chloroplast of *Chlamydomonas reinhardtii*

by

Seongjoon Kang, B.S.; B.S. Bio.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2015

Dedication

To my parents

Acknowledgements

First, I would like to thank my supervisor, Dr. David L. Herrin for his guidance, support, patience, and encouragement. Alll the pieces of advice he gave me are valuable to my life as an independent researcher. I am grateful to Drs. Schonna Manning and John W. La Claire II. They gave me the opportunity to work as an undergrate research assistant and introduced me to the lab of Dr. David L. Herrin. My research career at the University of Texas at Austin had not begun without their support.

I also thank lab members, especially, Dr. O.W. Odom for advice, technical supports and discussions. It is pleasant to have research talk and small talk with him.

I also appreciate my dissertation committee: Drs. Edward C. Theriot, G. Barrie Kitto, John W. La Claire II, and Stanley J. Roux for suggestions and supports.

I also thank my friends at UT who supported me emotionally. I had wonderful time in Austin and share good memories with them.

Finally, I want to thank my family: parents, brothers, sister-in-laws, and a niece. I would not have finished this dissertation without their support and help.

Toward mosquito control with *Chlamydomonas*: Expression of Cry genes from *Bacillus thuringiensis israelensis* in the chloroplast of *Chlamydomonas reinhardtii*

Seongjoon Kang, Ph.D.

The University of Texas at Austin, 2015

Supervisor: David L. Herrin

Although crop plants that express Cry genes have been developed and used in the field to help control insect pests, genes that are toxic to mosquito larvae have not been exploited successfully for mosquito control. *Chlamydomonas reinhardtii* is a feasible platform for *Bacillus thuringiensis israelensis* (Bti) toxin expression and deployment as a mosquito larvicide. *C. reinhardtii* chloroplast genome engineering is well established and ideal for this application.

The Bti Cry proteins Cry4Ba (128 kDa), Cry11Aa (72 kDa), and a truncated Cry4Aa ($Cry4A_{700}$; 74 kDa) were recoded and expressed from the chloroplast genome of *C. reinhardtii*. Since these proteins can be toxic to heterologous hosts, the inducible *Cyc6*:*Nac2-psbD* expression system was used. The codon-optimized Cry genes were outfitted with a modified *psbD* 5' region, and integrated into the chloroplast genome of the Ind41_18 strain; homoplasmicity of the transformants was verified by PCR. Western blots of total cell protein showed the accumulation of all three proteins under induction (i.e., minus Cu^{2+}) conditions, with relative protein expression in the order of $Cry4Aa_{700}$

>> Cry11Aa > Cry4Ba. A live-cell bioassay demonstrated the toxicity of the $Cry4Aa_{700}$ and Cry11Aa transformants against larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Surprisingly, the growth rates of the transformants under inducing and noninducing conditions were very similar, suggesting that these Cry gene constructs were not very toxic to these cells (Ind41_18 host strain under minus-Cu^{2+} conditions). Finally, RT-PCR analysis of the mRNAs suggested that Cry11Aa expression may be limited at the post-translational level.

Constitutive expression of Cry toxins is essential for field application of Bti-*Chlamydomonas*. The same synthetic genes that were inducibly expressed in the Ind41_18 strain (above) were transformed into a wild-type strain of *C. reinhardtii*, but only Cry4Ba and Cry11Aa transformants reached homoplasmicity. Western blotting results confirmed the accumulation of Cry11Aa in those transformants, and indicated that it was at least as high as in the inducible system. The Cry11Aa wild-type strain was also toxic to *A. aegypti* larvae in a live cell bioassay. These results lay the foundation for obtaining Bti-*Chlamydomonas* strains that are highly toxic to mosquito larvae, and safe for non-target organisms. Taken together with the environmental and genetic controls on this organism and its chloroplast genes should make it an attractive biocontrol agent.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1 Mosquito control

Mosquitoes threaten human health by transmitting a number of serious diseases, including malaria, yellow fever, dengue, Chikungunya, filariasis, West Nile, and encephalitis. According to the World Health Organization (WHO) there were approximately 207 million cases of malaria and approximately 627,000 deaths in 2012. About 90% of the deaths were in sub-Saharan Africa, and many were children under five years-old (WHO, 2013).

West Nile Virus is a mosquito-borne disease that has become endemic to the U.S., and there is currently no vaccine or treatment for this virus. The Centers for Disease Control indicated there were 2,374 cases of West Nile Virus in the U.S. in 2013, which resulted in 114 deaths. In Texas alone, there were 183 cases and 14 deaths (CDC, 2014).

One of the most effective ways to reduce the transmission of these diseases is to control the insect vector (Takken and Knols, 2009). Most mosquito control programs have made extensive use of chemical insecticides, and they can be very effective. For example, indoor residual spraying and insecticide-treated bednets can reduce malaria cases tremendously (WHO, 2013). However, there are also undesirable effects of chemical insecticides, which include environmental pollution, ecological effects, and human health problems (Margalit, 1989). Also, the evolution of chemically-resistant mosquitoes is increasing (Margalit, 1989); in fact, populations of mosquitoes have

become resistant to essentially all of the chemicals that have been used in the field (Raghavendra *et al*., 2011).

A good example of a pesticide family with these issues is pyrethroids, which are chemicals that have been used extensively for indoor residual spraying and in insecticide-treated bednets. Pyrethroids can nonspecifically effect other organisms, including mammals, fishes, and desirable insects, such as honey bees. They are neurotoxins and possible carcinogens in humans (Miyamoto *et al*., 1995), and pyrethroid resistance among malaria-vector mosquitoes (*Anopheles*) has been reported (Nauen, 2007).

The increasing concern over chemical pesticides has generated considerable interest in bio-insecticides that would be more target-specific, environmentally friendly, and cause less resistance than chemo-insecticides (Milam *et al*., 2000; Poopathi and Abidha, 2010; Raghavendra *et al*., 2011; Weil *et al*., 2003). Since this is not really a new goal, researchers have, for some time, explored potential bio-based agents for mosquito control, including fish, fungi, nematodes, bacteria, and viruses (Chapman, 1974). Among these, *Bacillus thuringiensis* subsp. *israelensis* (Bti), is one of the most suitable control agents, because of its ease of amplification, specificity to mosquito families, and safety for non-target organisms (Canan, 2013).

1.2 *Bacillus thuringiensis* subsp. *israelensis* (Bti)

1.2.1 Discovery and field application

Bti was first isolated from carcasses of mosquito larvae in the Negev desert of Israel in 1976 (Goldberg and Margalit, 1977), and was subsequently identified by de

Barjac (1978) as serotype H-14, based on its flagellar H antigens. Bti is a sporulating, facultative anaerobic, and gram-positive soil bacterium. During sporulation, it produces a parasporal body that contains larvicidal activity toward Dipterans, especially mosquitoes (*Anopheles, Aedes,* and *Culex* families) and black flies. Bti was thus different from the known subspecies of *Bacillus thuringiensis*, which were toxic mostly to lepidopteran insects (Margalit, 1989).

The parasporal body (PB) of Bti H-14 has a crystal-like structure and contains two types of larvacidal proteins: crystal (Cry) proteins and cytolysins (Cyt) (Figure 1.1). There are 4 major polypeptides, which according to the newer nomenclature are named Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa, and they have molecular weights (from the predicted sequences) of 134, 128, 72 and 28 kDa, respectively (Frankenhuyzen, 2009; Poopathi and Abidha, 2010; Bravo *et al*., 2011; Laurence *et al*., 2011). The Cry and Cyt toxin genes are found on a 128-kb plasmid named pBtoxis (Berry *et al*., 2002), and the genes' sizes are 3543 bp (1180 amino acids) for Cry4Aa, 3408 bp (1136 amino acids) for Cry4Ba, 1929 bp (643 amino acids) for Cry11Aa, and 744 bp (248 amino acids) for Cyt1Aa (Ben-Dov, 2014). Thus, the Cry genes are large (Cry11Aa) to very large (Cry4Aa and Cry4Ba), and the Cyt gene is a common size for bacterial proteins.

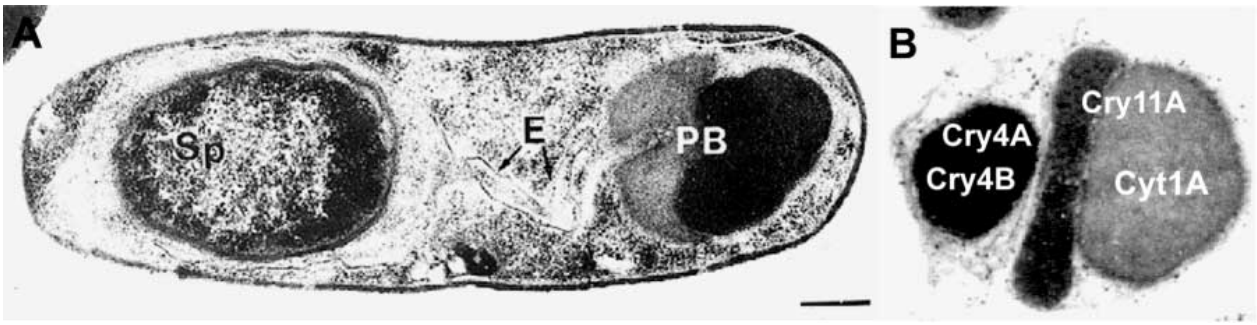

Figure 1.1 (A) *Bacillus thuringiensis* subsp. *israelensis* (Bti) containing spore (Sp) and parasporal body (PB). (B) A parasporal body of Bti containing 3 subinclusion bodies which are composed of Cry4A+Cry4B, Cry11A, and Cyt1A, respectively. (Adapted from Federici *et al.*, 2003)

Upon sporulation of the bacterium, the toxin genes on pBtoxis are expressed and the resulting proteins are assembled into the crystal-like PB (Ibarra and Federici, 1986). Cry4Aa+Cry4Ba, Cry11Aa, and Cyt1Aa are found as 3 distinct sub-inclusion bodies that are surrounded by a lamellar-like envelope (Figure 1.1) (Federici *et al.*, 2003). In addition, Cry10Aa, Cyt2Ba, and Cyt1Ca are minor toxins found in the PB (Ben-Dov, 2014). When sporulation is complete, the crystal endotoxin (PB) and the endospore are released from the mother cell. Ingestion of the crystals by mosquito and fly larvae can result in growth inhibition and death, with the effective toxicity being determined by a number of factors.

Bti was approved as a bio-mosquitocide by the US Environmental Protection Agency in 1981 (Becker, 2006), only 5 years after its first isolation in Israel. Since then, Bti has been used around the world for the control of mosquitoes and black flies, and without a single incident of highly resistant insects. For example, Bti application against black flies as part of the Onchocerciasis Control Programme (OCP) in West Africa rapidly reduced populations of this vector (Gullet *et al.*, 1990). In Germany, mosquitoes of the Upper Rhine Valley were reduced by 90% from 1981 to 1991 by intensive Bti treatments, and there were no significant effects on the environment, as reported by Becker (1997).

Using Bti as a biocontol agent has several advantages over chemical pesticides. First of all, Bti is considered a safe mosquito control agent (WHO, 1999) because its toxicity is highly specific to Dipterans. No substantial toxicity has been detected in the field against non-Dipteran organisms, including other insects and invertebrates, fish,

mammals and humans (Glare and O'Callaghan, 1998; Siegel, 2001). It is noted that chironomid midges were reported as being susceptible to the Bti toxin in a study of non-target organisms, but control of chironomid midges using Bti required seven-fold higher doses than for mosquitoes (Lacey and Merritt, 2003). When the Bti toxin was solubilized and injected at high doses into mice, some mortality was observed (Siegel and Shadduck, 1990). However, this toxicity by injection is not relevant to field applications, because the crystals are only solubilized at alkaline pH, whereas the mammalian gut is acidic. Moreover, the toxin proteins are activated by proteases in the larval midgut, and the Cry proteins bind to specific receptors in the microvilli cell membrane (Margalit, 1989; Ben-Dov, 2014).

Another property of Bti that makes it so attractive is that it does not induce strong resistance; several studies have reported no strong resistance of mosquito larvae to Bti crystals even after 30 years of application (Becker, 2000; Glare and O'Callaghan, 2000; Tetreau *et al*., 2013; Ben-Dov, 2014). Cyt1Aa in the PB is apparently the key suppressor of resistance in mosquito larvae, as strong resistance to individual Cry proteins has been detected in the laboratory and field (Tetreau *et al*., 2013; Ben-Dov, 2014).

Bti also costs approximately 200 times less than a chemical insecticide (c.a. US$ 500,000 vs c.a. US$ 20 million) to develop and register (Becker and Margalit, 1993).

1.2.2 Drawbacks to Bti

Although mosquitocidal products based on Bti are available on the open market, and are used in many mosquito control programs, the native toxin has several drawbacks, including sensitivity to sunlight (UV light), sinking out of the water column, and a lack of recycling (Margalit, 1989; Myasnik *et al*., 2001). Hence, control with Bti can require frequent applications, because of its short persistence. Also, when it sinks, it can be adsorbed by silt which lowers the accessibility of the toxin to mosquito larvae, especially *Anopheles*, which are known to be surface feeders (Otieno-Ayayo *et al*., 2008).

Several early field tests reported that the toxicity of sporal cultures of Bti lasted less than 24 hours (Ramoska *et al*., 1982). However, the toxin in the silt retained its activity for 22 days, though most filter feeding larvae could not consume it (Ohana *et al*., 1987). Floating briquette formulations of Bti have been developed that slowly release the toxin and extend its persistence (Fansiri *et al*., 2006). Other additives protect the toxin from sunlight (Vilarinhos and Monnerat, 2004); UV in sunlight degrades tryptophan residues causing loss of its toxicity (Pusztai *et al*., 1991; Liu *et al*., 1993). Despite these advances, Bti still does not recycle in most aquatic environments.

The Bti bacterium also produces an exotoxin that is a water-soluble metabolite(s). The exotoxin is less specific than the crystal endotoxin and can damage non-target organisms like Trematode Cercariae (parasitic flatworms) (Horák *et al*., 1996). Commercial preparations of Bti have to be tested for the exotoxin and there is a tolerance level that must not be exceeded.

1.2.3 Overall toxin function and activation

The Bti endotoxin can cause rapid mortality of target mosquito larvae. When the larvae were treated with the toxin crystals, they stopped feeding within an hour, moved slowly within two hours, and became paralyzed by six hours (Chilcott *et al*., 1990). Bti toxin causes death of target mosquito larvae by forming pores in the cell membranes of midgut microvilli; thus, the mode of action is similar to that of toxins from other *Bacillus thuringiensis* species (Bravo *et al*., 2007). All 4 of the major proteins exhibit toxicity to varying degrees, however, Cyt1Aa also possesses cytolytic (and hemolytic) activity (Butko *et al*., 1996; Butko, 2003).

The Cry proteins are produced in an inactive or protoxin form, while Cyt1Aa is produced in a partially active form. The Cry proteins are proteolytically activated in the gut, and Cyt1Aa is also processed there to increase its activity (Chilcott and Ellar, 1988; Al-yahyaee and Ellar, 1995). The Cry protoxins are subjected to N-terminal and C-terminal processing, and intramolecular cleavage, leaving a three-domain structure that confers toxicity (Schnepf *et al*., 1998). Much of the C-terminal half, and 30-50 amino acids of the N-terminus of Cry4Aa and Cry4Ba are cleaved off, yielding activated forms with a size of ~65 kDa (Ben-Dov, 2014). Further intramolecular cleavage produces two fragments, 20 and 45 kDa for Cry4Aa, and 18 and 45 kDa for Cry4Ba (Komano *et al*., 1998; Yamagiwa *et al*., 1999). For Cry11Aa, midgut proteases cleave off 28 residues at the N-terminus, and in the middle producing 34 and 32 kDa fragments (Dai and Gill, 1993) that remain associated with each other (Yamagiwa *et al*., 2004). Proteolytic

cleavage of Cry4Aa and Cry11Aa probably involves trypsin, and for Cry4Ba, chymotrypsin (Yamagiwa *et al*., 2002; Xu *et al*., 2014).

The 28 kDa Cyt1Aa is also cleaved by midgut proteases at both termini, leaving a ~25 kDa protein. Although it is a bacterial protease, proteinase K was reported to activate Cyt1Aa (Al-yahyaee and Ellar, 1995); the 24 kDa Cyt1Aa was approximately three times more effective than the protoxin (Butko *et al*., 1996). Also, the proteinase K-activated Cyt1Aa exhibited higher hemolytic activity than the trypsin-activated protein, owing to different cleavage sites of each enzyme (Al-yahyaee and Ellar, 1995).

1.2.4 Structure of Bti toxin proteins

1.2.4.1 Classification of Cry toxins

Cry proteins of *B. thuringiensis* have been classified based on size, homology of the amino acid sequence, and pathogenicity (Höfte and Whiteley, 1988, Crickmore *et al*., 1998). Based on the size of the protoxins, most Cry proteins can be put into two groups: ~130 kDa and ~70 kDa (Höfte and Whiteley, 1989). Cry4Aa and Cry4Ba belong to the former, while Cry11Aa belongs to the latter group. The 130-kDa proteins contain a highly conserved C-terminal region rich in cysteines, some of which are involved in disulfide bonds and formation of the inclusion body (Höfte and Whiteley, 1989); however, the N-terminal region confers toxicity. The 70-kDa group does not have the C-teminal region, but these proteins have structural similarities with the N-terminal region of the 130-kDa group proteins (Figure 1.2) (Jurat-Fuentes and Jackson, 2012).

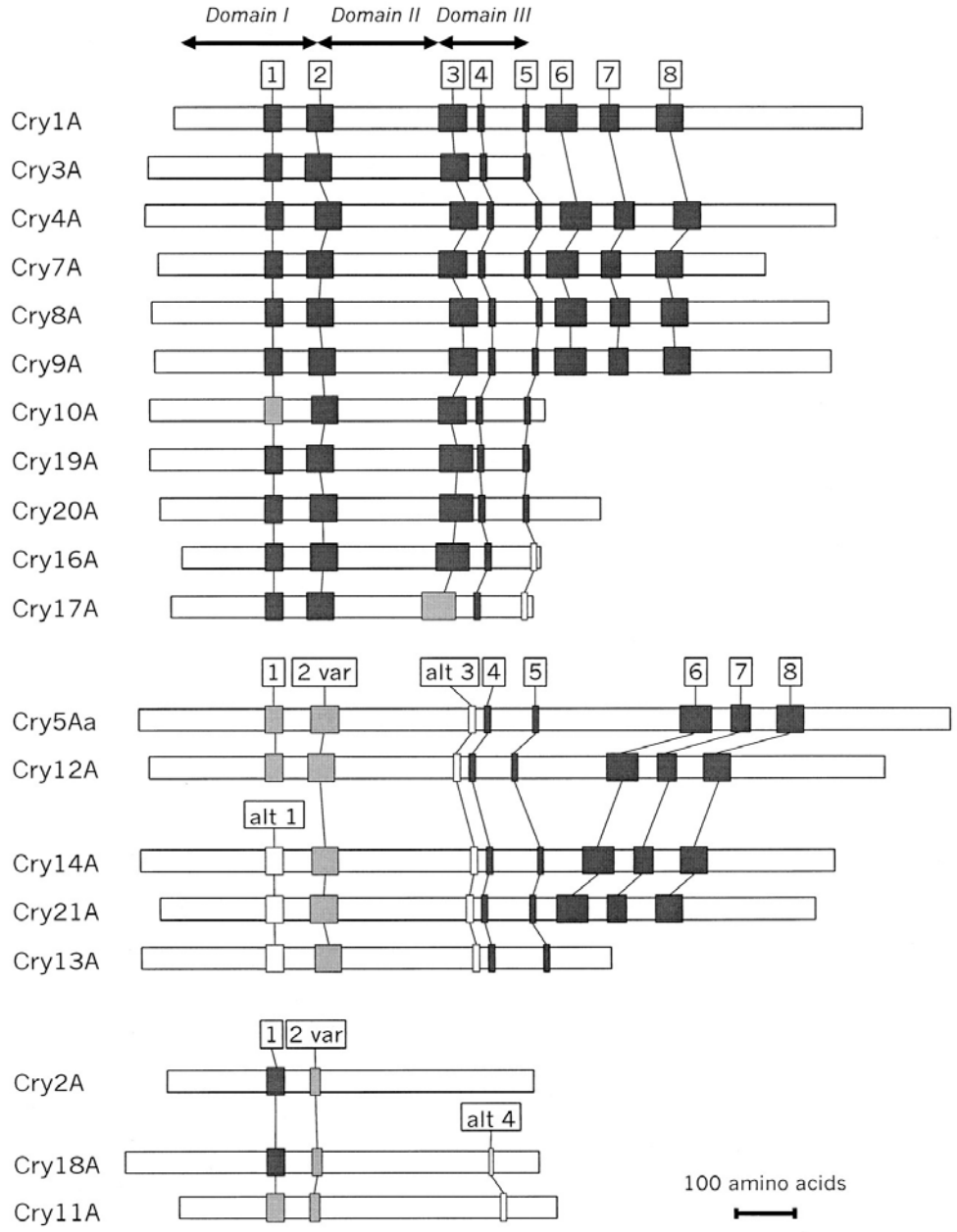

Figure 1.2 Cry structures with conserved blocks of amino acids. Each Cry protein has at least one conserved block. The darker color of the block indicates a higher degree of homology. Var, Variant; alt, alternate. Adapted from Schnepf *et al*. (1998).

1.2.4.2 Sequence similarity among Cry polypeptides

The Cry4Aa and Cry4Ba protoxins are closely related; the C-terminal regions (~465 amino acids) are highly homologous, and there is ~55% sequence similarity in their N-terminal regions (Höfte and Whiteley, 1989, Chungjatupornchai *et al*., 1988). The differences in their N-terminal region reflect toxin specificity. Cry11Aa and Cyt1Aa did not show a lot of aa sequence identity to each other, or to the Cry4Aa and Cr4Ba proteins (Höfte and Whiteley, 1989). However, sequence alignments using more Cry proteins have shown that conserved amino acid segments exist in all Cry toxins. Figure 1.2 shows the location of conserved blocks in Cry proteins (Höfte and Whiteley, 1989; Schnepf *et al*., 1998). These blocks are highly related to protein structure and toxicity (Boonserm *et al*., 2005). For example, the Cry proteins that share conserved blocks 1-5 are believed to have similar tertiary structure, even though the interblock regions show high divergence (Li *et al*., 1991; Xu *et al*., 2014). Although Cry4Aa and Cry4Ba have conserved blocks 1-5, Cry11A has only block 1; this region coincides with central helix α5 of Domain I and confers toxicity (Xu *et al*., 2014). Block 1 is rich in hydrophobic residues and is found in most Cry proteins (Boonserm *et al*., 2005).

1.2.4.3 Three-dimensional structures of Bti toxin proteins

The three-dimensional structures of the activated forms of Cry4Aa, Cry4Ba, and Cyt1Aa have been revealed by X-Ray crystallography (Figure 1.3) (Boonserm *et al*., 2005; Boonserm *et al*., 2006; Cohen *et al*., 2011). The structure of activated Cry11Aa

has not been determined, so far; the structure in Figure 1.3 was predicted using the structure of Cry2Aa, with which it shares homology (Fernández *et al*., 2005).

The activated Cry toxins consist of three Domains (I – III), which encompass conserved blocks 1-5 (Figure 1.2). Functionally, Domain I is involved in inserting into the membrane and forming a pore, while Domains II and III are responsible for receptor binding and toxin specificity (de Maagd *et al*., 2001). Domain I is composed of 5-7 alpha-helices (Xu *et al*., 2014), with a central hydrophobic helix (α5) surrounded by amphipathic helices (Boonserm *et al*., 2006; Leetachewa *et al*., 2006). Domain II is comprised of 3 antiparallel β-sheets (β-prism) in a "greek key" motif, with a hydrophobic core helix and three apical loops (Xu *et al*., 2014). Domain II has the most variable sequence, with the lengths and sequences of the exposed apical loops showing high divergence (Boonserm *et al*., 2005).

Functionally, the loops in Domain II are involved in interactions with the receptors, and determine much of the specificity. For example, Loop 2 in Domain II of Cry4Aa is essential for toxicity against C*ulex pipiens* (Howlader *et al*., 2009). Abdullah *et al*. (2003) replaced Loop 3 of Cry4Ba with Loop 3 of Cry4Aa and increased the toxicity of Cry4Ba against *Culex*; they also showed that Loops 1 and 2 are determinants of Cry4Ba activity against *Aedes* and *Anopheles*. In Cry11Aa, Loop α-8 is an epitope that interacts with gut receptors in *A. aegypti* (Fernández *et al*., 2005); Cry11Aa-receptor interactions also seem to involve β-4 and Loop 3 (Fernández *et al*., 2005).

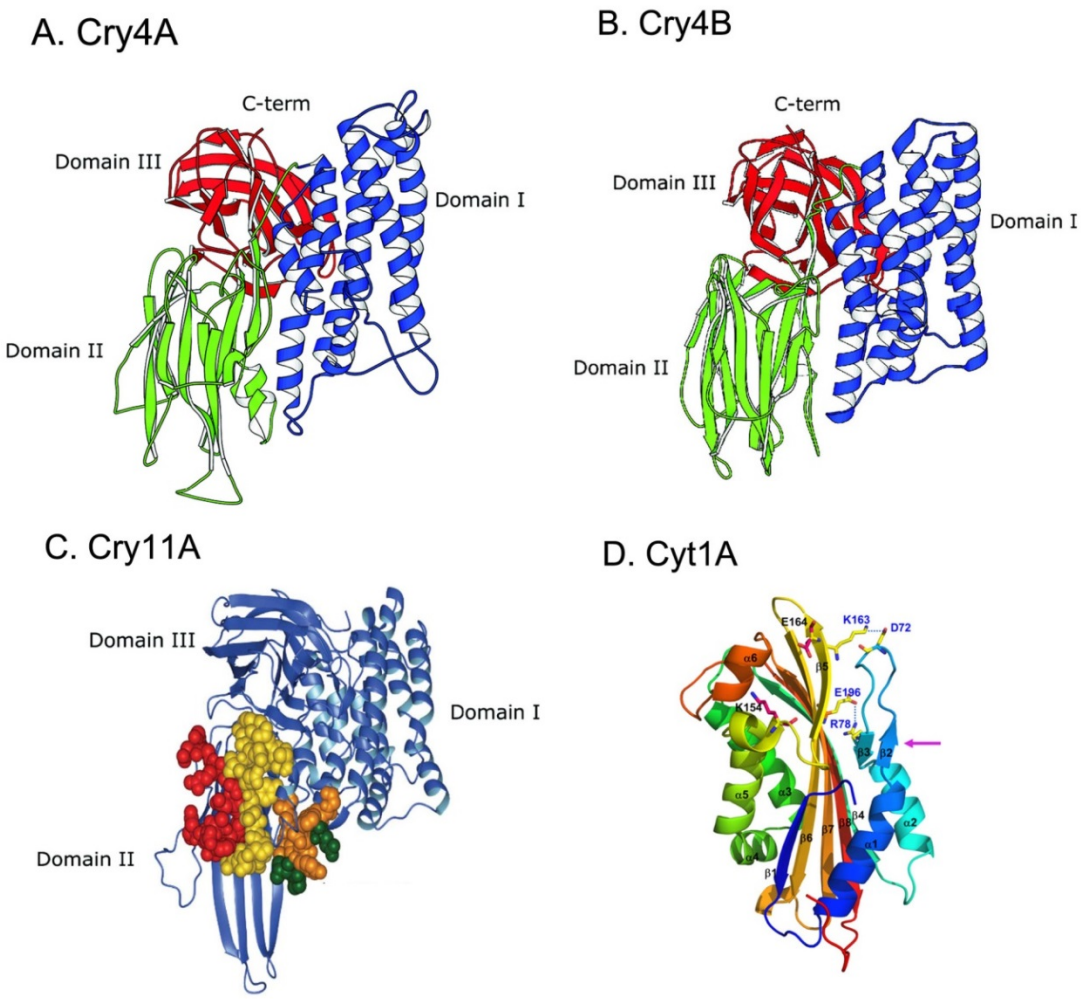

Figure 1.3 Tertiary structures of activated Bti toxins Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa. The 3 Cry proteins have a three domain structure: Domain I is the α-helix bundle; Domain II is named the β-prism, and Domain III is the β-sandwich. Cyt1A has a one-domain structure with α-helices flanking a β-sheet. Structures (A), (B), and (D) were determined from X-Ray crystallography, whereas structure (C) is an *in silico* model predicted by homology modeling with the three-dimensional structure of Cry2Aa. Adapted from Angsuthanasombat *et al*. (2004), Fernandez *et al*. (2005), and Cohen *et al*. (2011).

Domain III consists of two antiparallel β-sheets (β-sandwich) in a "Jelly roll" topology (Soberón *et al*., 2010; Xu *et al*., 2014). It is the most conserved region, with 3 conserved blocks (Figure 1.2). This domain was suggested to participate in membrane permeability or receptor binding and insect specificity (de Maagd *et al*., 2001).

Cyt1A is one-domain protein comprised of two α-helix layers surrounding a β-sheet (Figure 1.3) (Cohen *et al*., 2011; Bravo *et al*., 2011). Upon activation, α-helices A, B, C and D stay outside the membrane while β-strands 5, 6 and 7 enter the membrane forming a pore (Soberón *et al*., 2013). Stable folding and crystallization of Cyt1Aa in the PB in vivo is aided by P20, a chaperone located in the Cry11Aa operon (Visick and Whiteley, 1991; Dervyn *et al*., 1995)

1.2.5 Mechanisms of action

The activated Cry toxins bind to specific receptors on the midgut epithelial cells, which induce oligomerization and insertion into the plasma membrane (Xu *et al*., 2014). Hairpin helices α4-α5 in Domain I were suggested to be the hydrophobic core that penetrates the bilayer (Gazit *et al*, 1998). The pore-forming ability of the inserted proteins causes osmotic imbalance, degradation of the epithelia, and ultimately death (Bravo *et al*., 2011; Raghavendra *et al*., 2011).

Cyt1Aa has distinct mechanistic features compared to the Cry proteins. Toxicity of Cyt1Aa is not mediated by toxin-receptor interactions, but by toxin-phospholipid interactions (Butko, 2003). Importantly, it also acts as a receptor for the Cry toxins, especially Cry11Aa. Pérez *et al*. (2005) showed that Cyt1Aa binds Cry11Aa through two

exposed regions, loop β6-αE and part of β7. Interestingly, in Cry11Aa, Loop α8 and β-4 of Domain II bind to the exposed regions on Cyt1Aa and larval midgut receptors (Pérez *et al*., 2005). These data suggest how Cyt1Aa synergizes the toxicity of Cry proteins, and overcomes the resistance to Cry toxins of mosquito larvae (Wirth *et al*., 1997).

However, there are two models of how Cyt1Aa is toxic by itself: pore-forming and the detergent model (Butko, 2003). In pore-forming model, the two outer layers of α-helices contact the membrane and the β-strands penetrate, which promotes oligomerization and β-barrel (pore) formation (Li *et al*., 1996, Promdonkoy and Ellar, 2000). The detergent model suggests that Cyt toxins aggregate and absorb onto the lipid bilayer, causing its destabilization ((Butko, 2003; Manceva *et al*., 2005).

1.2.6 Target specificity of Bti toxins

Bti was reported to be toxic against larvae of 109 mosquito species; 40 species of *Aedes*, 27 species of *Anopheles*, and 19 species of *Culex* (Glare and O'Callaghan, 1998). Although Bti is toxic to a wide range of mosquito varieties, including those that are major disease vectors, the toxicity of specific protoxins varies significantly with the mosquito species. For example, Cry4Ba is highly toxic to *Anopheles* and *Aedes*, but weakly toxic to *Culex spp*., while Cry4Aa is highly active against *Culex* larvae. Cry11Aa is fairly lethal to all 3 genera. Cyt1Aa is weakly toxic to *Aedes* and *Culex*, and almost nontoxic to *Anopheles* (Frankenhuyzen, 2009, Poncet *et al*., 1995, Promdonkoy *et al*., 2005, Wu *et al*., 1994).

1.2.7 Receptors for Bti toxins

The leptopteran-specific Cry1A toxin of *B. thuringiensis* was found to bind to several receptors: a cadherin-like protein (CADR), a glycosylphosphatidyl inositol (GPI)-anchored aminopeptidase-N (APN), a GPI-anchored alkaline phosphatase (ALP), and a 270-kDa glycoconjugate (Brova *et al*., 2007). More recent studies of Bti have revealed that the dipteran-specific Cry toxins bind similar proteins to the aforementioned receptors. The receptors for Cry4Ba were identified as a GPI-anchored aminopeptidase-N (APN) and a GPI-anchored alkaline phosphatase (ALP) in *A. aegypti*, and an α-amylase in *Anopheles albimanus* (Saengwiman *et al*., 2011; Thammasittirong *et al*., 2011; Fernandez-Luna *et al*., 2010). The putative receptors for Cry11Aa are an aminopeptidase N (Chen *et al*., 2013), a GPI-anchored alkaline phosphatase (Fernandez *et al*., 2006), and a cadherin (Chen *et al*., 2009) in *A. aegypti*, and α-amylase in *Anopheles albimanus* (Fernandez-Luna *et al*., 2010). Receptors for Cry4Aa have not been identified.

1.2.8 Synergism among Bti toxins

Synergism among the Bti toxins contributes to the low chance of development of resistance in mosquito larvae (Ben-Dov, 2014). The native Bti crystal is more toxic than any single or multiple-gene combinations (Poncet *et al*., 1995). Mixtures of Cry4Aa and Cry4Ba were 5-fold more toxic than Cry4A or Cry4Ba alone (Angsuthanasombat *et al*., 1992). Cyt1Aa dramatically (> 5-fold) increased the toxicity of the Cry proteins, especially Cr11Aa, presumably by acting as a receptor at the cell membrane (Wu *et al*., 1994; Poncet *et al*., 1995; Promdonkoy *et al*., 2005; Frankenhuyzen, 2009).

1.3 Mosquitocidal toxins from other bacteria and *B. thuringiensis* subspecies

There have been several reports of mosquitocidal crystal proteins in other *B. thuringiensis* subspecies, and in one anaerobic bacterium.

1.3.1 Toxins of other *B. thuringiensis* subspecies

B. thuringiensis jegathesan produces 8 protoxins: Cry11Ba (81 kDa), Cry19Aa (75 kDa), Cyt2Bb (30 kDa), Cry24Aa (76 kDa), Cry25Aa (76 kDa), Cry30Ca (77 kDa), Cry60Aa (34 kDa), and Cry60Ba (35 kDa) (Sun *et al*., 2013). Several of these proteins are immunologically related to the protoxins of Bti, including Cry11Ba, which is related to Cry11Aa, and Cyt2Bb, which is related to Cyt1Aa (Delécluse *et al*. 1995, Delécluse *et al*., 2000). Among the Cry proteins, Cry11Ba exhibited the strongest toxicity against mosquito species *Aedes aegypti* (*A. aegypti*), *Culex pipiens*, and *Anopheles stephensii* (Delécluse *et al*., 1995). Cry19Aa was toxic to *Culex pipiens, Culex quinquefasciatus* (*C. quinquefasciatus*), and *Anopheles stephensii*, but only weakly to *A. aegypti* (Rosso and Delécluse, 1997). Interestingly, ORF2 in *jegathesan* promotes stability and crystallization of other Cry proteins, thus increasing toxicity (Sun *et al*., 2013).

B. thuringiensis medellin also produces several Cry proteins: Cry11Bb (94 kDa), Cry29Aa (74 kDA), Cry30Aa (78 kDa), Cyt1Ab (28 kDa), and Cyt2Ba (30 kDa) Delécluse *et al*., 2000). Cry29Aa and Cry30Aa exhibited no activity, but Cry11Bb, Cyt1Ab, and Cyt2Ba were toxic to mosquito larvae. Cry11Bb exhibited high toxicity against *A. aegypti, Anopheles albimanus* and *C. quinquefasciatus* larvae (Orduz, 1998).

1.3.2 *Clostridium* toxin

Clostridium bifermentrans malasya, which produces Cry16A and Cry17A, is the first anaerobic, non-*B. thuringiensis* organism with Cry proteins (Barloy *et al*., 1996). Cry16A and Cry17A were weakly toxic to *Anopheles*, *Aedes*, and *Culex* mosquito larvae (Qureshi *et al*., 2014).

1.3.3 *Bacillus sphaericus* toxin

Bacillus sphaericus (Bs) is a sporulating, aerobic, gram-positive soil bacterium (El-Bendary, 2006) that has also been employed for mosquito control since the late 1980s (Poopath and Abidha, 2010). The first mosquitocidal Bs strain, *neide*, was isolated from carcasses of mosquito larvae near Fresno, California in 1965 (Kellen *et al*., 1965); thereafter, hundreds of Bs strains were identified.

Bs produces several mosquitocidal toxins, but the binary toxin is the major one, and is produced in strains 1593 and 2362 (Peña-Montenegro and Dussán, 2013; Silva-Filha *et al*., 2004). During sporulation, Bs produces a parasporal body that contains the binary toxin, which is composed of BinA (42 kDa) and BinB (51 kDa). Its mode of action is similar to that of the Bti toxin (Poopathi and Abidha, 2010). Upon ingestion by larvae, the heterodimeric toxin is cleaved by proteases into active 39 kDa (BinA) and 43 kDa (BinB) proteins (Baumann *et al*., 1991; Canan, 2013) which act synergistically (Arapinis *et al*., 1988; Nicolas *et al*., 1993). Equal amounts of BinA and BinB provide maximum activity, and BinB is required for the activity of BinA (Baumann *et al*., 1991). BinB binds to a specific receptor, which is a 60-kDa α-glucosidase in *Culex pipiens*, while BinA is

involved in conferring toxicity (Darboux *et al*., 2001). The toxin is thought to participate in pore formation in the larval midgut (Schwartz *et al*., 2001). Bs has no reported toxicity against non-target organisms, including fish, mice, and humans (Shadduck *et al*., 1980; Grisolia *et al*., 2009; Oliveira-Filho *et al*, 2014).

The Bs binary toxin has some properties that are different from Bti crystals. The host range of Bs is more restrictive; it has high toxicity against *Culex*, but not *A. aegypti* or black flies (Wraight *et al*., 1987; Berry *et al*., 1993). Bs also acts more slowly than Bti (de Barjac, 1989), but the toxicity persists for longer periods and it can recycle in the field (Nicolas *et al*., 1987; Pantuwatana *et al*., 1989). The Bs toxin is also effective in polluted water, unlike Bti (Baumann *et al*., 1991; Wirth *et al*., 2010); however, it does engender resistance (Poopathi and Abidha, 2010). The resistance of *Culex* mosquito larvae to the Bs toxin has been reported in laboratory and field conditions (Silva-Filha *et al*., 1995; Wirth *et al*., 2000; Amorim *et al*., 2007). The main cause of this resistance seems related to the fact that, compared to Bti, it has a single major toxin with a relatively simple mode of action (Nielsen-Leroux *et al*., 1995). Co-expression of Cyt1Aa from Bti with the binary toxin has improved toxicity to resistant *Culex* larvae (Park *et al*., 2005; Wirth *et al*., 2010).

1.4 Transgenic Bti-modified organisms

To overcome the limitations of Bti, there have been attempts to produce Bti-modified organisms (Bti-organisms) that express the protoxins and either, reproduce in larval habitats (aquatic bacteria) or provide an alternate source of the toxins (yeast)

(Porter *et al*., 1993). Cry and/or Cyt1Aa genes were inserted into several gram-positive and gram-negative bacteria, including *Bacillus subtilis* (Ward *et al*., 1986), *Ancylobacter aquaticus* (Yap *et al*., 1994a), *Caulobacter crescentus* (Yap *et al*., 1994b), *Pseudomonas putida* (Xu *et al*., 2001), *E. coli* (Boonserm *et al*. 2004; Bukhari and Shakoori, 2009), and *B. sphaericus* (Federici *et al*., 2003). Also, several cyanobacterial species have been similarly engineered, including *Agmenellum quadruplicatum*, *Synechocystis PCC 6803*, *Synechococcus PCC 7942*, and *Anabaena* PCC 7120 (reviewed in Otieno-Ayayo *et al*., 2008). Cry protoxins have also been produced in two eukaryotic microorganisms: Cry11Aa was expressed in *Saccharomyces cerevisiae*, and Cry11Aa and a truncated Cry4Aa were expressed in *Pichia pastoris* (Quintana-Castro *et al.*, 2005; Borovsky *et al*., 2010). It should be said that none of these transgenic organisms have been used in the field for mosquito control to my knowledge.

There has been only one report with a higher plant, and that involved producing Cry11Aa in rice to make it resistant to bloodworms (Hughes, 2005). Since most insect pests of crops are not Dipterans, other classes of Cry toxins (such as Cry1A and Cry2A) - which are derived from different subspecies of *Bacillus thuringiensis* - have been expressed in crop plants (Kleter *et al*., 2007).

The most extensive effort to engineer a Bti-organism has been with the cyanobacterium *Anabaena* (Xiaoqiang *et al*., 1997; Boussiba *et al*., 2000; Khasdan *et al*., 2003). A strain expressing Cry4Aa, Cry11Aa, and Cyt1Aa was constructed, and shown to have high toxicity toward mosquito larvae. The transgenic *Anabaena* suppressed the inactivation of Bti by UV light (Manasherob *et al*., 2002) and the development of

resistance of the mosquito larvae to the toxin (Boussiba *et al*., 2000). However, the Bti-*Anabaena* has yet to be used in the field for mosquito control, in part because of concerns over the bacterial antibiotic-resistance genes that were co-introduced in the transformations (Zaritsky *et al*., 2010).

1.5 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii (*C. reinhardtii*) is a unicellular, eukaryotic green alga, widely-used in genetics and cell biology. There are two mating types (+ and −), as it can reproduce sexually or asexually (Pröschold *et al*., 2005). In sexual reproduction, which is critical for its survival in the wild, the vegetative cells differentiate into gametes, mate, and form a diploid zygote (Harris, 2001). The zygote is tough and resistant to hostile conditions and predation, but when conditions are good it germinates and divides into haploid zoospores (vegetative cells). *Chlamydomonas* is used to study photosynthesis, chloroplast biogenesis, circadian rhythms, cell cycle, adaptation, metabolism, motility and sexual reproduction (Harris, 2001; Silflow and Lefebvre, 2001).

C. reinhardtii is small for a eukarotic cell (averaging about 10 μm in diameter), and has a single chloroplast that is 40% of the cell volume (Rochaix, 1995). It also has two anterior flagella that are used for motility, and in mating (Figure 1.4) (Harris, 1989). Transformation of the nuclear, chloroplast, and mitochondrial genomes is possible (Kindle and Sodeinde, 1994), and the sequencing of those genomes has been completed for at least one reference strain (Maul *et al*., 2002; Merchant *et al*., 2007). *C. reinhardtii* can grow autotrophically in the light, heterotrophically in the dark, or mixotrophically if

both acetate (carbon source in the medium) and light are provided; this flexibility is key to being able to use non-photosynthetic mutants (Harris, 2001).

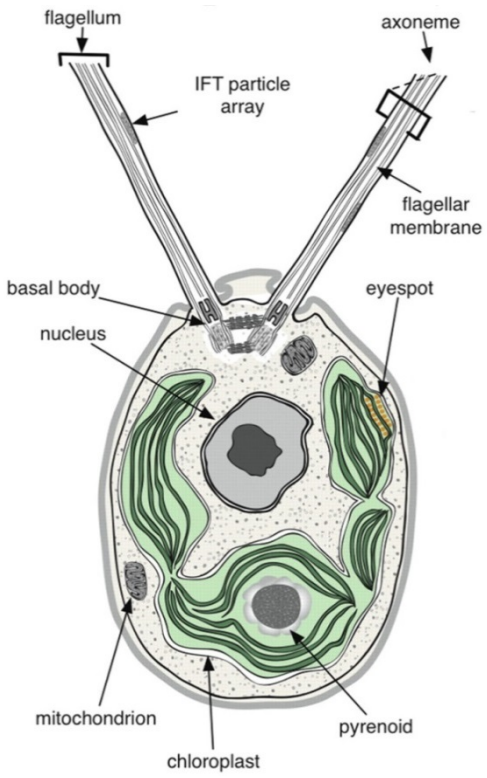

Figure 1.4 A schematic diagram of a *Chlamydomonas* cell based on transmission electron microscopic pictures. The cell has a cup-shaped chloroplast with a pyrenoid near the base, surrounded by starch granules, and an eyespot with carotenoids. The cell also has a nucleus, mitochondria, and two anterior flagella. Adapted from Merchant *et al*. (2007).

1.6 *C. reinhardtii* as a platform for recombinant protein production

It is recent that *C. reinhardtii* has become a reliable platform for biosynthesis of recombinant proteins, which so far include, human therapeutic proteins (Surzycki *et al*., 2009; Rasala *et al*., 2010; Tran *et al*., 2013), recombinant enzymes (Noth *et al*., 2013; Zedler *et al*., 2014), fluorescent proteins (Rasala *et al*., 2013) and food-derived bioactive peptides (Campos-Quevedo *et al*., 2013). There are several advantages for engineering *C. reinhardtii* for recombinant protein production. The expression of foreign genes in the nucleus (eukaryote-like expression) and the chloroplast (prokaryote-like expression) is well established (Boynton *et al*. 1988; Kindle and Sodeinde, 1994). Its doubling time is short (usually 8-12 hours), and large-scale culture costs are low (Harris, 2001). Moreover, recombinant proteins can be targeted to different organelles (nucleus, mitochondria, ER, and chloroplast), or secreted out of the cell (Lauersen *et al*., 2013; Rasala *et al*., 2014a). Also, *C. reinhardtii*, like many algae, is classified by U.S. FDA as a GRAS (generally regarded as safe) organism (Specht *et al*., 2010).

1.7 Chloroplast engineering in *C. reinhardtii*

The chloroplast has ~80 copies of a genome that is ~200 kb, and contains two inverted repeats of 21.2 kb and two single-copy regions of 80 kb and 78 kb (Maul *et al*., 2002). The genome encodes 99 genes, mostly involved in photosynthesis, transcription, and translation (Harris *et al*., 2009). The chloroplast, in both *C. reinhardtii* and land plants, has become an important location for foreign gene expression. The potential for high levels of expression, absence of gene silencing, unique expression signals, and

restrictive (uniparental) inheritance are all advantages of chloroplast engineering (Grant *et al*., 1980; Cerutti *et al*., 1997; Manuell and Mayfield, 2006). The relatively simple and efficient manipulation of the *C. reinhardtii* chloroplast genome using biolistic bombardment is also well established (Boynton *et al*., 1988); transgenes are inserted in a site-specific manner by homologous recombination, and are generally stable unless they are highly toxic to the host (Surzycki *et al*., 2009; Rasala and Mayfield, 2014b). Moreover, the chaperones and protein disulfide isomerases in the chloroplast of *C. reinhardtii* are capable of folding complex proteins (Rasala and Mayfield, 2014b).

1.8 Factors affecting recombinant protein expression in the chloroplast

1.8.1 Codon optimization

In the genetic code, most amino acids are encoded by more than one codon, and the differences in codon usage vary from species to species; this codon bias is usually related to an organism's tRNA pool (Gustafsson *et al*., 2004). The chloroplast of *C. reinhardtii* prefers adenine or uracil nucleotides in the wobble position, thus contributing to the high A-T content of the genome (Franklin *et al*., 2002; Rosales-Mendoza, 2011). A codon usage database for chloropast-encoded ORFs is readily available online (Nakamura *et al*., 2000); however, for this project I created my own using 8 highly expressed chloroplast genes.

The codon adaptation index (CAI) is a measure of codon usage bias, and can be used to predict whether heterologous genes will be expressed (Sharp and Li, 1987;

Surzycki, 2009). CAI values vary from 0 to 1, where 1 indicates that all codons in a gene are the most frequently used (Stenico *et al*., 1994).

Codon optimization is the process that changes all codons of a transgene into the most commonly used codons in a host organism (Gustafsson *et al*., 2004), so that the CAI value increases close to 1. In this dissertation, sequences of native Cry4Aa, Cry4Ba, and Cry11Aa genes were converted into codon-optimized sequences with Optimizer, a computer application developed by Puigbò *et al.* (2007). Expression of codon-optimized transgenes can increase protein levels dramatically, and has been successful in various hosts, including bacteria, plants, and mammals (Gustafsson *et al.*, 2004), and in the *C. reinhardtii* chloroplast. Franklin *et al*. (2002) claimed an 80-fold increase in GFP accumulation by re-synthesizing the *gfp* gene to agree with the codon bias of *C. reinhardtii* chloroplast genes. Codon-optimized luciferase reporter genes from *Vibrio harveyi* and firefly resulted also in high expression of the reporter gene (Mayfield and Schultz, 2004; Matsuo *et al.*, 2006).

1.8.2 Effect of the 5’ and 3’ untranslated regions on expression

The native chloroplast genes are regulated at transcriptional, post-transcriptional (RNA stability, processing, and splicing) and translational levels (Rochaix, 1996). Besides the transcriptional promoters, the 5’ and 3’ UTRs that flank the transgene are important determinants of expression; the 5’ UTR affects translation and sometimes mRNA stability, while the 3’ UTR mostly affects mRNA stability (Herrin and Nickelsen, 2004). Translational factors and ribosomes interact with the 5’ UTR in mediating

translation of an mRNA (Rochaix, 1996; Harris *et al*., 1994). The 5' UTR and especially the 3' UTR form stem-loop structures that bind proteins that protect the transcripts from exonucleases and determine the 3' end of the mRNA (Herrin and Nickelson, 2004).

There have been several studies on the relationship between transgene expression and the specific 5' and/or 3' UTR that is on the reporter gene (Ishikura *et al*., 1999; Barnes *et al*. 2005; Michelet *et al*., 2011; Rasala *et al*., 2011). For examples, Barnes *et al*. (2005) found that the 5' UTRs from the *atpA* and *psbD* genes gave higher levels of GFP than the 5' UTRs from the *rbcL* and *psbA* genes, but that various 3'-UTRs hardly affected GFP protein accumulation. Probably, the highest level of any foreign protein was obtained when the 5' and 3' expression signals on the transgene were from the *psbA* gene, and the transgene replaced the endogenous *psbA* gene (instead of an ectopic insertion) (Minai *et al*., 2006). Apparently, an autofeedback mechanism involving the psbA protein normally restricts translation (Minai *et al*., 2006; Manuell *et al*., 2007). The disadvantage of this approach, however, is the loss of photosynthesis caused by replacing native *psbA* with the transgene. To restore photosynthesis, the *psbA* gene with a non-native 5' UTR has to be inserted in another location. The lesson from these studies is that competition between endogenous genes and transgenes for limiting factors may limit protein expression levels.

1.8.3 Other factors that can affect expression

Light can regulate the translation of chloroplast transgenes that have a 5' UTR from photosynthesis genes. Synthesis of GFP driven by 5' UTRs from *psbA* or *psbD* was

increased under high light flux compared to cultures kept in darkness (Barnes *et al*., 2005; Rasala *et al*., 2010).

Other aspects of the coding region besides codon usage can affect translation efficiency (Herrin and Nickelsen, 2004), and of course, there is protein stability. Different foreign genes flanked by the same 5'/3' UTRs can vary greatly in the level of recombinant protein accumulation (Surzycki *et al*., 2009). An up to 3-fold higher level of bacterial β-glucuronidase (GUS) was achieved when the beginning of a native chloroplast gene was fused to the N-terminus of GUS (Kasai *et al*., 2003). Barnes *et al*. (2005) suggested that RNA-RNA interactions between the coding region and the 5' UTR might affect the local secondary structure and binding of translation factors. In at least one case, fusing a small protein to the C-terminus of the coding sequence enabled the accumulation of an apparently unstable recombinant protein (Rasala *et al*., 2010).

Lastly, the genetic background of the host strain can affect the level of transgene protein, at least for nuclear genes and probably for chloroplast genes (Fletcher *et al*., 2007). Two transformed host strains (137c and cc744) of *C. reinhardtii* exhibited different levels of luciferase accumulation with the same chloroplast transgene (Mayfield and Schultz, 2004).

1.9 *C. reinhardtii* as a novel platform for Bti modification

C. reinhardtii reproduces in most larval habitats, is highly digestible by the larvae (Marten, 1986), and can remain in the water column (unlike Bti crystals which tend to sink). In fact, an important study provided evidence that green algae like *C. reinhardtii*

were not only the preferred food for *Anopheline* larvae, but also were required for them to reach adulthood (Kaufman *et al*., 2006). Also, like other green algae, *C. reinhardtii* possesses UV screening compounds and pigments (Xiong *et al*., 1997; Gorton and Vogelmann, 2003; Holzinger and Lütz, 2006) that could prevent Bti proteins from damage by UV radiation.

C. reinhardtii - as a new platform for the delivery of Bti toxins - has several advantages over transgenic bacteria: 1) it is a eukaryotic alga that has sex, which places controls on the vegetative cells (the zygote is the "tough" stage); 2) bacterial antibiotic-resistance genes will not be present in the final transgenic strains (Newman *et al.* 1992; Fischer *et al*., 1996; Purton, 2007; Chen and Melis, 2013); and 3) unique aspects of chloroplast genes promote containment of the transgenes (Ferris *et al*., 1997; Lister *et al*., 2003; Harris *et al*., 2009); specifically that they will not express in bacteria, and will not pass into a local *Chlamydomonas* population (Lister *et al*., 2003; Pröschold *et al*., 2005). It is likely that a *Chlamydomonas* chloroplast-based biolarvacide – based on Bti protoxins - will stand a much better chance of getting approved for environmental use than will a transgenic bacterium.

1.10 Objectives of this dissertation

The ultimate goal of this line of research is to develop Bti-*Chlamydomonas* strains that can be used for mosquito control. My specific objectives were to: (1) design and obtain synthetic codon-optimized genes for the three major Bti Cry proteins, (2) generate chloroplast transformants that inducibly express each Cry gene and characterize them, and (3) create *C. reinhardtii* strains with constitutive (i.e., wild-type) production of Cry protoxins. Objectives 1 and 2 are described in Chapter 2, and the results for Objective 3 are described in Chapter 3. Together, these results indicate that it will be possible to generate healthy *Chlamydomonas* strains that are constitutively lethal to mosquito larvae, yet have the controls associated with chloroplast genes for photosynthesis.

Chapter 2. Inducible expression of Cry toxins of *Bacillus thuringiensis* subsp. *israelensis* (Bti) in the chloroplast of *Chlamydomonas reinhardtii*

2.1 Abstract

Although crop plants that express Cry genes have been developed and used in the field to help control insect pests, genes that are toxic to mosquito larvae have not been exploited successfully for mosquito control. Toward the development of a field-applicable organism that produces Bti Cry proteins, the Cry4Ba (128 kDa), Cry11Aa (72 kDa), and truncated Cry4Aa ($Cry4Aa_{700}$, 74 kDa) genes were expressed from the chloroplast genome of the eukaryotic green alga, *Chlamydomonas reinhardtii*. Since these proteins can be toxic to heterologous hosts, the inducible *Cyc6:Nac2-psbD* expression system was used. Codon-optimized Cry genes were synthesized, outfitted with a modified *psbD* 5' region, and integrated into the chloroplast genome of the Ind41_18 strain; homoplasmicity of the transformants was verified by PCR. Western blots of total cell protein showed the accumulation of all three proteins under induction (i.e., minus Cu^{2+}) conditions, with relative protein expression in the order of $Cry4Aa_{700}$ >> Cry11Aa > Cry4Ba. A live-cell bioassay demonstrated the toxicity of the $Cry4Aa_{700}$ and Cry11Aa transformants against larvae of *Aedes aegypti* and *Culex quinquefasciatus*. Surprisingly, the growth rates of the transformants under inducing and noninducing conditions were very similar, suggesting that these Cry gene constructs were not very toxic to these cells

(Ind41_18 host strain under minus-Cu^{2+} conditions). Finally, RT-PCR analysis of the mRNAs suggested that Cry11Aa expression may be limited at the post-translational level.

2.2 Introduction

Development of insect-resistant crops has been one of the most successful achievements of plant biotechnology. Cry genes from *Bacillus thuringiensis* were used to produce these plants using genetic engineering of the plant nucleus. Cry1A, which was obtained from *Bacillus thuringiensis* subsp. *kurstaki,* was one of the first Cry genes to be exploited, and extensive sequence recoding was necessary to obtain reasonale expression of this gene in the host plant. Cry1A is toxic to lepidopteran larvae and has now been successfully introduced into maize, rice, soybean and cotton (Halford, 2012). I propose that a similar approach could be used to control dipteran insects that are disease vectors by engineering a larval food source, the green alga *Chlamydomonas reinhardtii*, with genes from *Bacillus thuringiensis israelensis* (Bti).

Bti was isolated in the 1970s and was the first Bt strain shown to be specifically toxic to dipteran larva (Goldberg and Magalit, 1977). Even though Bti is a highly specific biolarvicide that has not caused strong resistance in >2 decades of field use, it has limited persistence as it is composed mainly of protein crystals (the spores do not recycle well in aquatic environments). In theory, this drawback could be overcome by transforming the Bti toxin genes into organisms that grow and persist in aquatic larval habitats. And one or more of the Bti toxin genes - Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa - have been introduced into some aquatic bacteria, including cyanobacteria (Reviewed in Margalith

and Ben-Dov, 1999; Boussiba *et al.*, 2000; Zaritsky *et al.*, 2010). However, little effort has been made to express Bti toxin genes in potential eukaryotic algal hosts (Kumar, 2010; Juntadech *et al.*, 2012), which are also common to these habitats.

The fact that algae and mosquito larvae reside in the same aquatic habitats, and that many algae are also excellent food sources for larvae suggest they could be a plausible platform for delivery of larvicidal Bti proteins (Marten, 1986; Kaufman *et al.*, 2006; Zaritsky *et al.*, 2010). In the most successful attempt to date, Zaritsky and colleagues in Israel developed a larvicidal cyanobacterium, *Anabaena*, which contained the Cry4Aa, Cry11Aa, and Cyt1Aa genes (Xiaoqiang *et al.*, 1997; Boussiba *et al.*, 2000; Zaritsky *et al.*, 2010). However, this strain has not been used for mosquito control yet, due partly to concerns over the bacterial antibiotic-resistance gene(s) that it contains (Zaritsky *et al.*, 2010). There may be other factors of concern, such as whether there are adequate controls on the potential spread of the organism and/or the genes it possesses. Using vegetative *Chlamydomonas* cells as the host for the Bti toxin does not bypass the natural controls that limit this organism, such as its inability to grow < 50 °F (Harris, 1989). Moreover, by using the chloroplast genome to express the Bti proteins, I can restrict the inheritance of the genes should the Bti-*Chlamydomonas* mate with a local *Chlamydomonas* strain; this is accomplished by using the minus (−) mating-type for toxin delivery (chloroplast genes are inherited from the (+) mating type). Finally, transformation technology in *Chlamydomonas* is such that the potential problem of undesirable bacterial antibiotic-resistance genes can also be avoided (Boynton and

Gillham, 1993; Fischer *et al.*, 1996; Odom *et al.*, 2001; Chen and Melis, 2013; Purton *et al.*, 2013; Economou *et al.*, 2014).

Transgenes expressed in the chloroplast have sometimes caused deleterious phenotypes (Magee *et al.*, 2004) and delayed plant development (Lössl *et al*, 2003; Chakrabarti *et al*, 2006). Oey *et al.* (2009) suggested that a high level of plastid transgene expression had exhausted the plastid translational capacity. So, one concern was that the Bti Cry genes could be toxic to *Chlamydomonas*. Also, the large sizes of the Bti Cry proteins, especially Cry4Aa and Cry4Ba (which are 134 and 128 kDa, respectively) could be problematic, since few, if any, foreign proteins of that size have been expressed in the chloroplast. I used synthetic, codon-adapted genes to increase my chances for successful expression of these large proteins. I also tried first to obtain transformants with strong constitutive expression of Cry proteins (Rasala *et al.*, 2010), but the transformants remained heteroplasmic, indicative of transgene toxicity. So, in order to obtain homoplasmic transformants, which are necessary for consistent results, I used an inducible chloroplast gene expression system.

Inducible expression systems have been the key to producing a variety of recombinant proteins in *E. coli* and other systems, especially if the proteins are potentially inhibitory. Fortunately, Rochaix and colleagues created an inducible expression system for the chloroplast (Surzycki *et al.*, 2007) by using a metal (Cu^{2+}) repressible promoter (from *Cyc6*) to control expression of the nuclear *NAC2* gene. The NAC2 protein goes to the chloroplast where it stabilizes any mRNA that has the 5' UTR from *psbD* (Fig 2.1). Hence, when their Ind41_18 host strain is used, removing copper

from the medium induces expression of the chloroplast transgene, which in this case are Cry genes. In this chapter, I describe the synthetic Cry genes that were created and their successful expression in the *Chlamydomonas* chloroplast using the Ind41_18 host strain (kindly provided by J-D Rochaix, U. of Geneva, Switzerland). The results set the stage for the next phase of the project, obtaining homoplasmic transformants with constitutive Cry gene expression.

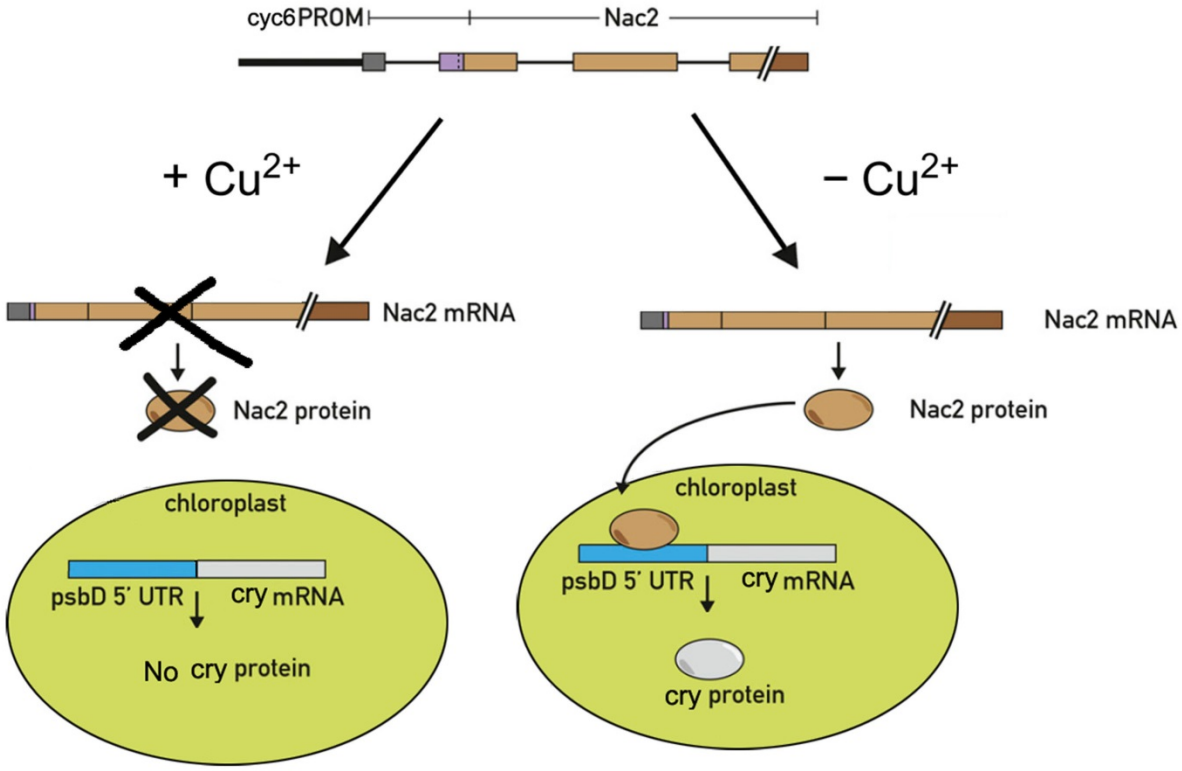

Figure 2.1 The inducible chloroplast gene expression system used to express Cry genes. In the Ind41_18 host strain of *Chlamydomonas*, expression of the Cry constructs is controlled by the nuclear *Cyc6:Nac2* gene, which is controlled by Cu^{2+} levels. The presence of Cu^{2+} inhibits the expression of the *Nac2* gene, which causes repression of the Cry gene flanked by *psbD* 5' UTR. When Cu^{2+} is removed, the NAC2 protein is made and binds to the *psbD* 5'-UTR of the chimeric Cry mRNA, stabilizing it. (The diagram was adapted from Ramundo *et al.*, 2013)

2.3 Materials and methods

2.3.1 *Chlamydomonas* strains and media

The Ind41_18 strain of *C. reinhardtii* was obtained from J-D Rochaix (U. of Geneva, Switzerland), and the wild-type 2137 strain (CC-1021) was from the *Chlamydomonas* Culture Center. The cultures were maintained by periodic transfer to fresh plates of Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) that were kept in the light at 23°C. TAP medium was also used as the $+Cu^{2+}$ medium (TAP + Cu^{2+}), and TAP minus copper (TAP − Cu^{2+}) was made by removing copper from the Hutner's trace elements solution. It was prepared as described by Quinn and Merchant (1998) and Harris (1989). A mixture of $ZnSO_4 \cdot 7H_2O$, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and $FeSO_4 \cdot 7H_2O$ was boiled, and then the EDTA solution was added. $CuSO_4 \cdot 5H_2O$, which was used for the normal Hutner's trace element solution, was not added for the $+Cu^{2+}$ medium. After cooling to 70°C, the pH was adjusted to 6.7 by adding hot 20% KOH. After adjusting the final vol to 1 L with MilliQ-water, the solution was allowed to stand for 1~2 weeks with daily shaking. During this time, the solution changed from orange-red to burgundy red. Liquid culture was in flasks that were ca. 40% full and mixed continuously on an orbital shaker at 125 rpm. Cell counts were made with a hemacytometer after killing the cells with iodine (5% (w/v) I_2, 10% (w/v) KI). Also, for the growth rate tests, total chlorophyll was used to estimate the number of cells/mL using the reference value of 4 mg chlorophyll per 1×10^9 cells (Harris, 1989).

2.3.2 Design and preparation of synthetic Cry genes

For codon optimization of Cry4Ba, Cry11Aa, and a truncated version of Cry4Aa containing only amino acids (aa) 1-700, $Cry4Aa_{700}$, I used the sequences of the corresponding Bti genes (Gene IDs were as follows: Cry4Aa – 5759905, Cry4Ba – 5759934, Cry11Aa - 5759849) and the program Optimizer (Puigbò *et al.*, 2007). The codon-usage table that was employed was one that I made from 8 highly expressed chloroplast genes; however, it was not that different from one that is based on all chloroplast-encoded ORFs (Kazusa University web site). The protein sequences also contained the 8-aa Flag peptide DYKDDDDK at the C-terminus to enable their detection on western blots with commercial Flag antibodies. After analyzing the predicted RNA structures at the 5' end of the genes using Mfold, the third codon in the optimized Cry4Ba sequence was changed from AAC to CAA, which changed the aa from asparagine (N) to glutamine (Q). This was done to prevent an unfavorable secondary structure that would have tied up the start codon in a paired region. The 3 genes were synthesized by Integrated DNA Technologies, who also confirmed the sequences and provided them to us as cloned plasmids. The DNA sequences of the synthetic genes are part of a patent disclosure that will be submitted in May 2015 by the University of Texas on behalf of the inventors (D.L Herrin, Seongjoon Kang and O.W. Odom).

2.3.3 Early attempts to express Cry genes constitutively

pD1-KanR (obtained from S Mayfield, University of California, San Diego) is a *Chlamydomonas* chloroplast transformation plasmid that can give one of the highest

levels of transgene expression. The foreign gene is expressed using 5' and 3' signals from *psbA* and the transgene actually replaces the endogenous *psbA* gene during transformation (Rasala *et al.*, 2010). The codon-adapted Cry4A$_{700}$, Cry4Ba, and Cry11Aa genes were excised from their original plasmids by NdeI + XbaI digestion and ligated into NdeI + XbaI-digested pD1-KanR to give plasmids pD1-4A, pD1-4B, and pD1-11A, respectively. These plasmids were transformed into the chloroplast of a wild-type strain (2137 mt+) using biolistic bombardment (section 2.3.5), and transformants were selected on kanamycin (100 µg/mL) plates incubated under dim light (ca. 4 µE m^{-2} sec^{-1}) at 23°C. Single colonies were re-streaked several times on plates containing 300 µg/mL kanamycin, before they were tested for homoplasmicity by PCR. All of the transformants remained heteroplasmic (i.e., they contained a mixture of transformed and untransformed copies of the chloroplast genome), indicating a certain level of toxicity to the cells.

I also considered using the 5' expression signals from two chloroplast ribosomal protein genes, *rps4* and *rps7*, to drive (constitutive) Cry11Aa expression in wild-type background. The idea of using ribosomal protein 5' expression signals was to try and direct synthesis of Cry proteins away from the thylakoid membrane, which might make it less toxic to the chloroplast. However, when I tried to clone the Cry genes into the *rps* expression plasmids (P-655 for *rps7* and P-657 for *rps4*; obtained from the Chlamydomonas Center, U. of Minnesota), the Cry4Aa$_{700}$ and Cry4Ba clones could not be established even in *E. coli*. Although Cry proteins are considered to be protoxins that become fully activated only in the larval gut, it is also clear that they do have toxicity

even as protoxins and that they can damage host cells if expression is too high (Manasherob *et al.*, 2003; Chakrabarti *et al.*, 2006; Chen *et al.*, 2014).

2.3.4 Constructing Cry plasmids for inducible expression

Construction of the plasmids for inducible Cry gene expression was carried out using *E. coli* DH5α (Invitrogen) as the host, and they were assembled in the low-copy pET-16b plasmid. The codon-adapted Cry genes (from IDT) were excised from the IDT plasmids by digestion with XbaI (on the 3' side), blunting with the Klenow DNA polymerase, and then digestion with NdeI (on the 5' side). The Cry4Aa$_{700}$ and Cry11Aa genes were ligated to pET-16b that had been cut with XhoI, blunted with Klenow, and then cut with NdeI. However, the NdeI digestion was incomplete and so the clones turned out to have 9 extra nucleotides (3 amino acids, MLD) at the beginning of the coding sequence that included an intact NdeI site. For Cry4Ba subcloning, pET-16B was digested with BamHI instead of XhoI, then blunted and digested with NdeI; so the Cry4Ba clone did not have extra nucleotides at the 5' end. The new plasmids were pET-4A$_{700}$, pET-4B, and pET-11A.

The 5' and 3' expression signals (from *psbD* and *psbA*, respectively) were added sequentially to the Cry genes as PCR products made with the high-fidelity Phusion DNA polymerase (from New England Biolabs) according to the manufacturer's instruction. The primers used for the PCR reactions are listed in Table 2.1, and the thermocycling program was as follows: 94°C for 3 minutes; 33 cycles of 52°C (1 minute), 72°C (3.5

minutes), and 94°C (30 seconds); 52°C (1 minute); and then 72°C (5 minutes). The PCR products were analyzed on 1% agarose gels before restriction digestion and cloning.

The 5' expression signals for the *psbD* gene, including the promoter and 5'-UTR, were amplified from plasmid p108-14 (Surzycki *et al.*, 2007) which has the EcoRI R3 fragment of the chloroplast genome of *C. reinhardtii*, and was obtained from Jean-David Rochaix (U of Geneva). The forward primer (847 in Table 2.1) contained overlapping NcoI and BamHI sites; the NcoI site was used to attach it to the coding regions (as a NcoI-NdeI fragment) and the BamHI site was used later to excise the whole gene for subcloning into the chloroplast transformation plasmid. The reverse primer (850 in Table 2.1) contained an NdeI site - for attaching it to the coding region - but also altered the possible Shine-Dalgano sequence at nucleotides -13 to -9 from GGAG to AAAG (Nickelsen *et al.* 1999); this mutation was introduced to block translation in *E.coli*. The altered 5' region was named $psbD_m$, and the resulting PCR product was double-digested with NcoI and NdeI and cloned into the NcoI + NdeI-digested pET-Cry plasmids (above); the new plasmids were named pET-5D4A$_{700}$, pET-5D4B, and pET-5D11A.

Table 2.1. Oligonucleotide sequences

ID no.	Name[a]	Sequence[b]
795	Cry4A F	GTCAACAAAACCAACAATACG
796	Cry4A R	TTAGTGTAGTCAGTACCTGAG
797	Cry4B F	AACGACTTACAAGGTTCAATG
798	Cry4B R	TGTCTGGGAATACGTCTACAG
799	Cry11A F	GGAAGACTCATCATTAGACAC
800	Cry11A R	AGTAGCAGTGTTGAAACCAGT
802	T7 prom pET	GAAATTAATACGACTCACTATAGG
803	T7 ter pET	GCTAGTTATTGCTCAGCGG
847	*psbD* 5' F	gctcccatggatccTCATAATAATAAAACCTTTATTCAT NcoI BamHI
850	*psbD* 5' R	ccggcatatgGTGTATCTTTTAAAATAAAAAAACAACTCATCGTTACG NdeI
860	*psbA* 3' F	cggggctgAGCTCAAACAACTAATTTTTTTTTAAAC BlpI
861	*psbA* 3' R	cagtgctcagcggaTCCTGCCAACTGCCTATGGTAGC BlpI BamHI
864	Integration site F	TGGAATTGGATATGGACTAG
865	Integration site R	GGTACTTGCATTTCATAAGT

[a] F, forward; R, reverse

[b] Upper case letters, Cry or chloroplast gene nucleotides; underlined letters, nucleotides used to generate restriction sites; lower case letters (not underlined), additional nucleotides added to increase digestion efficiency. Bold and gray-shaded TT nts in *psbD*5'R are substitutions of the normal CC nts, in order to eliminate the Shine-Dalgarno-like sequence.

To add the *psbA* 3' region, it was amplified from plasmid P-322 (Newman *et al.*, 1992; *Chlamydomonas* Culture Center) with primers 860 and 861 (Table 2.1). Both primers contained a BlpI site for subcloning the product downstream of the Cry coding regions, and the reverse primer (861) also contained a BamHI site for cloning the whole construct into a chloroplast transformation plasmid (see below). The PCR product was cut with BlpI and cloned into Bpu1102I-cut pET-5D4A$_{700}$, pET-5D4B, and pET-5D11A, where it attaches in only one direction. This added ~50 nucleotides of the vector to the end of the coding region, preceding the 3' UTR from *psbA*. The new plasmids were named pET-5D4A$_{700}$3A, pET-5D4B3A, and pET-5D11A3A. The *psbD$_m$*-Cry-*psbA* constructs were confirmed by sequencing.

To create the chloroplast transformation plasmids, the Cry gene constructs were excised with BamHI and cloned into the BamHI site of p322.1, which corresponds to the intergenic region between the *psbA* and the 23S rRNA genes (in the inverted repeat of CpDNA). The final plasmids were named pCry4A$_{700}$, pCry4B, and pCry11A.

2.3.5 Chloroplast transformation of the Ind41_18 strain

For transformation, the Ind41_18 strain was grown in liquid TAP medium under a light flux of ca. 40 µE m^{-2} sec^{-1} at 23°C. The cultures were mixed continuously on an orbital shaker (125 rpm) until they reached the late log/early stationary phase (2×10^6 - 4×10^6 cells/mL). The cells were collected by centrifugation, and resuspended in fresh TAP to a concentration of ~1×10^8 cells/mL; cell number was approximated from chlorophyll content (Arnon, 1949; Harris, 1989). 0.4 mL of the cells (~4×10^7) was

mixed with 0.4 mL of molten 0.25% agar in TAP minimal medium. 0.8 mL of the mixture was pipetted onto the center of a TAP-agar plate containing 100 μg/mL of ampicillin, and allowed to air dry.

Biolistic transformation of the Ind41_18 chloroplast with the Cry plasmids was performed as described by Odom *et al.* (2001) using co-transformation with plasmid pB4CC110. pB4CC110 harbors a 7-kb BamHI fragment of CpDNA that contains the spectinomycin-resistance marker, spr-u-1-6-2, in the 16S *rrn* gene (Harris, 1989; Newman *et al.*, 1990). 5 μg of pB4CC110 and an equal amount of one of the Cry plasmids were co-precipitated onto 3 mg of tungsten particles (M17, Bio-Rad), and about 600 ng of plasmid DNA was shot at each plate of cells embedded in a layer of soft agar (Boynton and Gillham, 1993). The bombarded plates were incubated overnight in dim light (ca. 2 μE m^{-2} sec^{-1}), then the cell layer of each was scraped off and split onto two TAP-agar plates containing 100 μg/mL spectinomycin. The selection plates were incubated under bright light (ca. 40 μE m^{-2} sec^{-1}) at 23°C, and spectinomycin-resistant colonies appeared in 2-4 weeks. The colonies were re-streaked and grown several times on TAP-agar containing 300 μg/mL spectinomycin until they reached homoplasmicity as judged by PCR.

2.3.6 PCR screening of chloroplast transformants

Cry transformants for PCR analysis were grown on a TAP-agar plate with 300 μg/mL spectinomycin, and total DNA was extracted as described by Kwon *et al.* (2014). To check the homoplasmicity of the chloroplast transformants, I used a set of primers

(864 + 865) (Table 2.1) that amplify the integration site in CpDNA; homoplasmicity was indicated by the absence of untransformed copies of the genome. PCR with gene-specific primers for each Cry gene (795 + 796 for Cry4Aa$_{700}$, 797 + 798 for Cry4Ba, and 799 + 800 for Cry11Aa) (Table 2.1) was also performed to confirm the presence of the Cry gene. Standard PCR procedures with *Taq* DNA polymerase (New England Biolabs) and the manufacturer's buffer were used. The thermocycle program for these amplifications was as follows: 94°C for 3 minutes; 33 cycles of 52°C (1 minute), 72°C (3.5 minutes), and 94°C (30 seconds); 52°C for 1 minute; and 72°C for 5 minutes. The PCR products were analyzed on 1% agarose gels.

2.3.7 Protein and chlorophyll extraction, and western blotting

The transformants and parental strain were grown in liquid TAP, which contains Cu^{2+} (uninducing condition), and in TAP − Cu^{2+} (inducing condition) at a light flux of ca. 40 µE m^{-2} sec^{-1} (23°C) with shaking until late log-early stationary phase (2×10^6 - 4×10^6 cells/mL). The erlenmeyer flasks and graduated cylinders (glass) used for the inducing culture were washed sequentially with 6 N hydrochloric acid, distilled water (7×), and MilliQ-water (3×) prior to use. For the extraction, 50 ml of culture was centrifuged and resuspended in 0.5 ml of leupeptin cocktail (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 µg/mL leupeptin, 0.5 mM EDTA, 2 mM DTT, 1 mM PMSF; Pognonec *et al.*, 1991); since PMSF is unstable in water, it was added right before use. Then, 0.5 ml of 2× lysis buffer (100 mM Tris-HCl pH 7.4, 4% SDS) was added, and the samples were

sonicated (2 × 30 seconds at 80% power on ice). The lysates were rocked at 37°C for 1 hour, centrifuged at 14,000 × g (RT) for 10 min, and the supernatant was saved.

Total chlorophyll was measured as described by Windermans and De Mots (1965). Cells were harvested from 1 mL of culture by centrifugation in a microfuge for 5 minutes, then the pellet was extracted with 1 mL of 95% EtOH. After centrifuging again for 2 minutes, the supernatant was removed and its absorption at 665 nm and 649 nm was used to calculate total chlorophyll in µg/mL.

For the western blots, the cell lysates were mixed with 3× SDS loading buffer (50 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 0.03% bromphenol blue, 0.3 M DTT) and boiled for 3 minutes. A prestained protein ladder (PageRuler from Fermentas) was used as size markers, and the samples were separated at RT in SDS-PAGE gels (either 12% or 6%) using an acrylamide/bisacrylamide ratio of 30:0.8 and Laemmli buffer (Laemmli, 1970). The gels were 16 cm in length, 1 mm thick, and the lane width was 6 mm. After electrophoresis, the gel was soaked in cold transfer buffer (25 mM Tris base, 192 mM glycine, 5% methanol) for ~15 min to allow equilibration. A PVDF membrane (Hybond-P, GE HealthCare) was soaked in 100% methanol for 10 seconds, washed with Milli-Q water for 5 minutes, and then transferred to the cold transfer buffer for ~10 minutes. The proteins were transferred to the PVDF membrane for 1.5 hours at 12 Volts and 4ºC using a Genie Blotter (Idea Scientific) (Memon *et al.* 1993). The blots were stained with Ponceau S to confirm the transfer of total protein, and then blocked for 1 hour with 5% nonfat dried milk in TBS-T (Tris-buffered saline plus 0.05% Tween 20). The blots were washed 2× with TBS-T for 5 minutes each with shaking. They were probed with an anti-

FLAG monoclonal antibody (M2) coupled to alkaline phosphatase (Sigma no. A9469) that was diluted 1:4,000 in TBS-T; the binding was for 1 hr at room temperature with shaking. The blots were washed 6× with TBS-T for 5 minutes each with shaking. The bound antibodies were detected using the Lumi-Phos WB Chemiluminescent substrate (Thermo Scientific) as described in the manufacturer's instructions. The chemiluminescence was detected by exposing the blots to X-Ray film, and developing them with SRX-101A (Konica Minolta). The developed films were scanned using an HP scanner and Silver Fast (LaserSoft Imaging) software. The quantification of relative western band intensity was performed using the ImageJ program (Version 1.46, National Institutes of Health, Bethesda, MD).

2.3.8 RT-PCR

Total nucleic acids (TNA) was extracted as described previously (Kwon *et al.*, 2014) from cultures (50 ml) grown in $+Cu^{2+}$ and $-Cu^{2+}$ TAP medium in the light until late log phase. To obtain the RNA fraction, 10 µg of the TNA preparations were treated with DNase (Turbo DNase from Ambion) in total volume of 55 µL to eliminate the DNA. 4 µL of each RNA sample was copied into cDNA using reverse transcriptase (Superscript III, Invitrogen) in a total volume of 20 µL at 65°C (5 minutes) and internal reverse primers, 796 (Table 2.1) for $Cry4Aa_{700}$ and 800 (Table 2.1) for Cry11Aa. One µL of the reverse transcription reaction (cDNA) was used as the template for the PCR reaction (total volume of 25 µL) with specific primer sets: 795 + 796 (Table 2.1) for $Cry4Aa_{700,}$ and 799 + 800 (Table 2.1) for Cry11Aa. *Taq* DNA Polymerase (New England

Biolabs) was used in a standard PCR program which was the same as described above (section 2.3.6), except that the number of cycles was lowered to 24. 10 µL (out of 25 µL) of amplified cDNA was analyzed by electrophoresis in 1% agarose gels.

2.3.9 Growth rate determinations

Growth rates of the Cry transformants and the parental (Ind41_18) strain under uninduced and induced conditions were determined in liqud medium. Agar cultures were used to inoculate liquid TAP medium and the cells were grown until near-stationary phase (4×10^6 cells/mL). Then, they were used to inoculate fresh 50-ml cultures of TAP + Cu^{2+} and TAP − Cu^{2+} to a cell concentration of 5×10^4 cells/mL, and grown as described above (see section 2.3.7). In order to estimate growth, aliquots were removed every 12 hours for determination of total chlorophyll (Harris, 1989).

2.3.10 Bioassay for larvacidal activity of Cry transformants

Bioassays with mosquito larvae were aided by Dr. Saravanan Thangamani (U of Texas Medical Branch-Galveston), and followed guidelines of the World Health Organization (2005) with some modifications. The $Cry4Aa_{700}$ and Cry11Aa transformants were grown under inducing and noninducing conditions, and Ind41_18 was grown under inducing conditions as described in section 2.3.7 until they reached stationary phase. Then, volumes equivalent to 5×10^6 cells, 2.5×10^7 cells, and 5×10^7 cells were centrifuged at ~1000 rpm (GH-3.7 rotor) for 5 minutes, and the pellets were washed with dH_2O and re-centrifuged. The final pellets were resuspended in 2 mL of

dH_2O. The bioassays were performed in triplicate at 27°C, under 12 hour:12 hour light/dark cycles, and in quadrant petri dishes (Pyrex); each quadrant contained 10 live mosquito larvae and *Chlamydomonas* in 5 mL of dH_2O. Two mL of concentrated *Chlamydomonas* cells (in dH_2O) was added to the larvae, which were in 3 mL of dH_2O, to make the final concentrations of 1×10^6 cells/mL (1×), 5×10^6 cells cells/mL (5×), and 1×10^7 cells cells/mL (10×). The dH_2O was used so the algae would not grow during the assay. The larvae were transferred to dH_2O the day before the assay and starved overnight. The larvae were 3rd instar *Culex quinquefasciatus* (*C. quinquefasciatus*) and 4th instar *Aedes aegypti (A. aegypti)*, and larval deaths were counted visually after 24, 48, and 72 hours with the live algae. For the determination of the median lethal concentration (LC_{50}) of the Cry11Aa transformant against 4th-instar *A. aegypti* larvae, 10 different concentrations of induced-Cry11Aa cells were used (in triplicate) in the bioassay. These were: 2.5×10^5 cells/mL, 3.76×10^5 cells/mL, 5×10^5 cells/mL, 7.5×10^5 cells/mL, 1×10^6 cells/mL, 1.5×10^6 cells/mL, 2×10^6 cells/mL, 2.5×10^6 cells/mL, and 5×10^6 cells/mL. The LC_{50} was calculated with Microsoft Excel using Probit analysis (Finney, 1971). In some cases, images of the larvae were captured using LAS EZ software on a stereomicroscope. The toxicity of the transformants against *Anopheles* was not tested because eggs of *Anopheles* are difficult to be obtained and stored.

2.4 Results

2.4.1 Synthetic Cry genes

An early attempt to express the native Cry11Aa gene in the *C. reinhardtii* chloroplast was unsuccessful in that no protein was detected using an anti-Cry11Aa antibody (OW Odom, personal communication). In recent years, it has become clear that codon bias is one of the limiting factors for expressing foreign genes in the *Chlamydomonas* chloroplast (Franklin *et al.*, 2002; Mayfield *et al.*, 2003). And though I successfully expressed a native luciferase gene in the chloroplast, that protein was only 35 kDa (Minko *et al.*, 1999), and the Cry proteins are 72 to 134 kDa. Thus, synthetic Cry4Aa$_{700}$, Cry4Ba, and Cry11Aa genes were designed based on the codon usage of 8 highly expressed chloroplast genes from *Chlamydomonas*, such that the Codon Adaptive Index increased from ~0.5 to 1 after optimization (Figure 2.2). Also, for Cry4Aa, only the first 700 amino acids were used in order to increase the chances of higher expression; larger proteins tend to be less abundant than smaller proteins (Bernaudat *et al.*, 2011). Lastly, the 8-amino acid Flag tag (Einhauer and Jungbauer, 2001) was added to the C-terminus of all three Cry proteins to make it possible to detect them with a commercial antibody (Figure 2.3). This tag was expected to have little effect on insect toxicity, since the terminal amino acids are cleaved off in the gut. Before attempting chloroplast expression, I expressed all 3 genes in *E. coli* using the inducible pET system (Studier *et al.*, 1990), and the expected protein sizes were obtained in all 3 cases (data not shown).

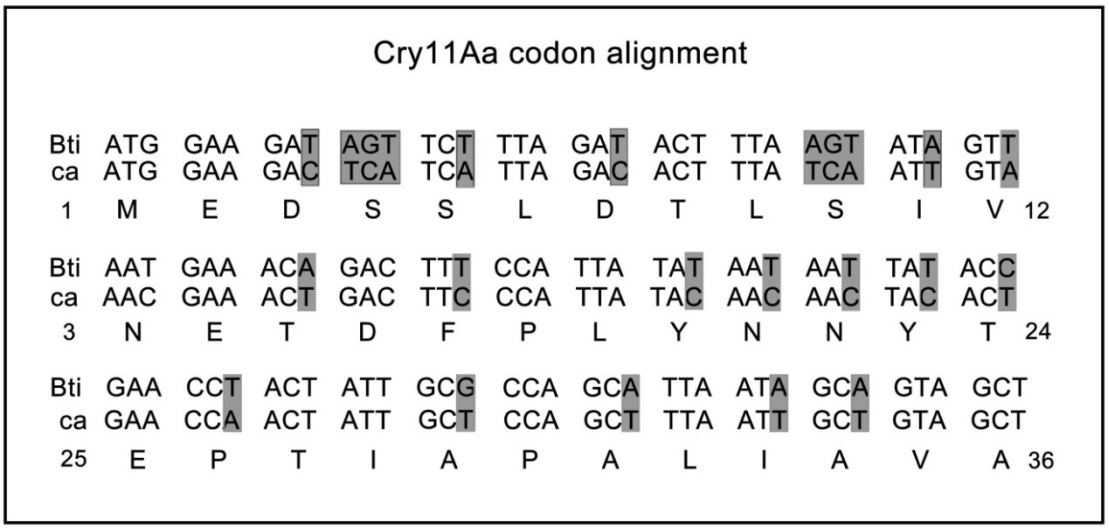

Figure 2.2 Representative **c**omparison between a native Bti toxin sequence (Bti) and a codon-adapted (ca) toxin sequence; this part of Cry11Aa corresponds to amino acids 1-36. Nucleotides that were changed are shaded.

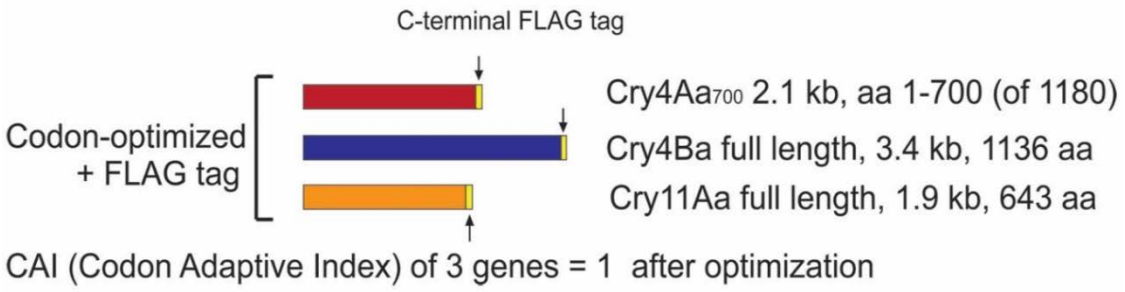

Figure 2.3 Synthetic codon-optimized Cry genes: Cry4Aa$_{700}$**, Cry4Ba, and Cry11Aa**. The genes were designed using the native toxin amino acid sequences, the program Optimizer, and a codon-usage table based on highly-expressed *Chlamydomonas* chloroplast genes; with optimization, the codon adaptive index (CAI) for each gene increased from ~0.5 to 1. The Flag epitope tag for antibody-detection was added to the C-terminus of all threee genes. The genes were synthesized for us by Integrated DNA Technologies.

2.4.2 Attempts to express the synthetic Cry genes using pD1-KanR, p655 and p657

In my first attempt to express the synthetic Cry genes in the *Chlamydomonas* chloroplast, the pD1-KanR vector (Rasala *et al.*, 2010) was used. With this vector, the transgene is expressed using the 5' and 3' control regions of the chloroplast *psbA* gene, and the transgene replaces the *psbA* gene; thus, the transformants are non-photosynthetic and kanamycin resistant. However, the transformants remained heteroplasmic despite repeated selective growth, suggesting that the proteins have some host cell toxicity. I also looked at expressing the Cry genes using the 5' control regions from the ribosomal protein genes *rps7* and *rps4*, with the 3' region coming from *rbcL*; however, most of these constructs were too toxic to *E. coli*, presumably because the *rps* 5' expression signals are functional in bacteria (Fargo *et al.*, 1998). Based on these results, I decided to use an inducible chloroplast expression system to express these Cry genes for the first time in the organelle.

2.4.3 Inducible Cry gene construction and chloroplast transformation

To get around the problem of host cell toxicity, I used the copper-repressible system developed in the Rochaix lab (Surzycki *et al.*, 2007), in which expression of the chloroplast transgene is controlled by the nuclear *Cyc6:NAC2* gene (Figure 2.1). With Cu^{2+} in the medium, the *Cyc6:NAC2* is repressed, which destabilizes the transgene mRNA in the chloroplast. When Cu^{2+} is removed from the medium, the *Cyc6:NAC2* is expressed and the NAC2 protein stabilizes the chloroplast transgene mRNA by binding to the *psbD* 5' UTR region. I made one modification to the native *psbD* sequence, a possible

Shine-Dalgarno sequence in the 5' UTR, GGAG, was mutated to AAAG (creating 5' *psbDm*) to decrease translation in *E. coli*; this change should have had little or no effect in the chloroplast (Nickelsen *et al.*, 1999). Also, to further minimize toxicity to *E. coli*, the Cry gene constructs were assembled in a low-copy plasmid (pET-16b).

The *psbDm:Cry:psbA* gene constructs were cloned into an intergenic site in p322.1 (Figure 2.4), and co-transformed into Ind41_18 with pB4C110, which contains a spectinomycin-resistant 16S rRNA gene; the inserts from both plasmids are from the inverted repeat region of CpDNA. Spectinomycin-resistant colonies were re-streaked on spectinomycin plates several times until they approached homoplasmicity as judged by PCR analysis of the CpDNA. Figure 2.5 shows the PCR analysis used to evaluate transformants. Primer pair 864/865 amplifies the integration site in CpDNA and was used to judge the homoplasmicity of transformants. The 864/865 pair gives a small product (~100 bp) from genome copies that have no Cry gene insert but a large product from copies that have been transformed; the absence of the small product indicates that all copies have been transformed (i.e., homoplasmic). Also, internal primer pairs were used to verify the presence of the specific Cry gene (795/796 for Cry4Aa$_{700}$, 797/798 for Cry4Ba, and 799/800 for Cry11Aa), and in every case they gave the correct size product (Figure 2.5). The results indicate that one of the two Cry4Aa$_{700}$ transformants is homoplasmic (4A-2 in Figure 2.5), while the other (4A-5) still had some untransformed copies. The only Cry4Ba transformant was homoplasmic (4B-1 in Figure 2.5), whereas one of the two Cry11Aa transformants is homoplasmic (11A-8), but the other (11A-6)

still had some untransformed CpDNA copies. It should be noted that the CpDNA in Ind41_18 and wild-type (WT) are the same in that region of the genome.

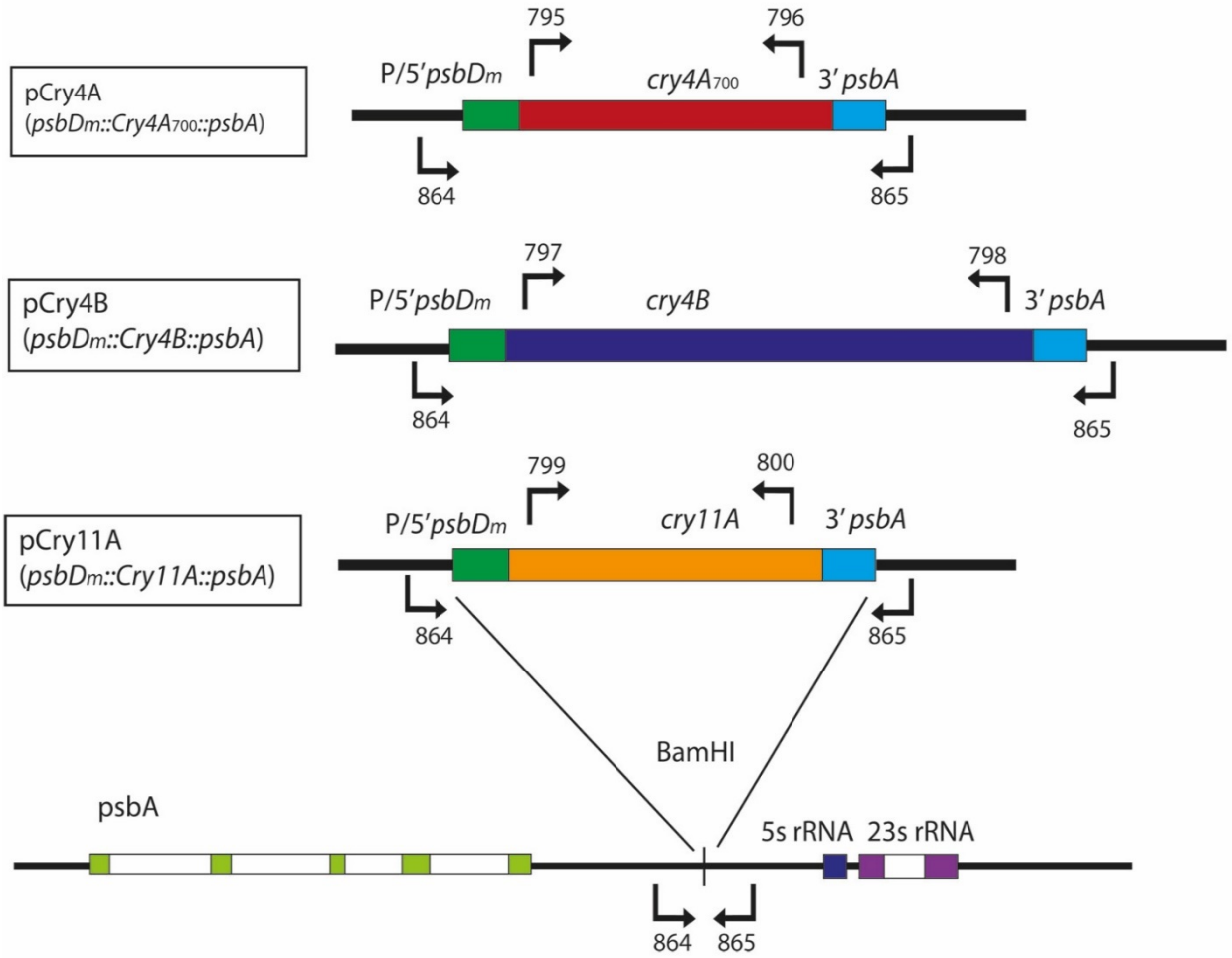

Figure 2.4 Diagrams of the Cry gene constructs and the site of integration in the chloroplast genome of Ind41_18. Expression of the Cry genes is controlled by a modified *psbD* promoter/5'-UTR ($psbD_m$) and 3' region from *psbA*. The locations of primers used for PCR screening of the transformants (Fig 2.5) are indicated. Note that primers 864 and 865 are located upstream and downstream, respectively, of the integration site in CpDNA. Some parts of the diagram are not drawn to scale.

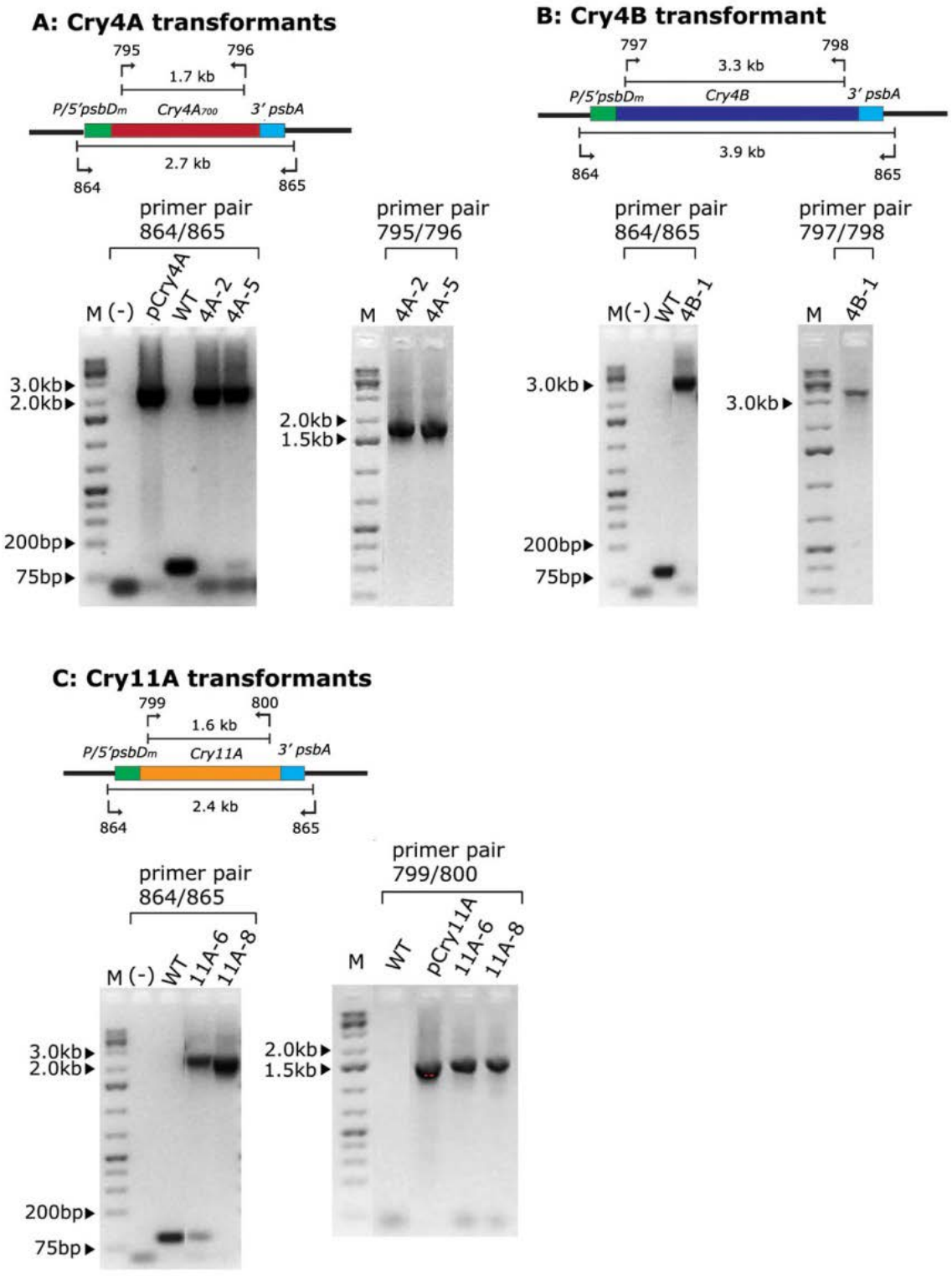

Figure 2.5 See next page for the legend.

Figure 2.5 PCR analysis of chloroplast transformants. Diagrams of the primers and expected sizes of the respective PCR products are indicated above the agarose gels, whose fluorescence images were inverted. **(A)** Analysis of two Cry4Aa$_{700}$ transformants. Wild-type DNA was used as the positive control for the integration site as it is identical to Ind41_18 in this region. pCry4A is the plasmid that was integrated into the chloroplast. The other lanes contained size markers (M) and a reaction with no DNA (−) as a negative control. **(B)** Analysis of a Cry4Ba transformant. The other lanes were similar to (**A**). **(C)** Analysis of two Cry11Aa transformants. Lanes WT, M and (–) were similar to (A), and lane pCry11A was the plasmid that was shot into the cells.

2.4.4 Western blot analysis of Cry protein accumulation

Accumulation of the Cry proteins in the transformants grown with Cu^{2+} (Uninduced) and without Cu^{2+} (Induced) was assessed using western blotting of total cell protein with an anti-Flag antibody (Figure 2.6). It should be noted that both of the Cry4Aa$_{700}$ transformants (Figure 2.5) gave similar results, as did both of the Cry11Aa transformants (Figure 2.5), so only the results with the homoplasmic Cry4Aa$_{700}$ and Cry11Aa transformants (4A-2 and 11A-8, respectively) are shown in Figure 2.6. The left panel is from a 12% gel, and contained all 3 types of transformants, whereas the right panel is from a 6% gel, which was used to better separate the very large Cry4Ba protein (~130 kDa predicted) from a non-specific protein band (NS) that appear with the Flag antibody (left panel). Proteins close to the predicted sizes for Cry4Aa$_{700}$ (74 kDa), Cry4Ba (130 kDa), and Cry11Aa (73 kDa) were obtained (or increased) under the induced conditions. There was also significant accumulation of Cry11Aa, and to a lesser extent Cry4Aa$_{700}$, in the uninduced condition. However, the induction of Cry4Aa$_{700}$ was quite strong (6-10-fold), whereas the increase in Cry11Aa under induction was only 2-2.5 fold. Quantification of three different blots provided an estimate of the relative expression of the Cry proteins under induction conditions as 3.5:1:0.75 for Cry4Aa$_{700}$:Cry11Aa:Cry4Ba.

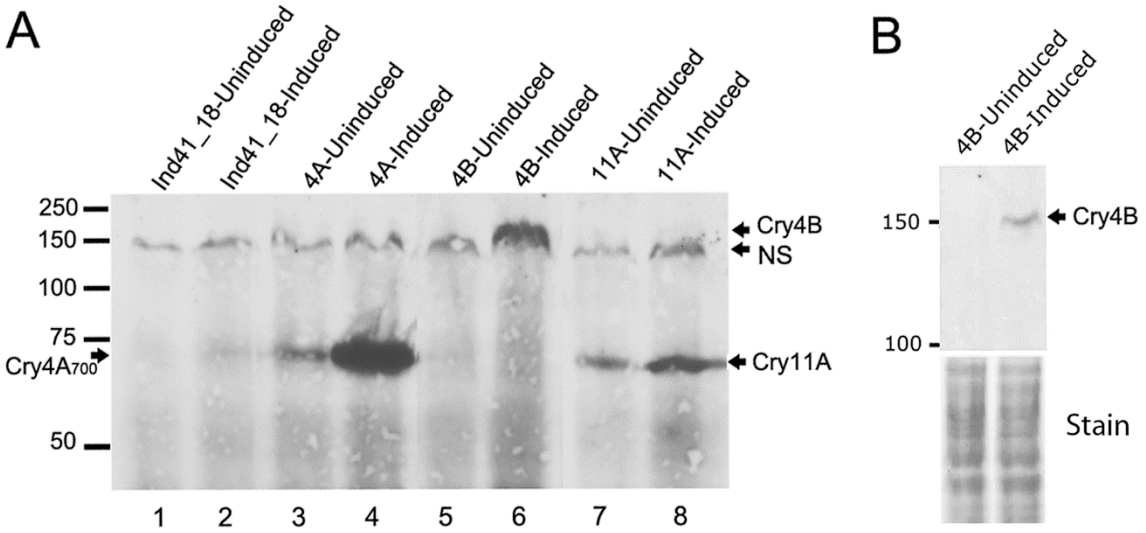

Figure 2.6 Western blot analysis of Cry transformants with the anti-Flag antibody. **(A)** Solubilized cells (20 μg chlorophyll) were separated on a 12% polyacrylamide gel, blotted and probed with the monoclonal anti-Flag antibody. The *Chlamydomonas* strains were: Ind41_18, parental; 4A, Cry4Aa$_{700}$ transformant 4A-2; 4B, Cry4Ba-1 transformant 4B-1; 11A, Cry11Aa transformant 11A-8. Each strain was grown under uninduced and induced conditions for ~72 hours. The non-specific band (NS) migrating at ~145 kDa in all the lanes serves as a loading control. (**B**) Solubilized cells (10 μg chlorophyll) from the 4B-1 transformant, grown as indicated, were separated on a 6% polyacrylamide gel. Duplicate lanes were either stained with coomassie (bottom panel) to verify the loading, or blotted and probed with the anti-Flag antibody (top panel).

2.4.5 RT-PCR analysis of $Cry4Aa_{700}$ and Cry11Aa expression

Although $Cry4Aa_{700}$ and Cry11Aa are similar-sized proteins, the $Cry4Aa_{700}$ protein level under inducing conditions was 3-4-fold higher than Cry11Aa, so I decided to examine the mRNAs by semi-quantitative RT-PCR. Figure 2.7 shows that both mRNAs were present without induction, but that both also increased substantially (3-5-fold) under induction conditions. The presence of the mRNAs without induction suggests that the absence of the NAC2 protein is not totally destabilizing for the mRNAs; moreover, it explains the presence of the Cry11Aa protein without induction. On the other hand, the results indicate a lack of correlation between the *psbDm:Cry11Aa:psbA* mRNA and the Cry11Aa protein with the mRNA induction being much stronger than the protein induction (~5-fold versus 2-fold). This suggests that Cry11Aa expression is limited at the level of translation or protein stability, at least under inducing ($-Cu^{2+}$) conditions.

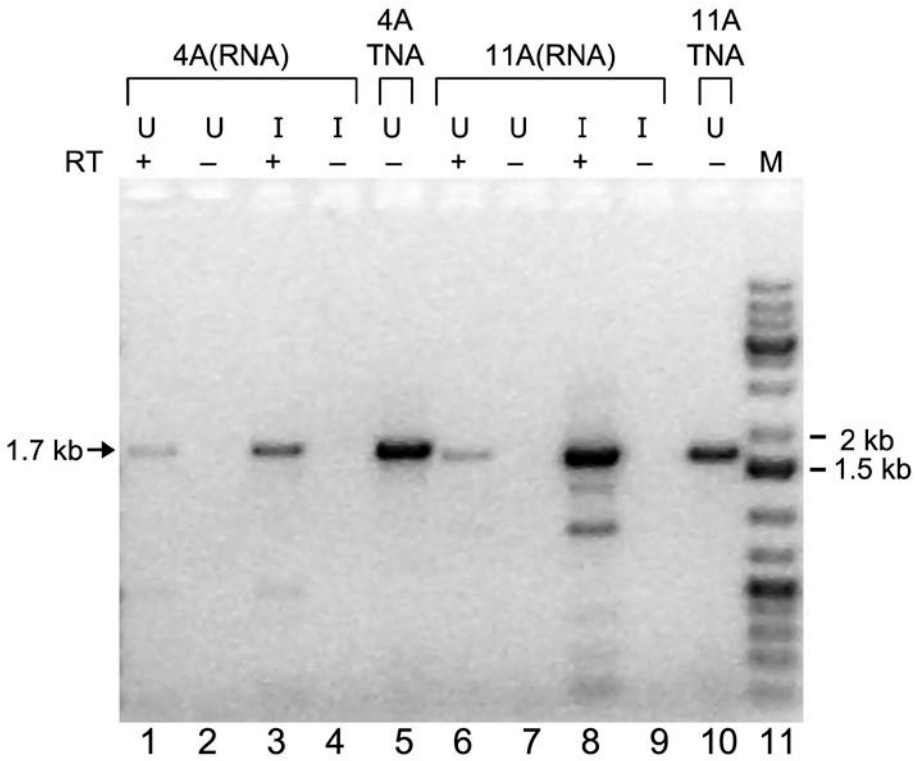

Figure 2.7 RT-PCR analysis of the Cry4Aa$_{700}$-2 (4A) and Cry11Aa-8 (11A) transformants. An equal amount of RNA from cultures grown for 72 hours under uninduced (U) and induced (I) conditions was used for reverse transcription with gene-specific primers; 796 for Cry4A$_{700}$ and 799 for Cry11A. The resulting cDNAs were amplified using primers 795 + 796 for Cry4Aa$_{700}$ and 799 + 800 for Cry11Aa. Reactions without reverse transcriptase in the RT step served as negative controls (lanes 2, 4, 7, 9). Also, PCR reactions with total nucleic acids (TNA) from both strains served as positive controls for the PCR step (lanes 5 and 10). Lane M contained size markers, and the gel image was inverted. RT, reverse transcriptase

2.4.6 Effect of inducing Cry genes on growth rates

To test for toxicity of the accumulated Cry proteins to *Chlamydomonas* cells, the growth rates of the transformants (and parental strain) under $-Cu^{2+}$ (Induced, I) and $+Cu^{2+}$ (Uninduced, U) conditions were examined (Figure 2.8). Surprisingly, the growth curves obtained under both conditions were quite similar for the $Cry4Aa_{700}$, Cry4Ba, and Cry11Aa transformants, suggesting that the proteins are not highly toxic when expressed under these conditions (i.e., with the *psbDm* control region, in the Ind41_18 host strain, and in minus-Cu^{2+} medium).

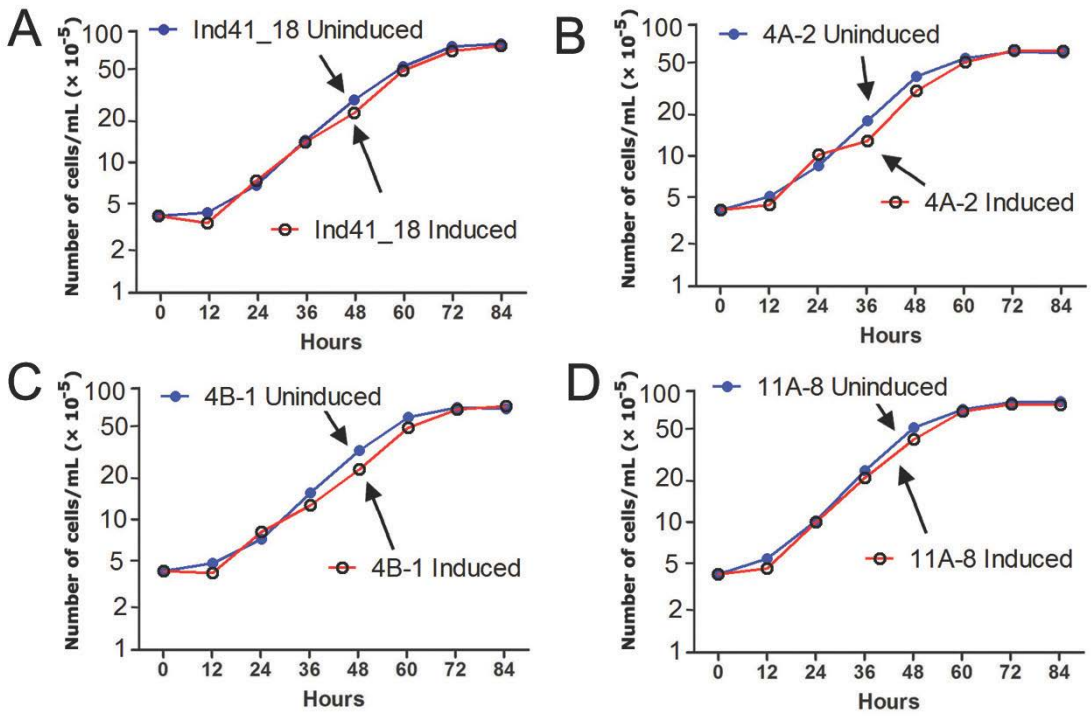

Figure 2.8 Effect of inducing Cry4Aa$_{700}$, Cry4Ba, and Cry11Aa on the growth rate of the transformants. The Ind41_18 parental strain (**A**) and the Cry4Aa$_{700}$-2 (**B**), Cry4Ba-1 (**C**) and Cry11Aa-8 (**D**) transformants were grown under uninduced (TAP + Cu^{2+}) and induced (TAP − Cu2+) conditions. Growth was estimated by measuring total chlorophyll and converting to numbers of cells.

2.4.7 Larvacidal activity of the Cry transformants

To test for activity of the Cry proteins/transformants, live cell bioassays with mosquito larvae were performed. I used 4th instar larvae of *A. aegypti* and 3rd instar larvae of *C. quinquefasciatus* - the larval stages were identified by morphology – and dH_2O was used as the medium, so the algae would not grow, but remain alive. When the larvae were raised on untransformed *Chlamydomonas* cells (Ind41_18), they were very active and developed into pupae and adults, confirming that *Chlamydomonas* can be used as sole food source (Marten, 1986; Kaufman *et al.*, 2006). Larvae feeding on induced $Cry4Aa_{700}$ and Cry11Aa transformants became sluggish and most eventually died; the dead larvae had dark bodies with poorly defined abdominal segments (Figure 2.9), and did not respond to physical stimuli.

Figure 2.9 *A. aegypti* larvae. (A) A typical healthy *A. aegypti* larva fed Ind41_18. (**B**) Dead *A. aegypti* larvae that were fed Cry11Aa-8 grown under inducing conditions. The images were captured 4 days after feeding.

Figure 2.10 shows the bioassay results with the Cry4Aa$_{700}$ and Cry11Aa transformants in terms of larval deaths (out of 10) after 48 hours for *A. aegypti* (Figure 2.10A) and *C. quinquefasciatus* (Figure 2.10B). Initial tests with the Cry4Ba transformant showed low toxicity against *A. aegypti*, and so it was not pursued further, as Cry4Ba is known to have low toxicity against *Culex sp*. (Angsuthanasombat *et al.*, 1992; Delécluse *et al.*, 1993). As Figure 2.10A shows, both the Cry4Aa$_{700}$ (4A) and Cry11Aa (11A) transformants were lethal to *A. aegypti* larvae, with Cry11Aa exhibiting ~3-fold greater toxicity at a cell concentration of 1×10^6 cells/mL (= 1×). The relatively low lethality of uninduced Cry11Aa at the 10× cell concentration - compared with the induced cells at the same cell concentration (10×) - was somewhat unexpected, since the uninduced cells contained only 2-2.5-fold less Cry11Aa than the induced. It should be noted that the comparative effects of the uninduced and induced Cry11Aa cells on *C. quinquefasciatus* (compare 11A-U (10×) with 11A-I (10×) in Figure 2.10B) were more consistent with the western blot data. As with *A. aegypti*, however, the Cry11Aa transformant was more lethal to *C. quinquefaciatus* than the Cry4Aa$_{700}$ transformant. The data also provide evidence of toxicity inhibition at the higher algal cell numbers (5× and 10×), an effect that is analogous to the suppressing effect that food has on Bti toxicity (Becker and Margalith, 1993; Saiful *et al.*, 2012). It is also noticeable that *C. quinquefasciatus* does not survive in dH_2O alone as well as *A. aegypti*, which is more resistant to starvation.

To estimate LC_{50} for the induced Cry11Aa transformant, a more extended series of cell concentrations were used in the bioassay with 4th instar *A. aegypti* larvae. After Probit analysis of the data, the LC_{50} was found to be 3.3×10^5 cells/mL.

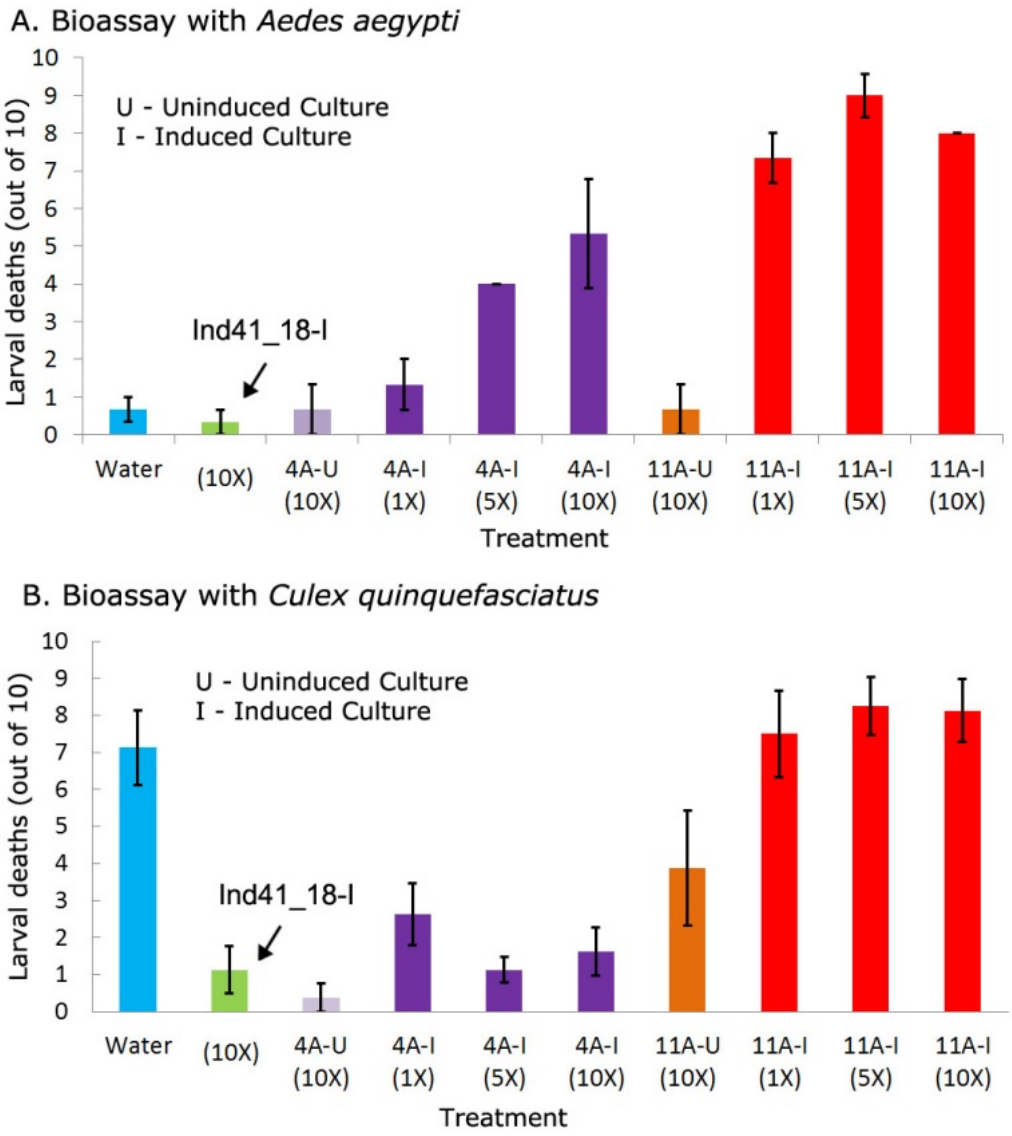

Figure 2.10 Lethality of the Cry4Aa$_{700}$ and Cry11Aa transformants to *A. aegypti* and *C. quinquefasciatus* larvae. The Cry4Aa$_{700}$ (4A-2) and Cry11Aa (11A-8) transformants were grown under uninduced (U) and induced (I) conditions, the parental strain (Ind41_18) only under induced conditions. The assays were performed in dH_2O to prevent the algae from growing, and a dH_20-only control (Water) was included. The assays were performed in triplicate with 10 larvae, either *A. aegypti* (**A**) or *C. quinquefasciatus* (**B**). Larval mortality was checked every 24 hours; the data are from 48 hours. 1× = 1 × 10^6 cells/mL.

2.5 Discussion

The goal for this part of the dissertation was to develop transgenic *Chlamydomonas* that inducibly express Cry toxin genes, in order to begin to show its potential as a host for Bti proteins. Since my first attempt to express these codon-optimized Cry genes in the chloroplast - under the control of *psbA* gene expression signals - produced only heteroplasmic transformants, I reasoned that high constitutive expression would be problematic due to toxicity to the host cells. Although most failures probably do not get published, there is evidence in the literature for toxicity of Bti protoxins to heterologous bacteria (Manasherob *et al.*, 2003; Chen *et al.*, 2014). So, in order to become stable, homoplasmic chloroplast transformants without having to try many types of expression signals and host strains, I used the inducible *Cyc6:NAC2* expression system (Surzycki *et al.*, 2007). With the inducible Ind41_18 strain as host, it was possible to produce all 3 of the major Bti Cry proteins in the chloroplast: a truncated form of Cry4Aa (Cry4Aa$_{700}$, containing amino acids 1-700); full-length Cry4Ba (native size = 128 kDa); and full-length Cry11Aa (native size = 72 kDa). Toxicity of the Cry4Aa$_{700}$ and Cry11Aa transformants to mosquito larvae was also demonstrated. This is the first report of successful expression of Cry proteins from Bti in a eukaryotic alga or organelle (chloroplast).

There have been at least three previous attempts to express a Bti Cry protein in *Chlamydomonas*, one by my lab with Cry11Aa (OW Odom and DL Herrin, unpublished results), and two attempts by other labs with Cry4B variants (Kumar, 2010; Juntadech *et al.*, 2012). In all 3 cases, the genes that were used were not resynthesized to optimize the

codons, and no Cry proteins were detected. In this study, codon-optimized genes were used and were designed based on the codon usage of 8 highly expressed chloroplast genes of *C. reinhardtii*. Also, one of the two very large Cry proteins, Cry4Aa, was truncated after 700 amino acids, while the other, Cry4Ba, was left intact, partly to see if the chloroplast could be engineered to produce such a large protein (130 kDa). The Cry4Aa truncation was based on the fact that larval toxicity has been shown to reside in amino acids 38-695, which are liberated from the 125-kD protoxin by proteases in the mid-gut (Yoshida *et al.*, 1989). Nonetheless, this is only the second time that a truncated Cry4Aa has been used successfully as the protoxin (Borovsky *et al.*, 2011). Finally, I would like to note that Cry4Ba has not been produced in a eukaryote before; only Cry4Aa and Cry11Aa were produced in yeasts (Quintana-Castro *et al.*, 2005; Borovsky *et al.*, 2010; Borovsky *et al.*, 2011).

Homoplasmic $Cry4Aa_{700}$, Cry4Ba, and Cry11Aa transformants allowed us to compare the expression levels of the proteins using the C-terminal Flag tag encoded in each synthetic gene. Moreover, since all 3 genes have the same 5' and 3' expression signals and were integrated at the same place in the genome, the relative protein levels were expected to be determined by the coding regions. The highest level was observed for $Cry4Aa_{700}$, which was substantially (3-4 fold) greater than Cry11Aa or Cry4Ba, which was the lowest. The relatively strong accumulation of $Cry4Aa_{700}$ suggests that the truncation was successful, although I did not have a full-length Cry4Aa transformant for a direct comparison. Truncating Cry1 proteins, which are also large, was successful in

increasing their expression and toxicity in higher plants (Barton *et al.*, 1987; Fishoff *et al.*, 1987; Vaeck *et al.*, 1987), and that was part of my inspiration for truncating Cry4Aa.

The relatively low accumulation of Cry4Ba might be due to the fact that I expressed the full length protein (Barton *et al.*, 1987). Jundatech *et al.* (2012), who also tried to express a full-length variant of Cry4Ba in the *Chlamydomonas* chloroplast, demonstrated successful production of the mRNA, but not the protein; thus confirming the importance of codon optimization for translating this large mRNA. A low level of Cry4Ba protein was also observed in transgenic cyanobacteria, even with strong promoters (Angsuthanasombat and Panyim, 1989; Soltes-Rak *et al.*, 1993; Soltes-Rak *et al.*, 1995; Khasdan *et al.*, 2003). Thus, the truncation of Cry4Ba along the line that I have pursued with Cry4Aa may improve its expression.

The Cry11Aa protein was detected even in uninduced cells, and so was its mRNA. Moreover, induction increased the level of the protein only about 2-2.5-fold, even though it increased the level of the mRNA at least 5-fold. This contrasts with the $Cry4Aa_{700}$ gene, which showed a strong increase in both mRNA and protein with induction conditions. The lack of correlation between mRNA and protein dynamics for Cry11Aa suggests that it is being controlled by translation efficiency and/or protein stability, at least in the Ind41_18 strain grown without Cu^{2+} (induction conditions). Although it remains to be seen if this gene (*psbDm:Cry11Aa:psbA*) is regulated the same way in a wild-type background - which does not require the removal of Cu^{2+} to stabilize the mRNA - this result could have implications for deciding how to increase Cry11Aa protein levels. And by that I mean that changing the 5' and/or 3' expression signals –

which is typically used to improve protein expression in the chloroplast (e.g., Barnes *et al.*, 2005) - may not have much effect if expression is limited entirely by the coding region.

The larvacidal activity of the $Cry4Aa_{700}$ and Cry11Aa transformants was demonstrated by feeding the cells to larvae of *C. quinquefasciatus* and *A. aegypti*. There was little evidence of lethality for the Cry4Ba transformant, and so it was not pursued further. The Cry11Aa strain (induced) showed 3-4-fold higher lethality against these larvae than the $Cry4Aa_{700}$ strain (also induced) at the lower cell concentration tested in Figure 2.8 (1×10^6 cells/mL); there is a plateau and/or a decreasing lethality at higher algal cell numbers that likely represents the effect of excess food on Cry toxicity (Becker and Margalit, 1993; Saiful *et al.*, 2012). According to the immunoblot assay, Cry11Aa was ~3.2-fold less abundant than $Cry4Aa_{700}$, so if the cells are 3-4-fold more potent that suggests that Cry11Aa is ~10-12-fold more effective in this bioassay than $Cry4Aa_{700}$. That result is remarkably consistent with a meta-analysis of previous assays with native Cry proteins, which also concluded that Cry11 was ~10-15 fold more toxic than the Cry4 proteins (Frankenhuysen, 2009). .

The LC_{50} for the induced Cry11Aa transformant against 4th instar *A. aegypti* was estimated to be 3.3×10^5 cells/mL by Probit analysis. Although direct comparisons are complicated by experimental and biological differences, this value appears to be in the range for Cry11Aa expressed in multiple cyanobacterial species (reviewed in Otieno-Ayayo *et al.*, 2008). Also, by converting cell number to total protein using the value of 27.7 ± 0.6 pg protein/cell measured by Bradley and Quarmby (2005), an LC_{50} estimate of

9.14 µg (total) protein/mL is obtained. *Bacillus thuringiensis* subsp. *kurstaki* cells expressing Cry11Aa gave an LC_{50} of 1.74 µg protein/mL at 48 hours with 4th instar *Aedes aegypti* larvae (Wirth *et al.*, 2004), and cell-free extracts from a transgenic *Saccharomyces cerevisiae* that over-produced Cry11Aa gave an LC_{50} of 4.10 µg protein/mL. Hence, the toxicity of the Cry11Aa transformant is also in the range of these engineered heterotrophic microorganisms.

Although the larval bioassays were performed in dH_2O in order to prevent the algae from growing during the extended assay period, this may not be the best medium for achieving toxicity. At some point, the lack of essential mineral nutrients in the medium would presumably become detrimental to not just growth, but also to survival and motility functions of the algal cells. Hence, it may be worth evaluating other ways of limiting algal growth in the bioassays, to see what effect that has on toxicity.

The growth rates of the Cry transformants under induced and uninduced conditions were quite similar, suggesting that the Cry proteins were not highly toxic to Ind41_18 growing without Cu^{2+}. This result was surprising since my initial attempts to engineer wild-type cells for constitutive Cry gene expression were unsuccessful and indicative of toxicity problems. There are multiple possible explanations. First, the Ind41_18 strain is not a wild-type strain and it does not grow as well as wild-type. Second, induction requires the removal of Cu^{2+} from the medium, which has been shown to change significantly the proteome and physiology of *C. reinhardtii* (Hsieh *et al.*, 2013). Third, the combination of gene expression signals used to drive the Cry genes in this study have not yet been tried in wild-type cells, and they may also be important to the

success reported here. In any case, these results are encouraging, and provide the impetus and information needed to successfully engineer the *Chlamydomonas* chloroplast to constitutively express Cry11Aa and Cry4Aa$_{700}$. My ultimate goal is a healthy *Chlamydomonas* strain that constitutively produces Cry11Aa, at least, and Cyt1Aa.

2.6 Acknowledgements

This work was supported by the Bill and Melinda Gates Foundation (OPP1024635). I am grateful to Dr. Jean-David Rochaix for providing the Ind41_18 strain, and BEI Resources, NIAID, NIH for *Culex quinquefasciatus*. I am also grateful to Dr. Saravanan Thangamani (University of Texas Medical Branch at Galveston) for advice, and for providing eggs of *Aedes aegypti*. I especially thank Dr. O.W. Odom for advice and technical assistance.

Chapter 3. A strain of *Chlamydomonas reinhardtii* that is constitutively lethal to mosquito larvae: Cry11Aa expression in the chloroplast of wild-type

3.1 Abstract

Synthetic genes for mosquitocidal Cry4Aa$_{700}$, Cry4Ba, and Cry11Aa proteins have been expressed in the chloroplast of an inducible *Chlamydomonas reinhardtii* strain, Ind41_18. Inducible expression is useful for evaluating synthetic genes, especially when there is a likelihood of host toxicity; however, constitutive expression is necessary for a biolarvicide. In this chapter, each of the 3 synthetic genes, which had been outfitted with *psbD$_m$* and *psbA* expression signals, were transformed into the chloroplast of a wild-type strain of *C. reinhardtii*. Homoplasmic Cry11Aa and Cry4Ba transformants were obtained. Western blotting confirmed the accumulation of Cry11Aa in the respective transformants, with a level that was at least as high as that obtained with the inducible system. Lethality of the Cry11Aa^{WT} strain to *Aedes aegypti* larvae was confirmed with a live-cell bioassay. The growth rate of the Cry11Aa^{WT} strain was indistinguishable from wild-type under my standard growth conditions. These results indicate the feasibility of producing *Chlamydomonas* strains that can be used for mosquito control.

3.2 Introduction

In Chapter 2, the three main Cry proteins found in Bti were expressed in the chloroplast of *C. reinhardtii* using the copper repressible system. The system requires removal of copper ion in the media upon induction. Eliminating the mineral from the media is possible in a lab environment, but not in the field. Controlling the copper level in larval habitats near people would be laborious and probably implausible.

Therefore, constitutive expression of Cry protoxins is required for field use of Bti-*Chlamydomonas* as a biocontrol agent. Constitutive, or normalized expression is achieved by placing the Cry protoxin genes - containing plastid expression signals - into a wild-type *Chlamydomonas* strain, since all chloroplast genes are expressed on a daily basis, mostly during the pre-dawn hours and throughout the daytime (Lee and Herrin, 2002; Misquitta and Herrin, 2005). Expressing heterologous proteins constitutively and stably (i.e., in a homoplasmic state), however, can usually be achieved only when the proteins do not have an overly deleterious effect on the host, and it is not always possible to predict which constructs or proteins will be toxic (Surzycki *et al*., 2009; Rasala and Mayfield, 2011). Successful accumulation of the Cry protoxins in an inducible strain of *C. reinhardtii* using the $psbD_m$ and *psbA* expression signals (Chapter 2) raised the possibility of constitutive expression of these constructs in a wild-type background.

So far, no eukaryotic algae or plants have been engineered to express Bti Cry protoxins in the chloroplast. Numerous prokaryotes have been engineered with Bti Cry transgenes; however, only a couple of successes in eukaryotes have been published, and both examples involved yeasts, *Saccharomyces cerevisiae* and *Pichia pastoris* (Quintana-

Castro *et al*., 2005; Borovsky *et al*., 2010). The transgenic yeast strains required carbon sources such as methanol, ethanol, or galactose for the induction of the Cry genes, making them unlikely to be useful in the field.

In this chapter, I report the successful establishment of a wild-type strain of *C. reinhardtii* that expresses Cry11Aa, without any manipulations of the culture conditions, and is toxic to mosquito larvae (*Aedes aegypti*).

3.3 Materials and methods

3.3.1 Strains and growth conditions

The wild-type strain of *C. reinhardtii*, 2137 (CC-1021 wild type mt+), was obtained from the *Chlamydomonas* Center (U. of Minnesota). The Cry11Aa transformant of Ind41_18 was described in Chapter 2. All strains were grown in TAP medium in the light (40 µE m^{-2} sec^{-1}) at 23°C with shaking (section 2.3.1). For the inductive growth of the Ind41_18/Cry11Aa transformant, Cu^{2+} was removed from the medium as described in section 2.3.1. Cell number for the wild-type transformants was estimated from total chlorophyll using the reference value of 4 mg chlorophyll per 1×10^9 cells (Harris, 1989). For the Ind41_18 transformant, cell number was counted with a hemacytometer.

3.3.2 Chloroplast transformation and PCR screening

The Cry gene plasmids used to transform the chloroplast of wild-type *C. reinhardtii* were described in Chapter 2; they contain the $Cry4Aa_{700}$, Cry4Ba, and Cry11Aa genes outfitted with $psbD_m$ and *psbA* expression signals, and integrated into

the chloroplast transformation vector p322.1 (section 2.3.4). Each one was co-transformed with pB4CC110, which confers spectinomycin resistance, as described in section 2.3.5. Transformants were selected on 100 µg/mL spectinomycin and re-streaked on 300 µg/mL spectinomycin until they were homplasmic as judged by PCR.

PCR was performed to check the integration of Cry constructs and verify that all copies of the genome had become homoplasmic for the Cry gene insertion. DNA extraction, PCR primers, and amplification conditions were the same as described in section 2.3.6.

3.3.3 Protein extraction and western blotting

Cell cultures in late log phase (2-4 × 10^6 cells/mL) were harvested, solubilized with SDS and sonication (section 2.3.7), and subjected to SDS-PAGE on 10% acrylamide gels. The proteins were electrotransferred to a PVDF membrane, and detected with a monoclonal anti-Flag antibody as described in section 2.3.7.

3.3.4 Chlorophyll measurement and cell number conversion

Total chlorophyll was measured by harvesting the cells from 1 mL of culture using centrifugation at 10,000 × g for 5 minutes, and then extracting the pellet with 1 mL of 95% EtOH. After centrifuging at 10,000 × g for 2 minutes, the supernatant was removed, and its absorption was read at 665 nm and 649 nm. Total chlorophyll, in µg/mL culture, was calculated as described in Windermans and De Mots (1965).

3.3.5 Bioassay for larvacidal activity

The bioassay was performed with 4th instar *Aedes aegypti* larvae as described in section 2.3.10. Ten larvae (per assay) were fed live wild-type and Cry transformant cells in dH_2O, and larval mortality was checked every 24 hours. When desired, images of the larvae were captured using LAS EZ software and a Leica EZ4 HD stereo microscope.

3.4 Results

3.4.1 Transformation of wild-type and DNA analysis

Figure 3.1 shows the Cry plasmids that were co-transformed into wild-type *C. reinhardtii*, and the site of integration between the *psbA* and 23S *rrn* genes. After primary selection, the transformants were restreaked on spectinomycin plates until they became homoplasmic, as judged by PCR of the integration site with primer pair 864/865; homoplasmicity is indicated by the absence of the ~100 bp product. As shown in Figure 3.2, at least 3 transformants were obtained for Cry11Aa and Cry4Ba, where all copies of the CpDNA have integrated Cry gene. PCR with internal primer pairs (799/800 for Cry11Aa and 797/798 for Cry4Ba) confirmed the presence of the respective Cry gene in each case (Figure 3.2). I did not obtain homoplasmic transformants with the $Cry4Aa_{700}$ plasmid, and it was not pursued further in this study.

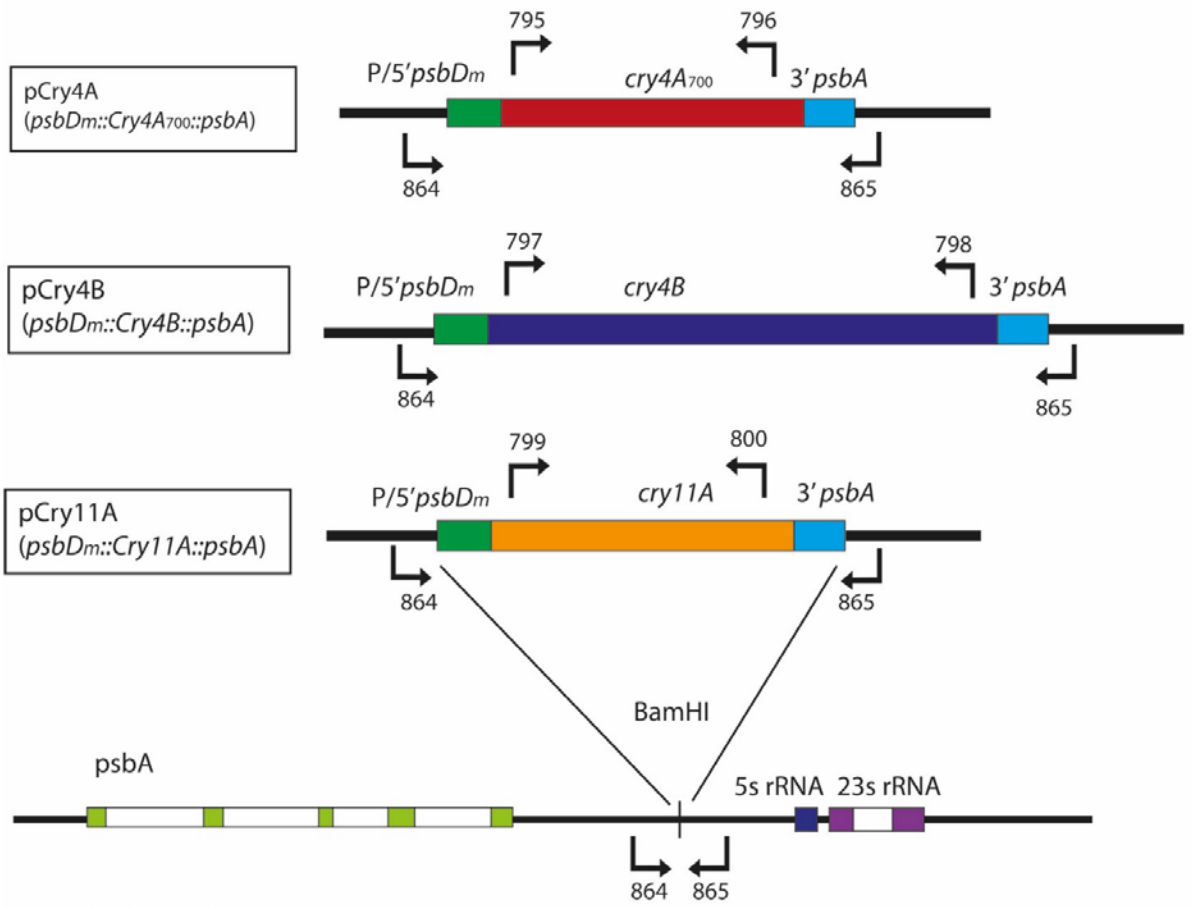

Figure 3.1 Diagram of pCry4A$_{700}$, pCry4B and pCry11A constructs and the site of integration in the chloroplast genome of wild-type *C. reinhardtii*. Each of the Cry genes have a Flag tag at the C-terminus, and are flanked by *psbD$_m$* and *psbA* control regions. The locations of primers used for PCR are indicated; note that 864 and 865 are located upstream and downstream, respectively, of the integration site in CpDNA. Some parts of the diagram are not drawn to scale.

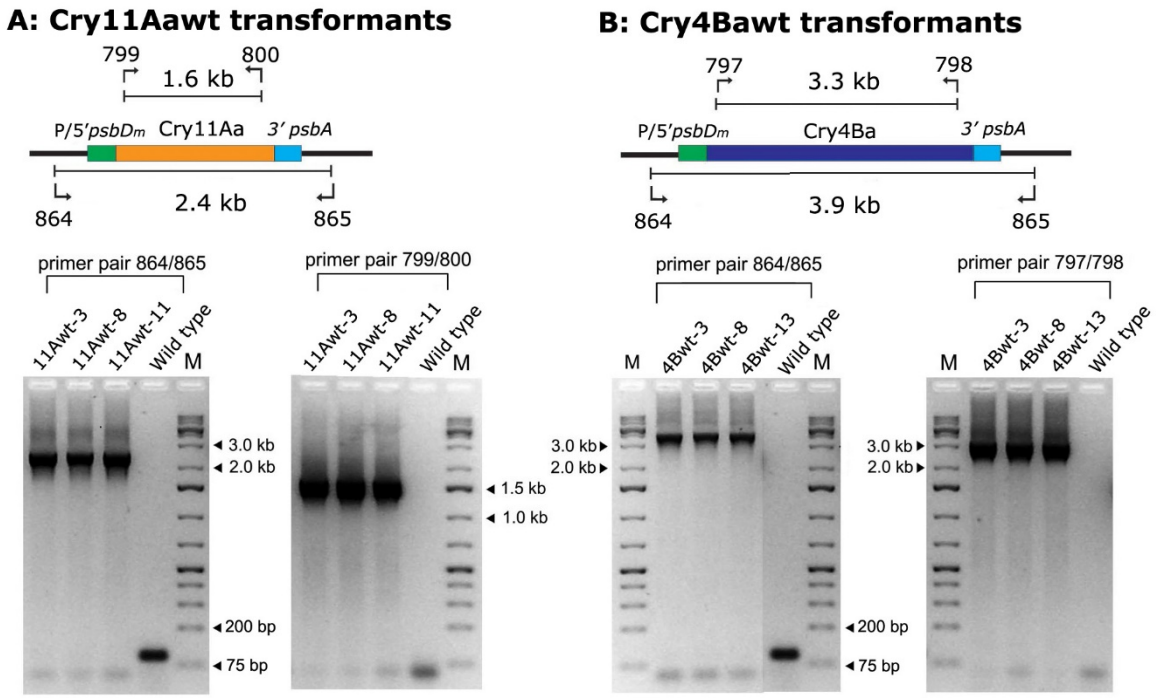

Figure 3.2 PCR analysis of chloroplast transformants in a wild-type host. Analysis of three independent transformants that were co-transformed with either pCry11A (**A**) or pCry4B (**B**) and selected on spectinomycin. Total DNA was used for PCR with primers that either flanked the integration site (864/865), or were internal and gene-specific (799/800 for Cry11Aa, and 797/798 for Cry4Ba). Reactions with wild-type DNA were included to evaluate homoplasmicity at the integration site (864/865). Lane M contained DNA size markers.

3.4.2 Protein analysis

With the Cry11Aa and Cry4Ba constructs in a wild-type background, special conditions are not required for expression, so the transformants were grown under standard conditions and total cell protein was subjected to western blot analysis with the anti-Flag antibody. The signal with the Cry4Ba transformants appeared to be even lower than with the inducible system (Chapter 2), and given the weaker inherent toxicity of the Cry4Ba protein, the Cry4Ba-wt transformants were not pursued further. However, western blot analysis of the Cry11Aa-wt transformants was fruitful, as they showed accumulation of Cry11Aa that was similar to that in the inducible system; the last lane of the blot in Figure 3.2 contained an equivalent amount of extract (based on chlorophyll) from the induced 11A-8 transformant (Chapter 2). For reasons that are not clear, a non-specific band at ~42 kDa that reacts with the anti-Flag antibody in wild-type extracts does not react in extracts from the inducible strain, Ind41-18. The western blot also shows that accumulation of Cry11Aa was similar in all three Cry11Aa-wt transformants, which is typical of the uniformity of homoplasmic chloroplast transformants, and that untransformed wild-type had no protein of the size of Cry11Aa (72 kDa). The Cry11Aawt-8 transformant was selected as representative of the 3 transformants and was used in the analysis of growth rates and in the bioasssy.

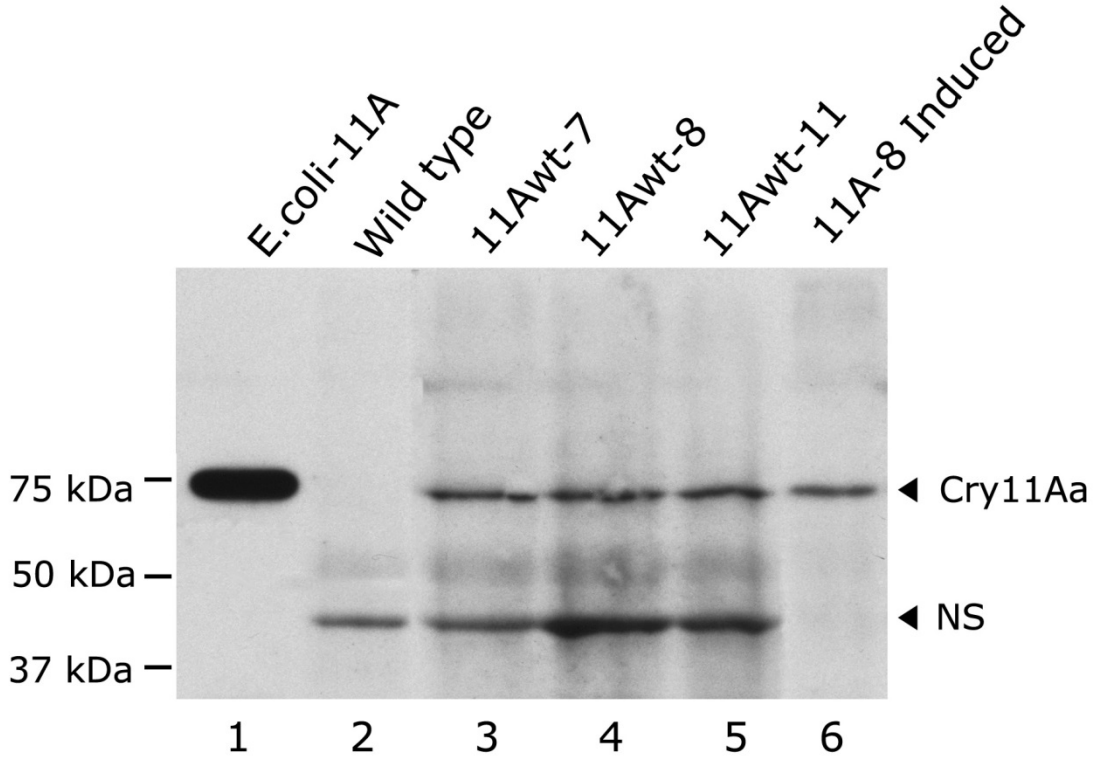

Figure 3.3 Western blot of the Cry11Aa wild-type transformants. The three Cry11Aa transformants from Figure 3.2 (11Awt-7, 11Awt-8, 11Awt-11), and the untransformed host strain (Wild type) were grown in TAP medium in the light. Also, the inducible Cry11Aa transformant 11A-8 was grown under induction conditions (lane 6). Equal total cell fractions (4 μg chlorophyll, ~60 μg protein) were loaded on the 10% gel, blotted and probed with the Flag antibody. *E. coli* expressing a His-tagged Cry11Aa (E. coli-11A) was included in lane 1 as a positive control. The positions of size markers are indicated to the left. The NS (Non-specific) band only appears with wild type cells (lanes 2-5), and not with Ind41-18, which is the host strain used for inducible expression (lane 6).

3.4.3 Growth rates

To determine if the *psbDm:Cry11Aa:psbA* gene and resulting Cry11Aa protein was detrimental to the growth of wild-type *C. reinhardtii*, growth curves were generated for Cry11Awt-8 and wild-type under standard conditions (Figure 3.4). The results indicate that the Cry11Aa gene and protein did not have a negative effect on the growth of this wild-type host strain, at least under standard growth conditions.

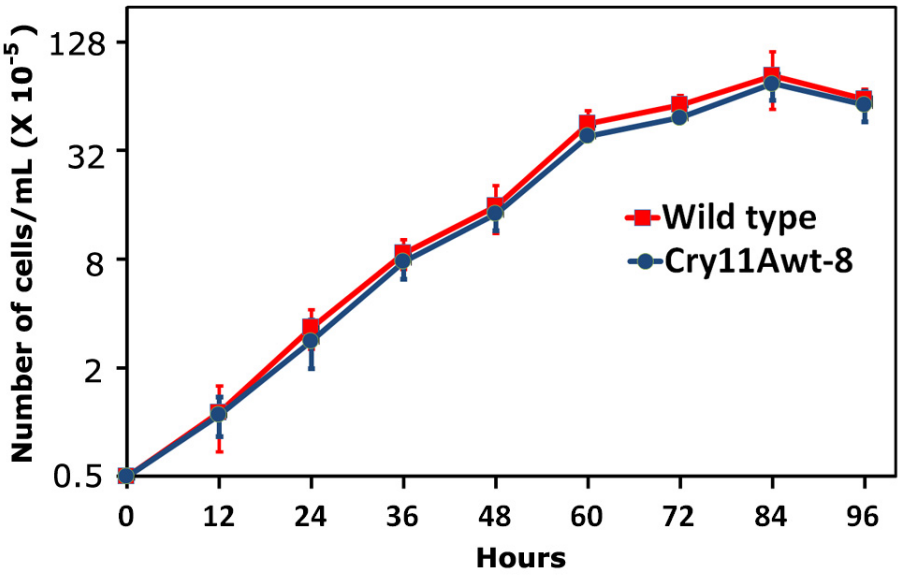

Figure 3.4 Growth curves of the Cry11Awt-8 transformant and host strain (Wild type). Cells were diluted to 5×10^4 cells/mL in TAP medium and incubated in the light with shaking. The number of cells was counted every 12 h. Plotted are the averages ± SEM from three independent trials.

3.4.4 Larvacidal activity

The toxicity of the Cry11Aa-wt transformant to mosquito larvae was verified using a live cell bioassay and 4th-instar larvae of *A. aegypti*. An example of the effects of feeding Cry11Awt-8 cells for 24 hours on 4th-instar larvae is shown in Figure 3.5. Live larvae have well-defined body segments (left panel in Figure 3.5) and active movement, whereas dead larvae have poor segment definition (right panel in Figure 3.5) and did not move or respond to a physical stimulus. Figure 3.6 shows the lethality of Cry11Aawt-8 cells in the bioassay with *A. aegypti* larvae, which is determined by the number of larval deaths, out of 10 per assay (n=3), after 48 hours in dH_2O. Although dH_2O may not be the optimum medium for toxicity, it prevents the algae from growing during the assay. The Cry11Aawt-8 cells are clearly toxic to the larvae, and the lethality (at 1×10^6 cells/mL) is only slightly less than the induced Ind41_18/Cry11Aa transformant (Chapter 2, Figure 2.10). The lack of an increase in lethality at 5× the cell number is most likely analogous to the suppressive effect that food has on the toxicity of Bti (Becker and Margalith, 1993; Saiful *et al.*, 2012).

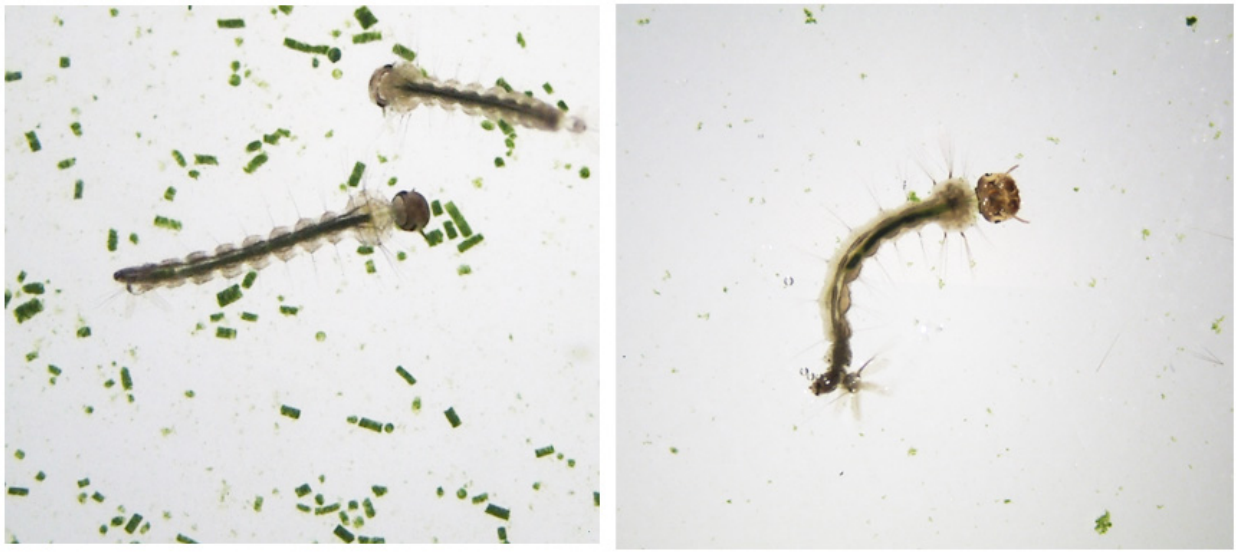

Figure 3.5 Representative live (left) and dead (right) *A. aegypti* larvae. The larvae on the left were fed wild type cells, whereas the larva on the right was fed Cry11Awt-8 cells.

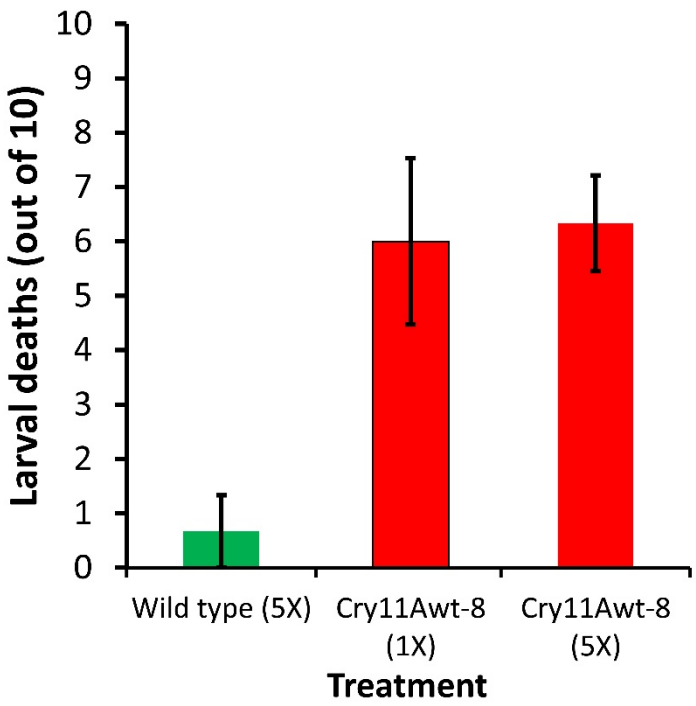

Figure 3.6 Larval bioassay with the Cry11Aa transformant (Cry11Awt-8) and host strain (Wild type) with *A. aegypti* larvae. Ten 4th-instar larvae and live algal cells (in dH_2O) were used in each assay, which was performed in triplicate (n = 3). Larval mortality was checked visually after 24 and 48 hours; the data are from 48 hours. 1× = 1 × 10^6 cells/mL.

3.5 Discussion

In this chapter, Cry11Aa-producing strains were established with wild-type *Chlamydomonas*, in order to achieve a line constitutively toxic to mosquito larvae. PCR analysis confirmed the homoplasmicity of the chloroplast transformants. Western blotting showed that Cry11Aa of the expected size accumulated under standard growth conditions, and that the level was similar to that obtained in the inducible system (Chapter 2). That is not surprising, perhaps, since the gene construct that was introduced into wild-type, *psbD$_m$:Cry11Aa:psbA*, is the same as that used in the chloroplast of the inducible Ind41_18 strain. The lethality of the Cry11Aa-wt cells toward *A. aegypti* larvae was tested with the live cell bioassay, and found to be similar, or slightly less than that of the inducible strain grown under induction conditions (Chapter 2). Incorporation of the *psbD$_m$:Cry11Aa:psbA* gene into the wild-type chloroplast had no apparent detrimental effect on the growth of the cells, at least under my standard conditions. These results show that it is possible to generate *C. reinhardtii* strains that are constitutively toxic to mosquito larvae via chloroplast gene engineering.

Unlike Cry11Aa, Cry4Ba accumulation in the wild-type transformants was undetectable on the western blot. In the inducible strain, Cry4Ba accumulation was the lowest of the three Cry proteins, but it was still detectable (Chapter 2). This result indicates that strain-to-strain variation in genetic background in *C. reinhardtii* can affect significantly the expression of an engineered Cry gene in the chloroplast. Perhaps by truncating Cry4Ba the way I did for Cry4Aa I could improve its expression in both systems, but given the lower toxicity of this protein to larvae (Crickmore *et al*., 1995;

Otieno-Ayayo *et al*., 2008), the increase in expression would have to be great, for it to be worthwhile. Consequently, I have shelved CryBa for the present.

Putative Cry4Aa$_{700}$ wild-type transformants did not survive serial re-streaking on high spectinomycin suggesting that they could not reach high enough levels of spectinomycin-resistant ribosomes, or that the Cry4Aa$_{700}$ construct was too toxic. Cry4Aa$_{700}$ accumulation in the inducible strain was substantially higher than Cry11Aa, so perhaps that level of Cry4Aa$_{700}$ is too high for the wild-type strain, or, given the strain-dependent expression of Cry4Ba mentioned above, perhaps Cry4Aa$_{700}$ expression was headed even higher in the wild-type background but was not sustainable.

One might ask, though, that if the Cry4Aa$_{700}$ gene construct is too toxic for wild-type, why did it not show evidence of toxicity to the Ind41_18 strain? The growth curve of the Cry4Aa$_{700}$ Ind41_18 transformant under inducing conditions was very similar to the growth curve under non-inducing conditions (Chapter 2). However, this is like comparing apples and oranges, since Ind41_18 is not a wild-type strain - in fact it grows significantly slower than wild-type - and induction requires removing Cu^{+2} from the medium. Besides altering photosynthetic electron transport, Cu^{2+} starvation also alters the levels of >100 proteins in *C. reinhardtii* (Hsieh *et al*., 2013). On the other hand, if I could understand why Cry4Aa$_{700}$ seems to be non-toxic to induced Ind41_18, it might help us reduce the toxicity to wild-type.

Even without such insight, however, there are a few things that could be tried to reduce toxicity to *Chlamydomonas* without reducing expression of Cry4Aa$_{700}$ or resorting to inducible control. Assuming that toxicity to *Chlamydomonas* involves membrane

effects, there are measures that can be taken to keep the Cry protein(s) away from chloroplast membranes. First, translation of the Cry mRNA could be directed away from the membrane by replacing the 5' UTR of *psbD* - which is translated on the thylakoid membrane (Herrin, *et al*., 1981) - with the 5' UTR from the *rbcL* gene. *RbcL* mRNA is translated at the pyrenoid (Uniacke and Zerges, 2009). Second, the Cry protoxin could be localized to the starch grains by adding a starch-binding domain to the C-terminus (Ji *et al*., 2003). These are relatively small (~100 amino acids) domains that would likely not interfere with protoxin processing and activity in the larvae. Third, the first and second suggestions could be combined, which should keep the Cry protoxin away from the membranes during and after translation.

Another approach that could help with $Cry4Aa_{700}$ toxicity, and possibly increase Cry11Aa expression if it is limited by protein stability, is to co-express the P20 chaperone from Bti. P20 is encoded on the pBtoxis plasmid in the Cry11Aa operon, and has been shown to specifically enhance the yield and crystallization of Cry4Aa, Cry11Aa, and Cyt1Aa via protein-protein interactions (Deng *et al*., 2014). Moreover, P20 alleviated the toxicity of Cyt1Aa to *E. coli* (Manasherob *et al*., 2001).

3.6 Future research

I were fortunate to obtain homoplasmic chloroplast transformants that express Cry11Aa constitutively (i.e., in a wild-type background), since Cry11Aa is the most potent of the Bti Cry proteins. The lethality of the Cry11Aa strains to mosquito larvae (LC_{50} is ~3-5 × 10^5 cells/mL) needs to be increased, however, so that lower cell numbers can be an effective larval control. My current target is larval lethality at ~10^4 cells/mL while keeping in mind that sublethal doses of Bti also harm mosquito larvae (Aïssaoui.and Boudjelida, 2014). Thus, the first goal for the future is to increase the toxicity of the Bti-*Chlamydomonas* about 50-fold. The second goal, which will also help achieve the first, is to co-express Cyt1Aa with Cry11Aa in the chloroplast. This will probably be the most effective way of increasing toxicity against mosquito larvae, because Cyt1Aa has a strong synergistic effect on the Cry protoxins, especially Cry11Aa (Crickmore *et al*., 1995). Moreover, Cyt1Aa prevents the development of strong resistance in larval populations (Wirth *et al*., 1997), one of the great benefits of Bti. Since Cyt1Aa is a small protein (27 kDa), the gene is also small, and I have already synthesized a codon-optimized version of the gene with an epitope tag at the C-terminus Cyt1Aa, and together with O.W. Odom have expressed it in *E. coli*. Further, I have synthesized a codon-optimized starch-binding domain in anticipation of using it to reduce Cry protein damage to the chloroplast.

Several strategies are being looked at to increase Cry11Aa expression without decreasing the fitness of the algae to an unacceptable amount. Most of them were discussed above as strategies that would potentially allow us to generate $Cry4Aa_{700}$

transformants in a wild-type background. However, an alternative strategy would be to try other Cry11 genes. Cry11Aa is not the only highly toxic Cry11 gene, as there are also Cry11B genes, and a particularly effective one against mosquito larvae is from the *jegathesan* subspecies of *Bacillus thuringiensis* (Delécluse *et al*., 1995).

References

Abdullah, M.A.F., Alzate, O., Mohammad, M., McNall, R.J., Adang, M.J., and Dean, D.H. (2003). Introduction of *Culex* toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. *Applied and Environmental Microbiology* **69**, 5343-5353.

Al-yahyaee, S.A.S., and Ellar, D.J. (1995). Maximal toxicity of cloned CytA δ-endotoxin from *Bacillus thuringiensis* subsp. *israelensis* requires proteolytic processing from both the N- and C-termini. *Microbiology* **141**, 3141-3148.

Amorim, L.B., de Oliveira, C.M.F., Rios, E.M., Regis, L., and Silva-Filha, M.H.N.L. (2007). Development of *Culex quinquefasciatus* resistance to *Bacillus sphaericus* strain IAB59 needs long term selection pressure. *Biological Control* **42**, 155-160.

Angsuthanasombat, C., and Panyim, S. (1989). Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium Agmenellum quadruplicatum PR-6. *Applied and Environmental Microbiology* **55**, 2428-2430.

Angsuthanasombat, C., Crickmore, N., and Ellar, D.J. (1992). Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. *FEMS Microbiology Letters* **94**, 63-68.

Angsuthanasombat, C., Uawithya, P., Leetachewa, S., Pornwiroon, W., Ounjai, P., Kerdcharoen, T., Katzenmeier, G., and Panyim, S. (2004). *Bacillus thuringiensis* Cry4A and Cry4B mosquito-larvicidal proteins: homology-based 3D model and implications for toxin activity. *Journal of Biochemistry and Molecular Biology* **37**, 304-313.

Aïssaoui, L. and Boudjelida, H. (2014). Larvicidal activity and influence of *Bacillus thuringiensis* (Vectobac G), on longevity and fecundity of mosquito species, *European Journal of Experimental Biology* **4**, 104-109

Arapinis, C., de la Torre, F., and Szulmajster, J. (1988). Nucleotide and deduced amino acid sequence of the *Bacillus sphaericus* 1593M gene encoding a 51.4 kD polypeptide which acts synergistically with the 42 kD protein for expression of the larvicidal toxin. *Nucleic Acids Research* **16**, 7731.

Arnon, D.I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24**, 1-15.

Barloy, F., Delécluse, A., Nicolas, L., and Lecadet, M.M. (1996). Cloning and expression of the first anaerobic toxin gene from *Clostridium bifermentans* subsp. *malaysia*, encoding a new mosquitocidal protein with homologies to *Bacillus thuringiensis* delta-endotoxins. *Journal of Bacteriology* **178**, 3099-3105.

Barnes, D., Franklin, S., Schultz, J., Henry, R., Brown, E., Coragliotti, A., and Mayfield, S. (2005). Contribution of 5′- and 3′-untranslated regions of plastid mRNAs to the

expression of *Chlamydomonas reinhardtii* chloroplast genes. *Molecular Genetics and Genomics* **274**, 625-636.

Barton, K.A., Whiteley, H.R., and Yang, N.-S. (1987). *Bacillus thuringiensis* §-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiology* **85**, 1103-1109.

Baumann, P., Clark, M.A., Baumann, L., and Broadwell, A.H. (1991). *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. *Microbiological Reviews* **55**, 425-436.

Becker, N., and Margalit, J. (1993). Use of *Bacillus thuringiensis israelensis* against mosquitoes and blackflies. In: *Bacillus thuringiensis: an environmental biopesticide : theory and practice* (Entwistle, P.F., Cory, J.S., Bailey, M.J., and Higgs, S., eds), 147-170, John Wiley & Sons, New York

Becker, N. (1997). Microbial control of mosquitoes: Management of the upper Rhine mosquito population as a model programme. *Parasitology Today* **13**, 485-487.

Becker, N. (2000). Bacterial control of vector-mosquitoes and black flies. In: *Entomopathogenic bacteria: from laboratory to field application* (Charles, J.F., Delécluse, A., and Nielsen-LeRoux, C., eds), 383-398, Kluwer, Dordrecht, The Netherlands.

Becker, N. (2006). Microbial control of mosquitoes: Management of the Upper Rhine mosquito population as a model programme. In: *An Ecological and Societal Approach to Biological Control* **2** (Eilenberg, J. and Hokkanen, H.M.T., eds), 227–245, Springer Verlag, Berlin.

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins* **6**, 1222-1243.

Bernaudat, F., Frelet-Barrand, A., Pochon, N., Dementin, S., Hivin, P., Boutigny, S., Rioux, J.-B., Salvi, D., Seigneurin-Berny, D., Richaud, P., Joyard, J., Pignol, D., Sabaty, M., Desnos, T., Pebay-Peyroula, E., Darrouzet, E., Vernet, T., and Rolland, N. (2011). Heterologous expression of membrane proteins: choosing the appropriate host. *PLoS ONE* **6**, e29191.

Berry, C., Hindley, J., Ehrhardt, A.F., Grounds, T., de Souza, I., and Davidson, E.W. (1993). Genetic determinants of host ranges of *Bacillus sphaericus* mosquito larvicidal toxins. *Journal of Bacteriology* **175**, 510-518.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D., Zaritsky, A., and Parkhill, J. (2002). Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology* **68**, 5082-5095.

Boonserm, P., Pornwiroon, W., Katzenmeier, G., Panyim, S., and Angsuthanasombat, C. (2004). Optimised expression in *Escherichia coli* and purification of the

functional form of the *Bacillus thuringiensis* Cry4Aa δ-endotoxin. *Protein Expression and Purification* **35**, 397-403.

Boonserm, P., Davis, P., Ellar, D.J., and Li, J. (2005). Crystal Structure of the Mosquito-larvicidal toxin Cry4Ba and Its biological implications. *Journal of Molecular Biology* **348**, 363-382.

Boonserm, P., Mo, M., Angsuthanasombat, C., and Lescar, J. (2006). Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-Angstrom resolution. *Journal of Bacteriology* **188**, 3391-3401.

Borovsky, D., Khasdan, V., Nauwelaers, S., Theunis, C., Bertie0r, L., Ben-Dov, E., and Zaritsky, A. (2010). Synergy between *Aedes aegypti* trypsin modulating oostatic factor and δ-Endotoxins. *The Open Toxinology Journal* **3**, 141-150.

Borovsky, D., Naywelaers, S., Mileghem, A., Meyvis, Y., Laeremans, A., Theunis, C., Bertie, L., and Boons, E. (2011). Control of mosquito larvae with TMOF and 60 kDa Cry4Aa expressed in *Pichia pastoris*. *Pestycydy/Pesticides* **1-4**, 5-15.

Boussiba, S., Wu, X.Q., Ben-Dov, E., Zarka, A., and Zaritsky, A. (2000). Nitrogen-fixing cyanobacteria as gene delivery system for expressing mosquitocidal toxins of *Bacillus thuringiensis* ssp. *israelensis*. *Journal of Applied Phycology* **12**, 461-467.

Boynton, J.E., Gillham, N.W., Harris, E., Hosler, J., Johnson, A., Jones, A., Randolph-Anderson, B., Robertson, D., Klein, T., Shark, K., and et, al. (1988). Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* **240**, 1534-1538.

Boynton, J.E., and Gillham, N.W. (1993). Chloroplast transformation in *Chlamydomonas*. In: *Methods for transforming animal and plant cells, Methods in enzymology* **217** (Ray, W., ed), 510-536, Academic Press, New York.

Bradley, B.A., and Quarmby, L.M. (2005). A NIMA-related kinase, Cnk2p, regulates both flagellar length and cell size in *Chlamydomonas. Journal of Cell Science* **118**, 3317-3326.

Bravo, A., Gill, S.S., and Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* **49**, 423-435.

Bravo, A., Likitvivatanavong, S., Gill, S.S., and Soberón, M. (2011). *Bacillus thuringiensis*: A story of a successful bioinsecticide. *Insect Biochemistry and Molecular Biology* **41**, 423-431.

Bukhari, D., and Shakoori, A. (2009). Cloning and expression of *Bacillus thuringiensis cry11* crystal protein gene in *Escherichia coli*. Mol Biol Rep 36, 1661-1670.

Butko, P., Huang, F., Pusztai-Carey, M., and Surewicz, W.K. (1996). Membrane permeabilization induced by cytolytic δ-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry* **35**, 11355-11360.

Butko, P. (2003). Cytolytic toxin Cyt1a and its mechanism of membrane damage: data and hypotheses. *Applied and Environmental Microbiology* **69**, 2415-2422.

Cahan, R., Friman, H., and Nitzan, Y. (2008). Antibacterial activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis*. *Microbiology* **154**, 3529-3536.

Campos-Quevedo, N., Rosales-Mendoza, S., Paz-Maldonado, L., Martínez-Salgado, L., Guevara-Arauza, J., and Soria-Guerra, R. (2013). Production of milk-derived bioactive peptides as precursor chimeric proteins in chloroplasts of *Chlamydomonas reinhardtii*. *Plant Cell, Tissue and Organ Culture* **113**, 217-225.

Canan, U. (2013). Microorganisms in Biological Pest Control — A Review (Bacterial Toxin Application and Effect of Environmental Factors). In: *Current Progress in Biological Research*, (Silva-Opps M, ed), InTech, DOI: 10.5772/55786. Available from: http://www.intechopen.com/books/current-progress-in-biological-research/microorganisms-in-biological-pest-control-a-review-bacterial-toxin-application-and-effect-of-environ

CDC (2014, June 2). West Nile Virus: final annual maps & data for 1999-2013 (GA, USA). Available from http://www.cdc.gov/westnile/statsMaps/finalMapsData/index.html

Cerutti, H., Johnson, A.M., Gillham, N.W., and Boynton, J.E. (1997). Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. *The Plant Cell* **9**, 925-945.

Chakrabarti, S., Lutz, K., Lertwiriyawong, B., Svab, Z., and Maliga, P. (2006). Expression of the *cry9Aa2* B.t. gene in tobacco chloroplasts confers resistance to potato tuber moth. *Transgenic Research* **15**, 481-488.

Chapman, H.C. (1974). Biological control of mosquito larvae. *Annual Review of Entomology* **19**, 33-59.

Chen, H.-C., and Melis, A. (2013). Marker-free genetic engineering of the chloroplast in the green microalga *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal* **11**, 818-828.

Chen, J., Aimanova, K.G., Fernandez, L.E., Bravo, A., Soberon, M., and Gill, S.S. (2009). *Aedes aegypti* cadherin serves as a putative receptor of the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Biochemical Journal* **424**, 191-200.

Chen, J., Likitvivatanavong, S., Aimanova, K.G., and Gill, S.S. (2013). A 104 kDa *Aedes aegypti* aminopeptidase N is a putative receptor for the Cry11Aa toxin from *Bacillus thuringiensis* subsp. *israelensis*. *Insect Biochemistry and Molecular Biology* **43**, 1201-1208.

Chen, S., Kaufman, M.G., Korir, M.L., and Walker, E.D. (2014). Ingestibility, Digestibility, and engineered biological control potential of *Flavobacterium*

hibernum, isolated from larval mosquito habitats. *Applied and Environmental Microbiology* **80**, 1150-1158.

Chilcott, C.N., and Ellar, D.J. (1988). Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. *Journal of General Microbiology* **134**, 2551-2558.

Chilcott, C. N., Knowles, B. H., Ellar, D. J., Drobniewski, F. A. (1990). Mechanism of action of *Bacillus thuringiensis israelensis* parasporal body. In: *Bacterial Control of Mosquitoes and* black flies: biochemistry, genetics, & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (De Barjac, H., and Sutherland, D., eds), 202-217, Rutgers University Press, New Brunswick, NJ.

Choquet, Y., and Wollman, F.-A. (2002). Translational regulations as specific traits of chloroplast gene expression. *FEBS Letters* **529**, 39-42.

Chungjatupornchai, W., HÖFte, H., Seurinck, J., Angsuthanasombat, C., and Vaeck, M. (1988). Common features of *Bacillus thuringiensis* toxins specific for Diptera and Lepidoptera. *European Journal of Biochemistry* **173**, 9-16.

Cohen, S., Albeck, S., Ben-Dov, E., Cahan, R., Firer, M., Zaritsky, A., and Dym, O. (2011). Cyt1Aa toxin: crystal structure reveals implications for its membrane-perforating function. *Journal of Molecular Biology* **413**, 804-814.

Coragliotti, A., Beligni, M., Franklin, S., and Mayfield, S. (2011). Molecular factors affecting the accumulation of recombinant proteins in the *Chlamydomonas reinhardtii* chloroplast. *Molecular Biotechnology* **48**, 60-75.

Crickmore, N., Bone, E.J., Williams, J.A., and Ellar, D.J. (1995). Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiology Letters* **131**, 249-254.

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., and Dean, D.H. (1998). Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* **62**, 807-813.

Dai, S.-M., and Gill, S.S. (1993). In vitro and in vivo proteolysis of the *Bacillus thuringiensis* subsp. *israelensis* CryIVD protein by *Culex quinquefasciatus* larval midgut proteases. *Insect Biochemistry and Molecular Biology* **23**, 273-283.

Darboux, I., Nielsen-LeRoux, C., Charles, J., and Pauron, D. (2001). The receptor of *Bacillus sphaericus* binary toxin in *Culex* pipiens (Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochemistry and Molecular Biology 31, 981-990.

de Barjac, H. (1978). A new subspecies of *Bacillus thuringiensis* very toxic for mosquitoes; *Bacillus thuringiensis* serotype H-14. *Compte Rendu Académie Sciences Paris Series D*. **286**, 797-800.

de Barjac, H. (1989). New facts and trends in bacteriological control of mosquitoes. *Memórias do Instituto Oswaldo Cruz* **84**, 101-105.

De Cosa, B., Moar, W., Lee, S.-B., Miller, M., and Daniell, H. (2001). Overexpression of the *Bt cry2*Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nature Biotechnology* **19**, 71-74.

de Maagd, R.A., Bravo, A., and Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends in Genetics* **17**, 193-199.

Delécluse, A., Poncet, S., Klier, A., and Rapoport, G. (1993). Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology* **59**, 3922-3927.

Delécluse, A., Rosso, M.L., and Ragni, A. (1995). Cloning and expression of a novel toxin gene from *Bacillus thuringiensis* subsp. *jegathesan* encoding a highly mosquitocidal protein. *Applied and Environmental Microbiology* **61**, 4230-4235.

Delécluse, A., Juárez-Pérez, V., and Berry, C. (2000). Vector-active toxins: structure and diversity. In: *Entomopathogenic Bacteria: from Laboratory to Field Application* (Charles, J.F., Delécluse, A., and Roux C.N., eds), 101-125, Springer Science & Business Media, The Netherland.

Deng, C., Peng, Q., Song, F., and Lereclus, D. (2014). Regulation of cry gene expression in *Bacillus thuringiensis*. *Toxins* **6**, 2194-2209.

Dervyn, E., Poncet, S., Klier, A., and Rapoport, G. (1995). Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology* **177**, 2283-2291.

Drapier, D., Suzuki, H., Levy, H., Rimbault, B., Kindle, K.L., Stern, D.B., and Wollman, F.-A. (1998). The chloroplast *atpa* gene cluster in *Chlamydomonas reinhardtii*. *Plant Physiology* **117**, 629-641.

Eberhard, S., Drapier, D., and Wollman, F.A. (2002). Searching limiting steps in the expression of chloroplast-encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of *Chlamydomonas reinhardtii*. *The Plant Journal* **31**, 149-160.

Economou, C., Wannathong, T., Szaub, J., and Purton, S. (2014). A simple, low-cost method for chloroplast transformation of the green alga *Chlamydomonas reinhardtii*. In: *Chloroplast Biotechnology* ,*Methods in Molecular Biology* **1132** (Maliga, P., ed), 401-411, Humana Press, New York,

Einhauer, A., and Jungbauer, A. (2001). The FLAG™ peptide, a versatile fusion tag for the purification of recombinant proteins. *Journal of Biochemical and Biophysical Methods* **49**, 455-465.

El-Bendary, M.A. (2006). *Bacillus thuringiensis* and *Bacillus sphaericus* biopesticides production. *Journal of Basic Microbiology* **46**, 158-170.

Erickson, J.M., Rahire, M., Malnoë, P., Girard-Bascou, J., Pierre, Y., Bennoun, P., and Rochaix, J.-D. (1986). Lack of the D2 protein in a *Chlamydomonas reinhardtii psbD* mutant affects photosystem II stability and D1 expression. *The EMBO Journal* **5**, 1745-1754.

Fansiri, T., Thavara, U., Tawatsin, A., Krasaesub, S., and Sithiprasasna, R. (2006). Laboratory and semi-field evaluation of mosquito dunks against *Aedes aegypti* and *Aedes albopictus* larvae (Diptera: Culicidae). *The Southeast Asian Journal of Tropical Medicine and Public Health* **37**, 62-66.

Fargo, D.C., Zhang, M., Gillham, N.W., and Boynton, J.E. (1998). Shine-Dalgarno-like sequences are not required for translation of chloroplast mRNAs in *Chlamydomonas reinhardtii* chloroplasts or in *Escherichia coli*. *Molecular Genetics and Genomics* **257**, 271-282.

Federici, B.A., Park, H.-W., Bideshi, D.K., Wirth, M.C., and Johnson, J.J. (2003). Recombinant bacteria for mosquito control. *Journal of Experimental Biology* **206**, 3877-3885.

Fernández, L.E., Pérez, C., Segovia, L., Rodríguez, M.H., Gill, S.S., Bravo, A., and Soberón, M. (2005). Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. *FEBS Letters* **579**, 3508-3514.

Fernández, L.E., Aimanova, K.G., Gill, S.S., Bravo, A., and Soberón, M. (2006). A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochemical Journal* **394**, 77-84.

Fernandez-Luna, M.T., Lanz-Mendoza, H., Gill, S.S., Bravo, A., Soberon, M., and Miranda-Rios, J. (2010). An α-amylase is a novel receptor for *Bacillus thuringiensis* ssp. *israelensis* Cry4Ba and Cry11Aa toxins in the malaria vector mosquito *Anopheles albimanus* (Diptera: Culicidae). *Environmental Microbiology* **12**, 746-757.

Ferris, P.J., Pavlovic, C., Fabry, S., and Goodenough, U.W. (1997). Rapid evolution of sex-related genes in *Chlamydomonas*. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 8634-8639.

Finney, D.J. (1977). Probit analysis, Cambridge University Press, Cambridge, U.K.

Fischer, N., Stampacchia, O., Redding, K., and Rochaix, J.-D. (1996). Selectable marker recycling in the chloroplast. *Molecular Genetics and Genomics* **251**, 373-380.

Fischer, R., Stoger, E., Schillberg, S., Christou, P., and Twyman, R.M. (2004). Plant-based production of biopharmaceuticals. *Current Opinion in Plant Biology* **7**, 152-158.

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G., and Fraley, R.T. (1987). Insect tolerant transgenic tomato plants. *Nature Biotechnology* **5**, 807-813.

Fletcher, S., Muto, M., and Mayfield, S. (2007). Optimization of recombinant protein expression in the chloroplasts of green algae. In: *Transgenic microalgae as green cell factories, Advances in Experimental Medicine and Biology* **616** (León, R., Galván, A., and Fernández, E., eds), 90-98 , Springer, New York,

Frankenhuyzen, K.V. (2009). Insecticidal activity of *Bacillus thuringiensis* crystal proteins. Journal of *Invertebrate Pathology* **101**, 1-16.

Franklin, S., Ngo, B., Efuet, E., and Mayfield, S.P. (2002). Development of a GFP reporter gene for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal* **30**, 733-744.

Gazit, E., Rocca, P.L., Sansom, M.S.P., and Shai, Y. (1998). The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin are consistent with an "umbrella-like" structure of the pore. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 12289-12294.

Geisel, N. (2011). Constitutive versus responsive gene expression strategies for growth in changing environments. *PLoS ONE* **6**, e27033.

Glare, T.R., and O'Callaghan, M. (1998). Environmental and health impacts of *Bacillus thuringiensis israelensis*, Report for The Ministry of Health.

Glare, T.R. and O'Callaghan, M. (2000) *Bacillus thuringiensis: Biology, Ecology and Safety*. J Wiley & Sons, West Sussex UK

Goldberg, L.J., and Margalit, J. (1977). A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. *Mosquito News* **37**, 355-358.

Goldschmidt-Clermont, M. (1991). Transgenic expression of aminoglycoside adenine transferase in the chloroplast: a selectable marker for site-directed transformation of *Chlamydomonas*. *Nucleic Acids Research* **19**, 4083-4089.

Gorman, D.S., and Levine, R.P. (1965). Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas* reinhardi. *Proceedings of the National Academy of Sciences of the United States of America* **54**, 1665-1669.

Gorton, H.L., and Vogelmann, T.C. (2003). Ultraviolet radiation and the snow alga *Chlamydomonas nivalis* (Bauer) Wille. *Photochemistry and Photobiology* **77**, 608-615.

Grant, D.M., Gillham, N.W., and Boynton, J.E. (1980). Inheritance of chloroplast DNA in *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences of the United States of America* **77**, 6067-6071.

Grisolia, C., Oliveira-Filho, E., Ramos, F., Lopes, M., Muniz, D., and Monnerat, R. (2009). Acute toxicity and cytotoxicity of *Bacillus thuringiensis* and *Bacillus sphaericus* strains on fish and mouse bone marrow. *Ecotoxicology* **18**, 22-26.

Gullet, P., Kurtak, D.C., Philippon, B., and Meyer, R. (1990) Use *of Bacillus thuringiensis israelensis* for Onchocerciasis control in west Africa, In: *Bacterial Control of Mosquitoes and black flies: biochemistry, genetics, & applications of Bacillus thuringiensis israelensis and Bacillus sphaericus* (De Barjac, H., and Sutherland, D., eds), 187-199, Rutgers University Press, New Brunswick, NJ.

Gustafsson, C., Govindarajan, S., and Minshull, J. (2004). Codon bias and heterologous protein expression. *Trends in Biotechnology* **22**, 346-353.

Halford, N.G. (2012). Toward two decades of plant biotechnology: successes, failures, and prospects. *Food and Energy Security* **1**, 9-28.

Harris, E.H. (1989). *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. Academic Press, San Diego.

Harris, E.H., Burkhart, B.D., Gillham, N.W., and Boynton, J.E. (1989). Antibiotic resistance mutations in the chloroplast 16S and 23S rRNA genes of *Chlamydomonas reinhardtii*: correlation of genetic and physical maps of the chloroplast genome. *Genetics* **123**, 281-292.

Harris, E.H., Boynton, J.E., and Gillham, N.W. (1994). Chloroplast ribosomes and protein synthesis. *Microbiological Reviews* **58**, 700-754.

Harris, E.H. (2001). *Chlamydomonas* as a model organism. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 363-406.

Harris, E.H. (2009). The *Chlamydomonas* Sourcebook, *Volume 1: Introduction to Chlamydomonas and its laboratory use*, 2nd edition, Academic Press, San Diego

Hayakawa, T., Howlader, M., Yamagiwa, M., and Sakai, H. (2008). Design and construction of a synthetic *Bacillus thuringiensis* Cry4Aa gene: Hyperexpression in *Escherichia coli*. *Applied Microbiology and Biotechnology* **80**, 1033-1037.

Herrin, D., Michaels, A., and Hickey, E. (1981). Synthesis of a chloroplast membrane polypeptide on thylakoid-bound ribosomes during the cell cycle of *Chlamydomonas* reinhardii 137+. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis* **655**, 136-145.

Herrin, D., and Schmidt, G. (1987). Chloroplast gene expression in chloroplast ribosome-deficient mutants of *Chlamydomonas Reinhardtii*. In: *Progress in Photosynthesis Research* (Biggins, J., ed), 645-648, Martinus Nijhoff, Dordrecht.

Herrin, D., and Nickelsen, J. (2004). Chloroplast RNA processing and stability. *Photosynthesis Research* **82**, 301-314.

Höfte, H., and Whiteley, H.R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* **53**, 242-255.

Holzinger, A., and Lütz, C. (2006). Algae and UV irradiation: Effects on ultrastructure and related metabolic functions. *Micron* **37**, 190-207.

Horák, P., Weiser, J., Mikeš, L., and Kolářová, L. (1996). The effect of *Bacillus thuringiensis* M-exotoxin on *Trematode Cercariae*. *Journal of Invertebrate Pathology* **68**, 41-49.

Howlader, M.T.H., Kagawa, Y., Sakai, H., and Hayakawa, T. (2009). Biological properties of loop-replaced mutants of *Bacillus thuringiensis* mosquitocidal Cry4Aa. J*ournal of Bioscience and Bioengineering* **108**, 179-183.

Hsieh, S.I., Castruita, M., Malasarn, D., Urzica, E., Erde, J., Page, M.D., Yamasaki, H., Casero, D., Pellegrini, M., Merchant, S.S., and Loo, J.A. (2013). The proteome of copper, iron, zinc, and manganese micronutrient deficiency in *Chlamydomonas reinhardtii*. *Molecular & Cellular Proteomics* **12**, 65-86.

Hughes, P. (2005). Bloodworm resistant rice, Rice CRC final report. Cooperative Research Centre for Sustainable Rice.

Ibarra, J.E., and Federici, B.A. (1986). Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology* **165**, 527-533.

Ishikura, K., Takaoka, Y., Kato, K., Sekine, M., Yoshida, K., and Shinmyo, A. (1999). Expression of a foreign gene in *Chlamydomonas reinhardtii* chloroplast. *Journal of Bioscience and Bioengineering* **87**, 307-314.

Ji, Q., Vincken, J.-P., Suurs, L.J.M., and Visser, R.F. (2003). Microbial starch-binding domains as a tool for targeting proteins to granules during starch biosynthesis. *Plant Molecular Biology* **51**, 789-801.

Juntadech T, Y.K., Tangphatsornruang S, Yap Y-K, Angsuthanasombat C. (2012). Efficient transcription of the larvicidal *cry4Ba* gene from *Bacillus thuringiensis* in transgenic chloroplasts of the green algal *Chlamydomonas reinhardtii*. *Advances in Bioscience and Biotechnology* **3**, 362–369.

Jurat-Fuentes, J.L., and Jackson, T.A. (2012). Bacterial Entomopathogens. In: *Insect Pathology*, 2nd edition (Vega, F.E. and Kaya, H.K., eds), 265-349, Academic Press, San Diego.

Kasai, S., Yoshimura, S., Ishikura, K., Takaoka, Y., Kobayashi, K., Kato, K., and Shinmyo, A. (2003). Effect of coding regions on chloroplast gene expression in *Chlamydomonas reinhardtii*. *Journal of Bioscience and Bioengineering* **95**, 276-282.

Kaufman, M.G., Wanja, E., Maknojia, S., Bayoh, M.N., Vulule, J.M., and Walker, E.D. (2006). Importance of algal biomass to growth and development of *Anopheles gambiae* larvae. *Journal of Medical Entomology* **43**, 669-676.

Kellen, W.R., Clark, T.B., Lindegren, J.E., and Ho, B.C. (1965). *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *Journal of Invertebrate Pathology* **7**, 442-448.

Khasdan, V., Ben-Dov, E., Manasherob, R., Boussiba, S., and Zaritsky, A. (2003). Mosquito larvicidal activity of transgenic *Anabaena* PCC 7120 expressing toxin genes from *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiology Letters* **227**, 189-195.

Kindle, K., and Sodeinde, O. (1994). Nuclear and chloroplast transformation in *Chlamydomonas reinhardtii*: strategies for genetic manipulation and gene expression. *Journal of Applied Phycology* **6**, 231-238.

Kleter, G.A., Bhula, R., Bodnaruk, K., Carazo, E., Felsot, A.S., Harris, C.A., Katayama, A., Kuiper, H.A., Racke, K.D., Rubin, B., Shevah, Y., Stephenson, G.R., Tanaka, K., Unsworth, J., Wauchope, R.D., and Wong, S.-S. (2007). Altered pesticide use on transgenic crops and the associated general impact from an environmental perspective. *Pest Management Science* **63**, 1107-1115.

Komano, T., Yamagiwa, M., Nishimoto, T., Yoshisue, H., Tanabe, K., Sen, K., and Sakai, H. (1998). Activation process of the insecticidal proteins CryIVA and CryIVB produced by *Bacillus thuringiensis* subsp. *israelensis*. *Israel Journal of Entomology* **32**, 184-198.

Kuchka, M.R., Goldschmidt-Clermont, M., van Dillewijn, J., and Rochaix, J.-D. (1989). Mutation at the *Chlamydomonas* nuclear NAC2 locus specifically affects stability of the chloroplast *psbD* transcript encoding polypeptide D2 of PS II. *Cell* **58**, 869-876.

Kumar, A. (2010). Optimization of transgene expression in *Chlamydomonas reinhardtii* and its biotechnological applications. Ph.D. dissertation. The Ohio State University.

Kumar, A., Wang, S., Ou, R., Samrakandi, M., Beerntsen, B.T., and Sayre, R.T. (2013). Development of an RNAi based microalgal larvicide to control mosquitoes. *Malarial World Journal* **4**, 1-6.

Kwon, T., Odom, O., Qiu, W., and Herrin, D. (2014). PCR analysis of chloroplast double-strand break (dsb) repair products induced by I-CreII in *Chlamydomonas*

and *Arabidopsis*. In: *Homing Endonucleases, Methods in Molecular Biology* **1123** (Edgell, D.R., ed), 77-86, Humana Press, New York.

Lacey, L., and Merritt, R. (2003). The safety of bacterial microbial agents used for black fly and mosquito control in aquatic environments. In: *Environmental Impacts of Microbial Insecticides, Progress in Biological Control,* **1** (Hokkanen, H.T. and Hajek, A., eds), 151-168, Kluwer Academic Press, The Netherlands.

Laemmli, U.K. (1970). Cleavage of Structural Proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lauersen, K.J., Berger, H., Mussgnug, J.H., and Kruse, O. (2013). Efficient recombinant protein production and secretion from nuclear transgenes in *Chlamydomonas reinhardtii*. *Journal of Biotechnology* **167**, 101-110.

Laurence, D.s., Christophe, L., and Roger, F. (2011). Using the bio-insecticide *Bacillus Thuringiensis israelensis* in mosquito control. In *Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment* (Stoytcheva, D.M., ed), InTech, DOI: 10.5772/948. Available from: http://www.intechopen.com/books/pesticides-in-the-modern-world-pests-control-and-pesticides-exposure-and-toxicity-assessment

Lee, J., and Herrin, D.L. (2002). Assessing the relative importance of light and the circadian clock in controlling chloroplast translation in *Chlamydomonas reinhardtii*. *Photosynthesis Research* **72**, 295-306.

Leetachewa, S., Katzenmeier, G., and Angsuthanasombat, C. (2006). Novel preparation and characterization of the α4-loop-α5 membrane-perturbing peptide from the *Bacillus thuringiensis* Cry4Ba δ-endotoxin. *Journal of biochemistry and molecular biology* **39**, 270-277.

Li, J., Carroll, J., and Ellar, D.J. (1991). Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature* **353**, 815-821.

Li, J., Koni, P.A., and Ellar, D.J. (1996). Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. k*yushuensis* and Implications for Membrane Pore Formation. *Journal of Molecular Biology* **257**, 129-152.

Lister, D.L., Bateman, J.M., Purton, S., and Howe, C.J. (2003). DNA transfer from chloroplast to nucleus is much rarer in *Chlamydomonas* than in tobacco. *Gene* **316**, 33-38.

Liu, Y.-T., Sui, M.-J., Ji, D.-D., Wu, I.H., Chou, C.-C., and Chen, C.-C. (1993). Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis*. *Journal of Invertebrate Pathology* **62**, 131-136.

Lössl, A., Eibl, C., Harloff, H.J., Jung, C., and Koop, H.U. (2003). Polyester synthesis in transplastomic tobacco (*Nicotiana tabacum* L.): significant contents of

polyhydroxybutyrate are associated with growth reduction. *Plant Cell Reports* **21**, 891-899.

Magee, A., Coyne, S., Murphy, D., Horvath, E., Medgyesy, P., and Kavanagh, T. (2004). T7 RNA polymerase-directed expression of an antibody fragment transgene in plastids causes a semi-lethal pale-green seedling phenotype. *Transgenic Research* **13**, 325-337.

Manasherob, R., Zaritsky, A., Ben-Dov, E., Saxena, D., Barak, Z.e., and Einav, M. (2001). Effect of Accessory Proteins P19 and P20 on Cytolytic Activity of Cyt1Aa from *Bacillus thuringiensis* subsp. *israelensis* in *Escherichia coli*. Current Microbiology **43**, 355-364.

Manasherob, R., Ben-Dov, E., Xiaoqiang, W., Boussiba, S., and Zaritsky, A. (2002). Protection from UV-B damage of mosquito larvicidal toxins from *Bacillus thuringiensis* subsp. *israelensis* expressed in *Anabaena* PCC 7120. *Current Microbiology* **45**, 217-220.

Manasherob, R., Zaritsky, A., Metzler, Y., Ben-Dov, E., Itsko, M., and Fishov, I. (2003). Compaction of the *Escherichia coli* nucleoid caused by Cyt1Aa. *Microbiology* **149**, 3553-3564.

Manceva, S.D., Pusztai-Carey, M., Russo, P.S., and Butko, P. (2005). A detergent-like mechanism of action of the cytolytic toxin Cyt1A from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry* **44**, 589-597.

Manuell, A., and Mayfield, S. (2006). A bright future for *Chlamydomonas*. *Genome Biology* **7**, 327.321-327.323.

Manuell, A.L., Beligni, M.V., Elder, J.H., Siefker, D.T., Tran, M., Weber, A., McDonald, T.L., and Mayfield, S.P. (2007). Robust expression of a bioactive mammalian protein in *Chlamydomonas* chloroplast. *Plant Biotechnology Journal* **5**, 402-412.

Margalit, Y. (1989). Biological control by *Bacillus thuringiensis* subsp. *israelensis* (Bti); history and present status. *Israel Journal of Entomology* **23**, 3-8.

Margalith, Y., and Ben-Dov, E. (1999). Biological control by *Bacillus thuringiensis* subsp. *israelensis*. In *Insect Pest Management: Techniques for Environmental Protection (*Rechcigl, J.E. and Rechcigl, N.A., eds), CRC Press, 244-303.

Marín-Navarro, J., Manuell, A., Wu, J., and P. Mayfield, S. (2007). Chloroplast translation regulation. *Photosynthesis Research* **94**, 359-374.

Marten, G.G. (1986). Mosquito control by plankton management: the potential of indigestible green algae. *The Journal of tropical medicine and hygiene* **89**, 213-222.

Matsuo, T., Onai, K., Okamoto, K., Minagawa, J., and Ishiura, M. (2006). Real-time monitoring of chloroplast gene expression by a luciferase reporter: evidence for

nuclear regulation of chloroplast circadian period. *Molecular and Cellular Biology* **26**, 863-870.

Maul, J.E., Lilly, J.W., Cui, L., dePamphilis, C.W., Miller, W., Harris, E.H., and Stern, D.B. (2002). The *Chlamydomonas reinhardtii* plastid chromosome: islands of genes in a sea of repeats. *The Plant Cell* **14**, 2659-2679.

Mayfield, S.P., Franklin, S.E., and Lerner, R.A. (2003). Expression and assembly of a fully active antibody in algae. *Proceedings of the National Academy of Sciences of the United States of America* 100, 438-442.

Mayfield, S.P., and Schultz, J. (2004). Development of a luciferase reporter gene, *luxCt*, for *Chlamydomonas reinhardtii* chloroplast. *The Plant Journal* **37**, 449-458.

Memon, A.R., Herrin, D.L., and Thompson Jr, G.A. (1993). Intracellular translocation of a 28 kDa GTP-binding protein during osmotic shock-induced cell volume regulation in *Dunaliella salina*. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1179**, 11-22.

Merchant, S.S., Prochnik, S.E., Vallon, O., Harris, E.H., Karpowicz, S.J., Witman, G.B., Terry, A., Salamov, A., Fritz-Laylin, L.K., Maréchal-Drouard, L., Marshall, W.F., Qu, L.-H., Nelson, D.R., Sanderfoot, A.A., Spalding, M.H., Kapitonov, V.V., Ren, Q., Ferris, P., Lindquist, E., Shapiro, H., Lucas, S.M., Grimwood, J., Schmutz, J., Cardol, P., Cerutti, H., Chanfreau, G., Chen, C.-L., Cognat, V., Croft, M.T., Dent, R., Dutcher, S., Fernández, E., Fukuzawa, H., González-Ballester, D., González-Halphen, D., Hallmann, A., Hanikenne, M., Hippler, M., Inwood, W., Jabbari, K., Kalanon, M., Kuras, R., Lefebvre, P.A., Lemaire, S.D., Lobanov, A.V., Lohr, M., Manuell, A., Meier, I., Mets, L., Mittag, M., Mittelmeier, T., Moroney, J.V., Moseley, J., Napoli, C., Nedelcu, A.M., Niyogi, K., Novoselov, S.V., Paulsen, I.T., Pazour, G., Purton, S., Ral, J.-P., Riaño-Pachón, D.M., Riekhof, W., Rymarquis, L., Schroda, M., Stern, D., Umen, J., Willows, R., Wilson, N., Zimmer, S.L., Allmer, J., Balk, J., Bisova, K., Chen, C.-J., Elias, M., Gendler, K., Hauser, C., Lamb, M.R., Ledford, H., Long, J.C., Minagawa, J., Page, M.D., Pan, J., Pootakham, W., Roje, S., Rose, A., Stahlberg, E., Terauchi, A.M., Yang, P., Ball, S., Bowler, C., Dieckmann, C.L., Gladyshev, V.N., Green, P., Jorgensen, R., Mayfield, S., Mueller-Roeber, B., Rajamani, S., Sayre, R.T., Brokstein, P., Dubchak, I., Goodstein, D., Hornick, L., Huang, Y.W., Jhaveri, J., Luo, Y., Martínez, D., Ngau, W.C.A., Otillar, B., Poliakov, A., Porter, A., Szajkowski, L., Werner, G., Zhou, K., Grigoriev, I.V., Rokhsar, D.S., and Grossman, A.R. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science* **318**, 245-250.

Michelet, L., Lefebvre-Legendre, L., Burr, S.E., Rochaix, J.-D., and Goldschmidt-Clermont, M. (2011). Enhanced chloroplast transgene expression in a nuclear mutant of *Chlamydomonas. Plant Biotechnology Journal* **9**, 565-574.

Milam, C.D., Farris, J.L., and Wilhide, J.D. (2000). Evaluating mosquito control pesticides for effect on target and nontarget organisms. *Archives of Environmental Contamination and Toxicology* **39**, 324-328.

Minai, L., Wostrikoff, K., Wollman, F.-A., and Choquet, Y. (2006). chloroplast biogenesis of photosystem II cores involves a series of assembly-controlled steps that regulate translation. *The Plant Cell 18*, 159-175.

Minko, I., Holloway, S.P., Nikaido, S., Carter, M., Odom, O.W., Johnson, C.H., and Herrin, D.L. (1999). Renilla luciferase as a vital reporter for chloroplast gene expression in *Chlamydomonas*. *Molecular Genetics and Genomics* **262**, 421-425.

Misquitta, R. and Herrin, D.L. (2005**)**. Circadian Regulation of Chloroplast Transcription: A Review. *Plant Tissue Culture* **15**, 83-101

Miyamoto, J., Kaneko, H., Tsuji, R., and Okuno, Y. (1995). Pyrethroids, nerve poisons: how their risks to human health should be assessed. *Toxicology Letters* **82–83**, 933-940.

Murray, C., Sutherland, P., Phung, M., Lester, M., Marshall, R., and Christeller, J. (2002). Expression of biotin-binding proteins, avidin and streptavidin, in plant tissues using plant vacuolar targeting sequences. *Transgenic Research* **11**, 199-214.

Myasnik, M., Manasherob, R., Ben-Dov, E., Zaritsky, A., Margalith, Y., and Barak, Z.E. (2001). Comparative sensitivity to UV-B radiation of two *Bacillus thuringiensis* subspecies and other *Bacillus* sp.. *Current Microbiology* **43**, 140-143.

Nakamura, Y., Gojobori, T., and Ikemura, T. (2000). Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic Acids Research* **28**, 292.

Nauen, R. (2007). Insecticide resistance in disease vectors of public health importance. *Pest Management Science* **63**, 628-633.

Newman, S.M., Boynton, J.E., Gillham, N.W., Randolph-Anderson, B.L., Johnson, A.M., and Harris, E.H. (1990). Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas*: molecular and genetic characterization of integration events. *Genetics* **126**, 875-888.

Newman, S.M., Harris, E.H., Johnson, A.M., Boynton, J.E., and Gillham, N.W. (1992). Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: evidence for a hotspot and an adjacent cold region. **Genetics** *132*, 413-429.

Nickelsen, J., Fleischmann, M., Boudreau, E., Rahire, M., and Rochaix, J.-D. (1999). Identification of cis-acting rna leader elements required for chloroplast *psbD* gene expression in *Chlamydomonas*. *The Plant Cell* **11**, 957-970.

Nicolas, L., Dossou-Yovo, J., and Hougard, J.-M. (1987). Persistence and recycling of *Bacillus sphaericus* 2362 spores in *Culex quinquefasciatus* breeding sites in west africa. *Applied Microbiology and Biotechnology* **25**, 341-345.

Nicolas, L., Nielsen-Leroux, C., Charles, J.-F., and Delécluse, A. (1993). Respective role of the 42- and 51-kDa components of the *Bacillus sphaericus* toxin overexpressed in *Bacillus thuringiensis*. *FEMS Microbiology Letters* **106**, 275-279.

Nielsen-Leroux, C., Charles, J.-F., Thiéry, I., and Georghiou, G.P. (1995). Resistance in a laboratory population of *Culex Quinquefasciatus* (Diptera: Culicidae) to *Bacillus Sphaericus* binary toxin is due to a change in the receptor on midgut brush-border membranes. *European Journal of Biochemistry* **228**, 206-210.

Noth, J., Krawietz, D., Hemschemeier, A., and Happe, T. (2013). Pyruvate: ferredoxin oxidoreductase is coupled to light-independent hydrogen production in *Chlamydomonas reinhardtii*. *Journal of Biological Chemistry* **288**, 4368-4377.

Odom, O.W., Holloway, S.P., Deshpande, N.N., Lee, J., and Herrin, D.L. (2001). Mobile self-splicing group I introns from the *psbA* gene of *Chlamydomonas reinhardtii*: highly efficient homing of an exogenous intron containing its own promoter. *Molecular and Cellular Biology* **21**, 3472-3481.

Odom, O.W., and Herrin, D.L. (2013). Reverse transcription of spliced *psbA* mRNA in Chlamydomonas spp. and its possible role in evolutionary intron loss. *Molecular Biology and Evolution* **30**, 2666-2675.

Oey, M., Lohse, M., Kreikemeyer, B., and Bock, R. (2009). Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *The Plant Journal* **57**, 436-445.

Ohana, B., Margalit, J., and Barak, Z.E. (1987). Fate of *Bacillus thuringiensis* subsp. *israelensis* under simulated field conditions. *Applied and Environmental Microbiology* **53**, 828-831.

Oliveira-Filho, E., Ramos, F., Miranda, B.G., Muniz, D.F., and Monnerat, R. (2014). evaluating the elimination of brazilian entomopathogenic *Bacillus* by non-target aquatic species: an experimental study. *Bulletin of Environmental Contamination and Toxicology* **93**, 461-464.

Orduz, S., Realpe, M., Arango, R., Murillo, L.A., and Dele¨cluse, A. (1998). Sequence of the *cry11Bb11* gene from *Bacillus thuringiensis* subsp. *medellin* and toxicity analysis of its encoded protein. *Biochimica et Biophysica Acta* **1388**, 267-272

Otieno-Ayayo, Z.N., Zaritsky, A., Wirth, M.C., Manasherob, R., Khasdan, V., Cahan, R., and Ben-Dov, E. (2008). Variations in the mosquito larvicidal activities of toxins

from *Bacillus thuringiensis* ssp. *israelensis*. *Environmental Microbiology* **10**, 2191-2199.

Pantuwatana, S., Maneeroj, R., and Upatham, E. (1989). Long residual activity of *Bacillus sphaericus* 1593 against *Culex quinquefasciatus* larvae in artificial pools. *Southeast Asian Journal of Tropical Medicine and Public Health* **20**, 421-427.

Park, H.-W., Bideshi, D.K., and Federici, B.A. (2000). Molecular genetic manipulation of truncated Cry1C Protein Synthesis in *Bacillus thuringiensis* to improve stability and yield. *Applied and Environmental Microbiology* **66**, 4449-4455.

Park, H.-W., Bideshi, D.K., Wirth, M.C., Johnson, J.J., Walton, W.E., and Federici, B.A. (2005). Recombinant larvicidal bacteria with markedly improved efficacy against *Culex* vectors of west nile virus. *The American Journal of Tropical Medicine and Hygiene* **72**, 732-738.

Peña-Montenegro, T.D., and Dussán, J. (2013). Genome sequence and description of the heavy metal tolerant bacterium *LysiniBacillus sphaericus* strain OT4b.31. *Standards in Genomic Sciences* **9**, 42-56.

Pérez, C., Fernandez, L.E., Sun, J., Folch, J.L., Gill, S.S., Soberón, M., and Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18303-18308.

Pognonec, P., Kato, H., Sumimoto, H., Kretzschmar, M., and Roeder, R.G. (1991). A quick procedure for purification of functional recombinant proteins over-expressed in *E. coli*. *Nucleic Acids Research* **19**, 6650.

Poncet, S., Delécluse, A., Klier, A., and Rapoport, G. (1995). Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD Toxic Components of *B. thuringiensis* subsp. *israelensis* Crystals. *Journal of Invertebrate Pathology* **66**, 131-135.

Poopathi, S., and Abidha, S. (2010). Mosquitocidal bacterial toxins (*Bacillus sphaericus* and *B. thuringiensis* serovar *israelensis*): Mode of action, cytopathological effects and mechanism of resistance. *Journal of Physiology and Pathophysiology* **1**, 22-38.

Porter, A.G., Davidson, E.W., and Liu, J.W. (1993). Mosquitocidal toxins of bacilli and their genetic manipulation for effective biological control of mosquitoes. *Microbiological Reviews* **57**, 838-861.

Promdonkoy, B., and Ellar, D.J. (2000). Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. *Biochemical Journal* **350**, 275-282.

Promdonkoy, B., Promdonkoy, P., and Panyim, S. (2005). Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism

against *Aedes aegypti* and *Culex quinquefasciatus* larvae. *FEMS Microbiology Letters* **252**, 121-126.

Pröschold, T., Harris, E.H., and Coleman, A.W. (2005). Portrait of a Species: *Chlamydomonas reinhardtii*. *Genetics* **170**, 1601-1610.

Puigbò, P., Guzmán, E., Romeu, A., and Garcia-Vallvé, S. (2007). OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. *Nucleic Acids Research* **35**, W126-W131.

Purton, S. (2007). Tools and techniques for chloroplast Transformation of *Chlamydomonas*. In: *Transgenic microalgae as green cell factories* (León, R., Galván, A., and Fernández, E., eds), *Advances in Experimental Medicine and Biology* **616,** 34-45, Springer, New York.

Purton, S., Szaub, J.B., Wannathong, T., Young, R., and Economou, C.K. (2013). Genetic engineering of algal chloroplasts: Progress and prospects. *Russian Journal of Plant Physiology* **60**, 491-499.

Pusztai, M., Fast, P., Gringorten, L., Kaplan, H., Lessard, T., and Carey, P.R. (1991). The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals. *Biochemical Journal* **273**, 43-47.

Quinn, J.M., and Merchant, S. (1998). Copper-responsive gene expression during adaptation to copper deficiency. In: *Photosynthesis: Molecular Biology of Energy Capture, Methods in Enzymology*, **297,** 263-279 (Lee, M., ed), Academic Press, New York,.

Quintana-Castro, R., Ramírez-Suero, M., Moreno-Sanz, F., and Ramírez-Lepe, M. (2005). Expression of the *cry11A* gene of *Bacillus thuringiensis* ssp. *israelensis* in *Saccharomyces cerevisiae*. *Canadian Journal of Microbiology* **51**, 165-170.

Qureshi, N., Chawla, S., Likitvivatanavong, S., Lee, H.L., and Gill, S.S. (2014). The cry toxin operon of *Clostridium bifermentans* subsp. *malaysia* is highly toxic to *Aedes* larval mosquitoes. *Applied and Environmental Microbiology* **80**, 5689-5697.

Raghavendra, K., Barik, T., Reddy, B., Sharma, P., and Dash, A. (2011). Malaria vector control: from past to future. *Parasitology Research* **108**, 757-779.

Ramoska, W.A., Watts, S., and Rodriguez, R.E. (1982). Influence of suspended particulates on the activity of *Bacillus thuringiensis* serotype h-14 against mosquito larvae. *Journal of Economic Entomology* **75**, 1-4.

Ramundo, S., Rahire, M., Schaad, O., and Rochaix, J.-D. (2013). Repression of essential chloroplast genes reveals new signaling pathways and regulatory feedback loops in *Chlamydomonas*. *The Plant Cell* **25**, 167-186.

Rasala, B.A., Muto, M., Lee, P.A., Jager, M., Cardoso, R.M.F., Behnke, C.A., Kirk, P., Hokanson, C.A., Crea, R., Mendez, M., and Mayfield, S.P. (2010). Production of therapeutic proteins in algae, analysis of expression of seven human proteins in

the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnology Journal* **8**, 719-733.

Rasala, B.A., Muto, M., Sullivan, J., and Mayfield, S.P. (2011). Improved heterologous protein expression in the chloroplast of *Chlamydomonas reinhardtii* through promoter and 5′ untranslated region optimization. *Plant Biotechnology Journal* **9**, 674-683.

Rasala, B.A., Barrera, D.J., Ng, J., Plucinak, T.M., Rosenberg, J.N., Weeks, D.P., Oyler, G.A., Peterson, T.C., Haerizadeh, F., and Mayfield, S.P. (2013). Expanding the spectral palette of fluorescent proteins for the green microalga *Chlamydomonas reinhardtii*. *The Plant Journal* **74**, 545-556.

Rasala, B.A., Chao, S.-S., Pier, M., Barrera, D.J., and Mayfield, S.P. (2014a). Enhanced genetic tools for engineering multigene traits into green algae. *PLoS ONE* **9**, e94028.

Rasala, B.A., and Mayfield, S. (2014b). Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses. *Photosynthesis Research* **123**, 1-13.

Rochaix, J.-D. (1995). *Chlamydomonas Reinhardtii* as the photosynthetic yeast. *Annual Review of Genetics* **29**, 209-230.

Rochaix, J.-D. (1996). Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. *Plant Molecular Biology* **32**, 327-341.

Rosales-Mendoza, S., Paz-Maldonado, L., and Soria-Guerra, R. (2011). *Chlamydomonas reinhardtii* as a viable platform for the production of recombinant proteins: current status and perspectives. *Plant Cell Reports* **31**, 479-494.

Rosso, M.L., and Delécluse, A. (1997). Contribution of the 65-kilodalton protein encoded by the cloned gene cry19A to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *jegathesan*. *Applied and Environmental Microbiology* **63**, 4449-4455.

Saengwiman, S., Aroonkesorn, A., Dedvisitsakul, P., Sakdee, S., Leetachewa, S., Angsuthanasombat, C., and Pootanakit, K. (2011). In vivo identification of *Bacillus thuringiensis* Cry4Ba toxin receptors by RNA interference knockdown of glycosylphosphatidylinositol-linked aminopeptidase N transcripts in *Aedes aegypti* larvae. *Biochemical and Biophysical Research Communications* **407**, 708-713.

Saiful, A.N., Lau, M.S., Sulaiman, S., and Hidayatulfathi, O. (2012). Residual effects of TMOF-Bti formulations against 1st instar *Aedes aegypti* Linnaeus larvae outside laboratory. *Asian Pacific Journal of Tropical Biomedicine* **2**, 315-319.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., and Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* **62**, 775-806.

Schwartz, J.L., Potvin, L., Coux, F., Charles, J.F., Berry, C., Humphreys, M.J., Jones, A.F., Bernhart, I., Dalla Serra, M., and Menestrina, G. (2001). Permeabilization of model lipid membranes by *bacillus sphaericus* mosquitocidal binary toxin and its individual components. *The Journal of Membrane Biology* **184**, 171-183.

Shadduck, J.A., Singer, S., and Lause, S. (1980). Lack of mammalian pathogenicity of entomocidal isolates of *Bacillus sphaericus*. *Environmental Entomology* 9, 403-407

Sharp, P.M., and Li, W.H. (1987). The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research* **15**, 1281-1295.

Siegel, J.P. and Shadduck, J. (1990) Mammalian safety of *Bacillus thuringiensis israelensis.* In: *Bacterial Control of Mosquitoes and* black flies: biochemistry, genetics, & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* (De Barjac, H., and Sutherland, D., eds), 202-217, Rutgers University Press, New Brunswick, NJ.

Siegel, J.P. (2001). The Mammalian safety of *Bacillus thuringiensis*- based insecticides. *Journal of Invertebrate Pathology* **77**, 13-21.

Silflow, C.D., and Lefebvre, P.A. (2001). Assembly and motility of eukaryotic cilia and flagella. lessons from *Chlamydomonas reinhardtii*. *Plant Physiology* **127**, 1500-1507.

Silva-Filha, M.-H., Regis, L., Nielsen-Leroux, C., and Charles, J.-F. (1995). Low-level resistance to *Bacillus sphaericus* in a field-treated population of *Culex quinquefasciatus* (Diptera: Culicidae). *Insecticide Resistance and Resistance Management* **88**, 525-530

Silva-Filha, M.H.N.L., Fontes de Oliveira, C.M., Regis, L., Yuan, Z., Rico, C.M., and Nielsen-LeRoux, C. (2004). Two *Bacillus sphaericus* binary toxins share the midgut receptor binding site: implications for resistance of *Culex pipiens* complex (Diptera: Culicidae) larvae. *FEMS Microbiology Letters* **241**, 185-191.

Soberón, M., Pardo, L., Muñóz-Garay, C., Sánchez, J., Gómez, I., Porta, H., and Bravo, A. (2010). Pore formation by Cry toxins. In: *Proteins: Membrane Binding and Pore Formation, Advances in Experimental Medicine and Biology* **677** (Anderluh, G. and Lakey, J., eds), 127-142, Landes Bioscience, Austin, Texas.

Soberón, M., López-Díaz, J.A., and Bravo, A. (2013). Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms. *Peptides* **41**, 87-93.

Soltes-Rak, E., Kushner, D.J., Williams, D.D., and Coleman, J.R. (1993). Effect of promoter modification on mosquitocidal *cryIVB* gene expression in

Synechococcus sp. strain PCC 7942. *Applied and Environmental Microbiology* **59**, 2404-2410.

Soltes-Rak, E., Kushner, D., Williams, D.D., and Coleman, J. (1995). Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC 7942. *Molecular Genetics and Genomics* **246**, 301-308.

Specht, E., Miyake-Stoner, S., and Mayfield, S. (2010). Micro-algae come of age as a platform for recombinant protein production. *Biotechnology Letters* **32**, 1373-1383.

Stenico, M., Lloyd, A.T., and Sharp, P.M. (1994). Codon usage in *Caenorhabditis elegans*: delineation of translational selection and mutational biases. *Nucleic Acids Research* **22**, 2437-2446.

Studier, W., , F., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. In: *Gene Expression Technology*, *Methods in Enzymology* **185** (David. V.G., ed), 60-89, Academic Press, San Diego.

Sun, Y., Zhao, Q., Xia, L., Ding, X., Hu, Q., Federici, B.A., and Park, H.-W. (2013). Identification and characterization of three previously undescribed crystal proteins from *Bacillus thuringiensis* subsp. *jegathesan*. *Applied and Environmental Microbiology* **79**, 3364-3370.

Surzycki, R., Cournac, L., Peltier, G., and Rochaix, J.-D. (2007). Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 17548-17553.

Surzycki, R., Greenham, K., Kitayama, K., Dibal, F., Wagner, R., Rochaix, J.-D., Ajam, T., and Surzycki, S. (2009). Factors effecting expression of vaccines in microalgae. *Biologicals* **37**, 133-138.

Takken, W., and Knols, B.G.J. (2009). Malaria vector control: current and future strategies. *Trends in Parasitology* **25**, 101-104.

Tetreau, G., Stalinski, R., David, J.-P., and Després, L. (2013). Monitoring resistance to *Bacillus thuringiensis* subsp. *israelensis* in the field by performing bioassays with each Cry toxin separately. *Memórias do Instituto Oswaldo Cruz* **108**, 894-900.

Thammasittirong, A., Dechklar, M., Leetachewa, S., Pootanakit, K., and Angsuthanasombat, C. (2011). *Aedes aegypti* Membrane-bound alkaline phosphatase expressed in *Escherichia coli* retains high-affinity binding for *Bacillus thuringiensis* Cry4Ba toxin. *Applied and Environmental Microbiology* **77**, 6836-6840.

Tran, M., Van, C., Barrera, D.J., Pettersson, P.L., Peinado, C.D., Bui, J., and Mayfield, S.P. (2013). Production of unique immunotoxin cancer therapeutics in algal

chloroplasts. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E15–E22.

Uniacke, J., and Zerges, W. (2009). Chloroplast protein targeting involves localized translation in *Chlamydomonas*. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 1439-1444.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Montagu, M.V., and Leemans, J. (1987). Transgenic plants protected from insect attack. *Nature* **328**, 33-37.

Vilarinhos, P., and Monnerat, R. (2004). Larvicidal persistence of formulations of *Bacillus thuringiensis* var. *israelensis* to control larval *Aedes aegypti*. *Journal of the American Mosquito Control Association* **20**, 311-314.

Visick, J.E., and Whiteley, H.R. (1991). Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on production of the CytA protein by *Escherichia coli*. *Journal of Bacteriology* **173**, 1748-1756.

Ward, E.S., Ridley, A.R., Ellar, D.J., and Todd, J.A. (1986). *Bacillus thuringiensis* var. *israelensis* δ-endotoxin: Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus* subtilis. *Journal of Molecular Biology* **191**, 13-22.

Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., Berticat, C., Pasteur, N., Philips, A., Fort, P., and Raymond, M. (2003). Comparative genomics: Insecticide resistance in mosquito vetors. *Nature* **423**, 136-137.

WHO. (1999). Microbial pest control agent: *Bacillus thuringiensis*. World Health Organization, Geneva Switzerland, Available from: Available from: http://apps.who.int/iris/handle/10665/42242

WHO. (2005). Guidelines for laboratory and field testing of mosquito larvicides. World Health Organization, Geneva, Available from: http://apps.who.int/iris/handle/10665/69101

WHO. (2013). World Malaria Report 2013. World Health Organization, Geneva Switzerland, Available from: http://www.who.int/malaria/publications/world_malaria_report_2013/en/

Wintermans, J.F.G.M., and De Mots, A. (1965). Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. *Biochimica et Biophysica Acta (BBA) - Biophysics including Photosynthesis* **109**, 448-453.

Wirth, M.C., Georghiou, G.P., and Federici, B.A. (1997). CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10536-10540.

Wirth, M.C., Georghiou, G.P., Malik, J.I., and Hussain, G. (2000). Laboratory selection for resistance to *Bacillus sphaericus*in*Culex quinquefasciatus* (Diptera: Culicidae) from California, USA. *Journal of Medical Entomology* **37**, 534-540.

Wirth, M.C., Jiannino, J.A., Federici, B.A., and Walton, W.E. (2004). Synergy between Toxins of *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*. *Journal of Medical Entomology* **41**, 935-941.

Wirth, M.C., Walton, W.E., and Federici, B.A. (2010). Evolution of resistance to the *Bacillus sphaericus* Bin toxin is phenotypically masked by combination with the mosquitocidal proteins of *Bacillus thuringiensis* subspecies *israelensis*. *Environmental Microbiology* **12**, 1154-1160.

Wraight, S.P., Molloy, D.P., and Singer, S. (1987). Studies on the culicine mosquito host range of *Bacillus sphaericus* and *Bacillus thuringiensis* var *israelensis* with notes on the effects of temperature and instar on bacterial efficacy. *Journal of Invertebrate Pathology* **49**, 291-302.

Wu, D., Johnson, J.J., and Federici, B.A. (1994). Synergism of mosquitocidal toxicity between CytA and CrylVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. *Molecular Microbiology* **13**, 965-972.

Xiaoqiang, W., Vennison, S.J., Huirong, L., Ben-Dov, E., Zaritsky, A., and Boussiba, S. (1997). Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. *Applied and Environmental Microbiology* **63**, 4971-4974.

Xiong, F., Komenda, J., Kopecký, J., and Nedbal, L. (1997). Strategies of ultraviolet-B protection in microscopic algae. *Physiologia Plantarum* **100**, 378-388.

Xu, C., Wang, B.-C., Yu, Z., and Sun, M. (2014). Structural insights into *Bacillus thuringiensis* Cry, Cyt and Parasporin Toxins. *Toxins* **6**, 2732-2770.

Xu, Y., Nagai, M., Bagdasarian, M., Smith, T.W., and Walker, E.D. (2001). Expression of the p20 gene from *Bacillus thuringiensis* H-14 Increases Cry11A toxin production and enhances mosquito-larvicidal activity in recombinant gram-negative bacteria. *Applied and Environmental Microbiology* **67**, 3010-3015.

Yamagiwa, M., Esaki, M., Otake, K., Inagaki, M., Komano, T., Amachi, T., and Sakai, H. (1999). Activation process of dipteran-specific insecticidal protein produced by *Bacillus thuringiensis*subsp. *israelensis*. *Applied and Environmental Microbiology* **65**, 3464-3469.

Yamagiwa, M., Ogawa, R., Yasuda, K., Natsuyama, H., Sen, K., and Sakai, H. (2002). Active form of dipteran-Specific insecticidal protein Cry11A produced by *Bacillus thuringiensis* subsp. *israelensis*. *Bioscience, Biotechnology, and Biochemistry* **66**, 516-522.

Yamagiwa, M., Sakagawa, K., and Sakai, H. (2004). Functional analysis of two processed fragments of *Bacillus thuringiensis* Cry11A toxin. *Bioscience, Biotechnology, and Biochemistry* **68**, 523-528.

Yap, W.H., Thanabalu, T., and Porter, A.G. (1994a). Expression of mosquitocidal toxin genes in a gas-vacuolated strain of *Ancylobacter aquaticus*. *Applied and Environmental Microbiology* **60**, 4199-4202.

Yap, W.H., Thanabalu, T., and Porter, A.G. (1994b). Influence of transcriptional and translational control sequences on the expression of foreign genes in *Caulobacter crescentus*. *Journal of Bacteriology* **176**, 2603-2610.

Yoshida, K., Matsushima, Y., K. Sen, H.S., and Komano, T. (1989). Insecticidal activity of a peptide containing the 30th to 695th amino acid residues of the 130-kDa protein of *Bacillus thuringiensis* var. *israelensis*. Agricultural and Biological Chemistry **53**, 2121-2127

Zaritsky, A., Ben-Dov, E., Borovsky, D., Boussiba, S., Einav, M., Gindin, G., Horowitz, A.R., Kolot, M., Melnikov, O., Mendel, Z., and Yagil, E. (2010). Transgenic organisms expressing genes from *Bacillus thuringiensis* to combat insect pests. *Bioengineered Bugs* **1**, 341-344.

Zedler, J.Z., Gangl, D., Hamberger, B., Purton, S., and Robinson, C. (2014). Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*. *Journal of Applied Phycology* **26**, 1-7.