



Published in final edited form as:

J Appl Phycol. 2017 June ; 29(3): 1377–1389. doi:10.1007/s10811-016-1008-z.

Toward mosquito control with a green alga: Expression of Cry toxins of *Bacillus thuringiensis* subsp. *israelensis* (Bti) in the chloroplast of *Chlamydomonas*

Seongjoon Kang^{1,2}, Obed W. Odom¹, Saravanan Thangamani³, and David L. Herrin^{1,2}

¹Dept. of Molecular Biosciences, University of Texas at Austin, Austin, TX 78712, USA

²Pond Life Technologies LLC, Cedar Park, TX 78613, USA

³Dept. of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA

Abstract

We are developing *Chlamydomonas* strains that can be used for safe and sustainable control of mosquitoes, because they produce proteins from *Bacillus thuringiensis* subsp. *israelensis* (Bti) in the chloroplast. *Chlamydomonas* has a number of advantages for this approach, including genetic controls that are not generally available with industrial algae. The Bti toxin has been used for mosquito control for > 30 years and does not engender resistance; it contains three Cry proteins, Cry4Aa (135 kDa), Cry4Ba (128 kDa) and Cry11Aa (72 kDa), and Cyt1Aa (25 kDa). To express the Cry proteins in the chloroplast, the three genes were resynthesized and *cry4Aa* was truncated to the first 700 amino acids (*cry4Aa*₇₀₀); also, since they can be toxic to host cells, the inducible *Cyc6:Nac2-psbD* expression system was used. Western blots of total protein from the chloroplast transformants showed accumulation of the intact polypeptides, and the relative expression level was Cry11Aa > Cry4Aa₇₀₀ > Cry4Ba. Quantitative western blots with purified Cry11Aa as a standard showed that Cry11Aa accumulated to 0.35% of total cell protein. Live cell bioassays in dH₂O demonstrated toxicity of the *cry4Aa*₇₀₀ and *cry11Aa* transformants to larvae of *Aedes aegypti* and *Culex quinquefasciatus*. These results demonstrate that the Cry proteins that are most toxic to *Aedes* and *Culex* mosquitoes, Cry4Aa and Cry11Aa, can be successfully expressed in the chloroplast of *Chlamydomonas*.

Keywords

green algae; *Bacillus thuringiensis* subsp. *israelensis*; *Chlamydomonas*; Cry toxins; mosquito control; West Nile; Zika

Introduction

The re-emergence of dengue and the spread of West Nile, chikungunya and now Zika arboviruses signals an increasing global danger of vector-borne infectious diseases, driven

Author for correspondence: David L. Herrin, 205 W. 24th St STOP A6700, Austin, TX 78712, USA; herrin@utexas.edu.

Conflict of interest statement: The senior author (DLH) is the founder and owner of Pond Life Technologies LLC. Also, a patent application based in part on this work has been filed with the US Patent Office (15/142,865).

by factors such as globalization and climate change (Morens and Fauci 2013). In the absence of treatments for the aforementioned viral diseases, vector control is the principal means of limiting these diseases. Mosquito control is a proven approach that is uniquely powerful, because it can block the transmission of multiple diseases simultaneously. The preferred strategy is integrated pest management (IPM), which uses a combination of practices based on data, and targets all stages of the insect life cycle. An important part of IPM is the use of pesticides to bring down damaging pest populations. In most places, the pesticides used most often are chemicals that kill the adult insects (e.g., DDT, Malathion and Naled), although methoprene is used to block larval development. The problems with these compounds are their toxicity to non-target organisms, such as honey bees, and the increasing resistance to them among mosquito populations (e.g., Ibrahim et al. 2013; Kioulos et al. 2014; Koou et al. 2014); indeed, all the chemical pesticides eventually fail due to the development of resistance. In addition, there are growing concerns about effects that long-term low-dose exposure to chemical pesticides may have on human beings (Kamel 2013). Thus, many leading public health organizations have called for new approaches and products for mosquito control.

Unbeknownst to most outside of the vector control arena, there are safe biolarvacides made from the bacterium *Bacillus thuringiensis* subsp. *israelensis* (Bti) that are also unique in that they have not produced strong resistance in > 30 years of use against mosquitoes (Ben-Dov 2014). Moreover, controlling mosquitoes at the larval stage, before they become disease-transmitting adults, has obvious advantages. However, Bti has drawbacks that have limited its use: mainly it does not persist very long in many environments, and in polluted or organic-rich waters its efficacy is diminished requiring much higher amounts (Ohana et al. 1987; de Barjac and Sutherland 1990; Liu et al. 1993; Glare and O'Callaghan 2000; Bravo et al. 2011; Tetreau et al. 2012). These drawbacks arise because the Bti toxin is a protein complex that is produced during sporulation but is outside of the spore, in what is known as a parasporal body. The Bti spores themselves do not reproduce well in most aquatic habitats.

The fact that algae and mosquito larvae co-occur in the same habitats (Laird 1988), and that most algae are excellent food sources for larvae (Marten 1986; Kaufman et al. 2006) raises the possibility of using them as a biocontrol mechanism. In an early effort, algae that are indigestible for mosquito larvae were identified, with the idea of using them to starve the larvae (Marten 1986); this study also identified many algae that could sustain larval development, including *Chlamydomonas reinhardtii*. Recently, *Chlamydomonas* strains expressing double-stranded RNA (RNAi) for a segment of a gene from *Anopheles gambiae* were shown to be detrimental to *Anopheles stephensi* when they were fed to the emerging larvae (Kumar et al. 2013). Whether or not the RNAi will effect non-target species, or if the mosquitoes will develop resistance to RNAi are not known. A marine chlorophyte, *Chlorella denticulata*, was stably engineered to produce a regulatory peptide (trypsin modulating oostatic factor) that represses trypsin in the larval gut (Borovsky et al. 2016). The transgenic *Chlorella* strains were lethal to *A. aegypti* larvae; however, the assays had extraordinarily high algal cell numbers (3×10^8 cells mL⁻¹).

The most effort has been based on modifying cyanobacteria to synthesize the larvicidal proteins of Bti (Angsuthanasombat and Panyim 1989; Stevens et al. 1994; Soltes-Rak et al.

1995; Zaritsky et al. 2010). In the most successful series to date, Zaritsky and colleagues developed a larvicidal *Anabaena* strain with chromosomally-expressed (ie, not plasmid-born) genes for *cry4Aa* and *cry11Aa* that was quite toxic to *Aedes* and *Culex* larvae (Wu et al. 1997). However, the *Anabaena* strain has not, to our knowledge, been employed for mosquito control, due partly to concerns over the bacterial antibiotic-resistance gene(s) that it contains (Zaritsky et al. 2010). It also lacked the *cyt1Aa* gene needed to prevent the development of resistance, although *cyt1Aa* gene could be present on a plasmid with these genes in other strains (Khasdan et al. 2003).

Our approach to this problem is to develop a biological platform for mosquito control based on the eukaryotic green alga *Chlamydomonas reinhardtii*, and, at least initially, the Bti larvicide. *Chlamydomonas* is an edible alga that can swim and multiply in larval habitats, but also depends on sexual reproduction to survive in the environment, as the zygote is the tough stage of the life cycle (Harris 1989). The sexual phase of its life cycle has a genetic feature that we can use to prevent the formation of genetically modified progeny should it mate with a local indigenous strain, namely that the chloroplast genome is inherited from only one parent (Harris 1989). Also, chloroplast engineering in *Chlamydomonas* can be accomplished without leaving behind a bacterial antibiotic-resistance gene (Boynton and Gillham 1993; Fischer et al. 1996; Odom et al. 2001; Chen and Melis 2013; reviewed in Purton et al. 2013). Besides their greater ability to recycle, algal strains producing the Bti proteins would be more effective than Bti in organic-rich and/or polluted waters, because the toxin will be intracellular. Moreover, they should be cheaper to produce, since they would not require processing, and could be grown locally anywhere.

The Bti toxin is a crystal-like inclusion composed of 4 main proteins - three Cry proteins (Cry4Aa, Cry4Ba, Cry11Aa) and Cyt1Aa - that act synergistically to destroy the gut membrane following ingestion by the larvae. Each of the individual proteins have activity, but the Cry proteins are considerably more toxic than Cyt1Aa; however, Cyt1Aa synergizes the Cry proteins and blocks the development of strong resistance. The Cry proteins differ considerably in their toxicity toward different mosquito genera; e.g., Cry4Ba is highly toxic to *Anopheles*, whereas Cry11Aa and Cry4Aa are very active against *Aedes* and *Culex*. These differences largely reflect the specific binding properties of the Cry proteins to receptors in the larval gut membrane (reviewed in Ben-Dov 2014).

The Bti toxin genes can be quite challenging to express in other organisms, as the Cry proteins are all large (ranging from 75 to 130 kDa) and can be toxic to host cells at high levels, even though they are considered to be protoxins in the bacterium. Hence, as a first step, we have used an inducible expression system (Surzycki et al. 2007) to produce the three Cry proteins in the chloroplast of *Chlamydomonas*. The inducible system relies on a metal (Cu^{2+}) repressible protein that goes into the chloroplast and activates the introduced cry gene via its 5' UTR, which is from the *psbD* gene. With this system, we could express all 3 Cry proteins from Bti in the chloroplast, and we demonstrated larvicidal activity for the Cry11Aa and Cry4Aa-producing strains.

Materials and methods

Chlamydomonas strains, growth, and chlorophyll determination

The Ind41_18 strain of *C. reinhardtii* was obtained from Jean-David Rochaix (University of Geneva, Switzerland). The cultures were maintained on agar plates of Tris-acetate-phosphate medium (TAP) (Gorman and Levine 1965) in light at 23°C. Since TAP contains copper it was used as the +Cu²⁺ repression medium (TAP + Cu²⁺), while the inducing medium (TAP – Cu²⁺) was made by removing copper sulfate from the Hutner's trace solution. The flasks and graduate cylinders (glass) used to make the – Cu²⁺ medium were washed sequentially with 6 N HCl, dH₂O (7×), and MilliQ-water (3×) before use (Quinn and Merchant 1998).

Liquid cultures were in flasks that were ~40% full and mixed continuously on an orbital shaker at 125 rpm. Cell counts were made with a hemocytometer after killing the cells with iodine, or by estimating total chlorophyll (below) and using the reference value of 4 mg chlorophyll per 1 × 10⁹ cells (Harris 1989). Culture wet weight were measured in some cases after centrifuging cell cultures in a 50 mL tube, and weighing of the cell pellets.

Growth rates of the Cry transformants and the parental Ind41_18 strain under uninduced and induced conditions were determined in liquid medium. First, agar cultures were used to inoculate flasks of TAP medium, and the cells were grown to near-stationary phase (4 × 10⁶ cells mL⁻¹). These cultures were used to inoculate, in parallel, 50-ml cultures of TAP and TAP – Cu²⁺ at 5 × 10⁴ cells mL⁻¹, and grown as described above. Aliquots were removed every 12 h for determination of total chlorophyll.

Total chlorophyll was measured as described by Wintermans and De Mots (1965). Culture aliquots (1 mL) were centrifuged in a microfuge for 5 min, and the resulting cell pellet was extracted with 1 mL of 95% EtOH and mixing. After recentrifugation (2 min), the supernatant was removed and its absorbance at 665 and 649 nm was measured and used to calculate total chlorophyll.

Design and preparation of synthetic cry genes

We elected to express the *cry4Ba* and *cry11Aa* genes as full-length and to truncate *cry4Aa* after amino acid 700 (*cry4A*₇₀₀), since studies showed that the toxicity resided in the first 700 amino acids (Yoshida et al. 1989). The FLAG peptide DYKDDDDK was added to the C-terminus of each protein. The sequences of the native Bti genes (gene IDs: *cry4Aa* - 5759905, *cry4Ba* - 5759934, *cry11Aa* - 5759849) were optimized with the program Optimizer (Puigbò et al., 2007) using a custom codon-usage table. After analyzing the predicted RNA structures at the 5' end using Mfold, the third codon in *cry4Ba* was changed from AAC to CAA, which changed the amino acid from asparagine (N) to glutamine (Q); this was done to prevent the start codon from being in a predicted paired region. The genes were synthesized commercially (Integrated DNA Technologies) and we obtained them as cloned plasmids.

Construction of Cry plasmids

The plasmids were carried in *E. coli* host strain DH5 α (Invitrogen), and were assembled in the low-copy pET-16b vector. The Cry genes were excised by digesting with *Xba*I (on the 3' side), blunting with Klenow DNA polymerase, and then digesting with *Nde*I (on the 5' side). To accept *cry4Aa*₇₀₀ and *cry11Aa*, pET-16b was cut with *Xho*I, blunted with Klenow, and then cut with *Nde*I. However, the *Nde*I digestion was incomplete, 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 *Nde*I site. For *cry4Ba*, pET-16B was digested with *Bam*HI before blunting and digestion with *Nde*I, so the *cry4Ba* clone did not have extra nucleotides at the 5' end. The new plasmids were pET-4A₇₀₀, 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 (New England Biolabs) according to the manufacturer's instructions. The thermocycling program was as follows: 94°C (3 min); 33 cycles of 52°C (1 min), 72°C (3.5 min), and 94°C (30 sec); 52°C (1 min); and then 72°C (5 min). And the PCR products were analyzed on 1% agarose gels before restriction digestion and cloning.

The 5' region from *psbD*, including the promoter and 5' UTR, was amplified from plasmid p108-14 (Surzycki et al. 2007), obtained from Jean-David Rochaix (University of Geneva). The forward primer (847 in Table 1) contained overlapping *Nco*I and *Bam*HI sites; the *Nco*I site was used to attach it to the coding regions (as a *Nco*I-*Nde*I fragment) and the *Bam*HI site was used later to excise the whole gene. The reverse primer (850 in Table 1) contained the *Nde*I site, but also altered a possible Shine-Dalgarno 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 *Nco*I and *Nde*I and cloned into the *Nco*I+*Nde*I-digested pET-Cry plasmids (above); the new plasmids were named pET-5D4A₇₀₀, pET-5D4B, and pET-5D11A. The *psbA* 3' region was amplified from plasmid P-322 (Newman et al. 1992) with primers 860 and 861; both primers contained a *Blp*I site and the reverse primer (861) contained a *Bam*HI site for subcloning the whole construct into a chloroplast transformation plasmid (below). The PCR product was cut with *Blp*I and cloned into *Bpu*1102I-cut pET-5D4A₇₀₀, pET-5D4B, and pET-5D11A, where it attaches in only one direction. This added ~50 nucleotides of vector sequence between the coding region and the 3' UTR-proper from *psbA*. The *psbD_m*-Cry-*psbA* constructs were confirmed by sequencing of the new plasmids (pET-5D4A₇₀₀3A, pET-5D4B3A, and pET-5D11A3A). To create the chloroplast transformation plasmids, the Cry gene constructs were excised with *Bam*HI and cloned into the *Bam*HI site of p322.1, creating pCry4A₇₀₀, pCry4B, and pCry11A.

Chloroplast transformation

The Ind41_18 strain was grown in TAP under a light flux of ~40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ until the culture reached $2 - 4 \times 10^6$ cells mL^{-1} . The cells were harvested by centrifugation and then biolistically transformed with the Cry plasmids as described previously (Odom et al. 2001) using co-transformation with plasmid pB4CC110; pB4CC110 contains the spectinomycin-resistance marker, *spr-u-1-6-2*, in the 16S *rnn* gene (Newman et al. 1990).

Typically, 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 particles containing ~ 600 ng of DNA was shot at each plate of cells embedded in 0.25% soft agar (Boynton and Gillham, 1993). The bombarded plates were incubated overnight in dim light ($\sim 2 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$), then the cell layer was scraped off and split onto two TAP-agar plates containing $100 \mu\text{g mL}^{-1}$ spectinomycin. These selection plates were incubated under bright light ($\sim 40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) 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 $300 \mu\text{g mL}^{-1}$ spectinomycin until they reached homoplasmy as judged by PCR.

Transformants for PCR analysis were grown on TAP-agar plates with $300 \mu\text{g mL}^{-1}$ spectinomycin, and total DNA was extracted as described by Kwon et al. (2014). To check for homoplasmy, primers 864 and 865 (Table 1), which amplify the site of integration site in chloroplast DNA (CpDNA) were used; homoplasmy was indicated by the absence of untransformed copies of the genome. PCR with gene-specific primers was also performed using the following oligos: 795 and 796 for *cry4Aa700*, 797 and 798 for *cry4Ba*, and 799 and 800 *cry11Aa* (Table 1). Standard PCR procedures with *Taq* DNA polymerase (NEB) and the manufacturer's buffer were used. The thermocycle program was: 94°C (3 min); 33 cycles of 52°C (1 min), 72°C (3.5 min), and 94°C (30 sec); 52°C (1 min); and 72°C (5 min).

Protein extraction, measurement, and western blotting

For the extraction of total cellular protein, 50 mL of culture was pelleted by centrifugation at $900 \times g$ for 10 min at RT, and resuspended in 1 mL of 100 mM Tris-HCl pH 8.5, 100 mM DTT, 7 mM benzimidazole, and 5 mM EDTA pH 8.0. Then, 0.6 mL was added to 0.4 mL of LDS solution (5% lithium dodecylsulfate, 30% sucrose, 0.025% bromophenol blue), and the lysates were stored at -70°C . Protein was determined with the Bradford reagent (Bio-Rad) on the remaining 0.4 mL of cells, after removing the chlorophyll. The cells were pelleted by centrifugation at $14000 \times g$ for 5 min (RT), and then resuspended in 90% acetone. After extraction for 2-3 min, the tubes were recentrifuged, and the protein pellet was resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA plus 1% SDS, by heating at 60°C for 3 min. Protein was measured with the Bradford assay (Bio-Rad) using IgG for the standard curve.

For SDS-PAGE, the solubilized cells and purified Cry11A (from *E. coli*) in 17 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 0.1 M DTT were heated to 100°C for 3 min. A prestained protein ladder (PageRuler, Fermentas) was used as size markers, and the samples were separated at RT in 10% acrylamide SDS-PAGE gels (Laemmli 1970). After electrophoresis, the gels were soaked in cold transfer buffer (25 mM Tris base, 192 mM glycine, 5% methanol) for 15 min and then electrotransferred to a PVDF membrane (Hybond-P, GE HealthCare) for 1.5 h at 4°C (Memon et al. 1993). The blots were stained with ponceau S to check for even protein transfer, blocked for 1 h with 5% nonfat dried milk in TBS-T (20 mM Tris-HCl pH 7.6, 150 mM NaCl plus 0.05% Tween 20), and washed $2\times$ with TBS-T for 5 min each (with shaking). The blots were incubated for 1 h at RT with an anti-FLAG monoclonal antibody (M2) coupled to alkaline phosphatase (Sigma A9469) that was diluted 1:4,000 in TBS-T. The blots were washed $6\times$ (for 5 min each) by agitation with TBS-T. The bound antibodies were detected using a chemiluminescent reaction with Lumi-

Phos WB (Thermo Scientific) or CDP-Star (Invitrogen) substrates as described by the manufacturer. The chemiluminescence was detected by exposing the blots to X-Ray film and/or detection with a Fluorchem Q imager (Protein Simple), and images were quantified with Image J (NIH).

Production and purification of a Cry11Aa standard for quantitative western blots

The codon-adapted *cry11Aa* gene was also expressed in *E. coli* and purified by virtue of an N-terminal His tag. Plasmid pET-11A_{ca} (above) was transformed into *E. coli* Rosetta (DE3) carrying pLysS/RARE (Novagen), and the transformants selected on carbenicillin (200 µg mL⁻¹) and chloramphenicol (34 µg mL⁻¹). For expression, a transformant was grown in LB plus the same antibiotics at 37°C and induced with IPTG (1 mM) for 3 h. The cells were pelleted, washed with cold 10 mM Tris-HCl pH 7.4, 150 mM NaCl and frozen at -70°C. After thawing, the cell pellet was resuspended in 50 mM NaH₂PO₄ pH 8, 0.3 M NaCl plus Triton X-100 (0.1%), and sonicated in 1-mL aliquots (on ice) using twenty 30-s pulses. The lysate was centrifuged at 10,000 × g for 30 min (4°C), the supernatant was removed and the pellet was saved. Cry11Aa was purified from the pellet (after solubilization with 8 M urea) on a Ni²⁺ affinity column (PrepEase, USB) under denaturing conditions as described by the manufacturer. Analysis by SDS-PAGE showed the protein to be at least 95% pure, and the concentration was estimated by SDS-PAGE and the Bradford assay (Bio-Rad) using BSA as standard.

RT-PCR

Total nucleic acids (TNA) were extracted as described previously (Kwon et al. 2014) from cultures (50 mL) grown in +Cu²⁺ and -Cu²⁺ medium (in the light), and 10 µg of each was treated with Turbo DNase (Ambion) in total volume of 55 µL. Subsequently, 4 µL aliquots were used for reverse transcription with Superscript III (Invitrogen) and internal reverse primers (796 for *cry4Aa700* and 800 for *cry11Aa*) in a total volume of 20 µL at 65°C (for 5 min). Then, 1 µL aliquots of the cDNA products were used in PCR reactions (25 µL total volume) with *Taq* DNA polymerase and primer sets specific for *cry4Aa700* (795/796, 1001/796, 795/1003) and *cry11Aa* (799/800, 1002/800, 799/1004) (Table 1). The thermocycle program was similar to that described above for analyzing transformants, except the number of cycles was varied so as to evaluate product appearance with time.

Larval bioassays

Bioassays with mosquito larvae followed guidelines of the World Health Organization (2005) with some modifications. The *cry4Aa700* and *cry11Aa* transformants were grown under inducing and noninducing conditions, and Ind41_18 was grown under inducing conditions (described above) until they reached stationary phase. The algal cells were harvested by centrifugation (1000 × g), washed with dH₂O, recentrifuged, and resuspended in dH₂O. The bioassays were performed in triplicate at 27°C under 12 h:12 h light/dark cycles with 10 live mosquito larvae in 5 mL of dH₂O plus/minus *Chlamydomonas* cells; dH₂O was used so the algae would not grow during the assay. The larvae, 3rd instar *Culex quinquefasciatus* (*C. quinquefasciatus*) and 4th instar *Aedes aegypti* (*A. aegypti*), were transferred to dH₂O the day before the assay and starved overnight; larval deaths were counted visually after 24, 48, and 72 h with the live algae. For the determination of the

median lethal concentration (LC₅₀) of the *cry11Aa* transformant against 4th-instar *A. aegypti* larvae, 10 different concentrations of induced-*cry11Aa* cells were used (in triplicate) in the bioassay, and the LC₅₀ was calculated with Microsoft Excel using Probit analysis (Finney 1971).

Results

The Cry genes

Early on, we attempted to express the native *cry11Aa* gene in the chloroplast and no protein was detected with an anti-*Cry11Aa* antibody (unpublished). Also, Juntadech et al. (2012) expressed a *cry4Ba* gene in the *Chlamydomonas* chloroplast with a similar result. Since codon bias is one of the limiting factors for expressing foreign genes in the *Chlamydomonas* chloroplast (Franklin et al. 2002; Mayfield et al. 2003), codon-optimized genes for the 3 Cry proteins of Bti were synthesized. Also, since there is a negative correlation between protein size and expression level (Raghava and Han 2005), *cry4Aa* (which is 1180 amino acids) was C-terminally truncated, containing only amino acids 1-700; the 3 essential domains (I-III) found in many Cry proteins are contained in this region (Yoshida et al. 1989). Although the Cry4Ba protein is nearly as gigantic as Cry4Aa, we did not truncate it, in part to find out if the chloroplast could produce a foreign protein of that size. The 8-amino acid FLAG tag (Einhauser and Jungbauer 2001) was added to the C-terminus of all three proteins; it was expected to have little effect on toxicity as both ends of Cry protoxins are cleaved in the gut. Before attempting chloroplast expression, we 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).

Using the inducible expression system

Initially, we tried to express the Cry genes using a high-expression system developed by Steve Mayfield and colleagues (Rasala et al. 2010), where the *psbA* gene's coding region is replaced with the transgene coding region. However, the transformants remained heteroplasmic, indicative of host cell toxicity. Although the knock-out of photosynthesis that accompanies this expression system could have been a major part of the problem, we decided to use an inducible system (Surzycki et al. 2007) to express these genes in the chloroplast.

To use the copper-repressible system (Surzycki et al. 2007), a modified *psbD* 5' region was added to the cry genes, to enable control by the nuclear *Cyc6:NAC2* gene, and the 3' region from *psbA* was added to stabilize the mRNA. The *psbD_m:Cry:psbA* gene constructs were cloned into an intergenic site in p322.1 (Fig 2), which when co-transformed into Ind41_18 with pB4C110 (which confers spectinomycin resistance) places the Cry gene in the inverted repeat region of CpDNA. Spectinomycin-resistant colonies were restreaked on spectinomycin plates several times prior to analysis of their CpDNA by PCR (Fig. 3). Primer pair 864/865 amplifies the integration site in CpDNA and was used to judge the homoplasmy of transformants: a 100-bp product with these primers is indicative of genome copies that have not been transformed, whereas the large product is from copies that have been transformed; thus, the absence of the small product indicates homoplasmy.

Also, internal primer pairs were used to verify the presence of the specific cry gene and in every case they gave the correct-size product (data not shown). The results indicate that: one of the two *cry4Aa*₇₀₀ transformants (Fig. 3a) was homoplasmic (4A-2), while the other (4A-5) still had some untransformed copies; the *cry4Ba* transformant (Fig. 3b) was homoplasmic; and one of the two *Cry11Aa* transformants (Fig. 3c) was homoplasmic (11A-8), while the other (11A-6) still had some untransformed CpDNA copies. Thus, we obtained at least one homoplasmic transformant for each cry gene. It should also be noted that the wild-type (WT) CpDNA that was used as the control DNA in Fig. 3, is the same as Ind41_18 in that region of the genome.

Analysis of Cry protein accumulation

Accumulation of the Cry proteins in the transformants grown with (U, Uninduced) and without (I, Induced) Cu²⁺ was assessed using western blotting of total cell protein with an anti-FLAG antibody (Fig. 4). Since both *cry4Aa*₇₀₀ transformants (Fig. 3a) gave similar results, as did both *cry11Aa* transformants (Fig. 3b), only results with the homoplasmic transformants are shown in Fig. 4. A protein of the predicted size (74 kDa for Cry4Aa₇₀₀, 130 kDa for Cry4Ba, and 73 kDa for Cry11Aa) was induced, or increased under inducing (I) conditions (– Cu²⁺), for each of the Cry gene transformants. In Fig. 4a,b, the same western blot that was exposed to X-Ray film for the image in Fig. 4a, was then imaged with a CCD-based imager in Fig. 4b; the film was more sensitive and facilitated the visualization of Cry4Ba, whereas the imager had a greater dynamic range and gave a more accurate Cry11Aa signal. Cry11Aa accumulation was significantly greater than Cry4Aa₇₀₀ and much greater (at least 5-fold) than Cry4Ba. It should be noted that because very large proteins like Cry4Ba do not transfer efficiently during blotting, its accumulation could be underestimated as much as 2-fold. The figure also shows that there was significant accumulation of Cry11Aa even without induction, although the protein level did increase ~2.5 fold with induction.

To obtain an absolute measure of Cry11Aa accumulation, we used the FLAG-tagged Cry11Aa protein purified from an *E. coli* culture as a standard, and quantitative western blotting (Fig. 4c). From 3 of these blots we estimated that Cry11Aa was accumulating to 0.33% ± 0.04% of total protein, or 90 µg g⁻¹ wet weight of cells.

RT-PCR analysis of *Cry4Aa*₇₀₀ and *Cry11Aa* gene expression

Although Cry4Aa₇₀₀ and Cry11Aa are similar size proteins and the genes had the same expression signals and chromosomal location, there were significant differences in protein accumulation and induction (Fig. 4). So, we decided to examine the mRNA expression by RT-PCR, by using primers that amplified either most of the mRNA (Fig. 5a), as a measure of mRNA intactness, or primers that gave short products (Fig. 5b), which can be more quantitative. Both analyses indicated that the levels of *cry11Aa* mRNA (11A in Fig. 5a,b) were higher than *cry4Aa*₇₀₀ mRNA, which is consistent with the protein findings. The data also shows that both mRNAs were present in the uninduced cells, as were the proteins (Fig. 3), suggesting that the accumulation and translation of these mRNAs is not totally dependent on the NAC2 protein (which is repressed by Cu²⁺). The results also indicate an incongruous correlation between *cry11Aa* mRNA and Cry11Aa protein, with the mRNA induction being

stronger than the protein induction. Taken together with the significant level of Cry11Aa protein in the uninduced cells with low mRNA, the data suggests that *cry11Aa* mRNA is translated more efficiently at low mRNA levels, and less efficiently at higher mRNA levels after induction.

Effect of Cry gene expression on *Chlamydomonas* growth rates

As a test of possible toxicity of the Cry proteins to the host *Chlamydomonas* cells, the growth rates of the transformants (and parental strain) under induced ($-Cu^{2+}$) and uninduced ($+Cu^{2+}$) conditions were examined. Fig. 6 shows that the growth curves obtained under both conditions were quite similar for the *cry4Aa700*, *cry4Ba*, and *cry11Aa* transformants, suggesting that the proteins are not very toxic to the Ind41_18 cells when expressed under these conditions.

Larvicidal activity of the Cry transformants

To determine whether the algae were larvicidal, live cell bioassays with larvae of *Culex quinquefasciatus* (3rd instar stage) and *Aedes aegypti* (4th instar stage) were performed. The bioassay was performed in dH₂O to prevent the algae from growing during the assay period. Preliminary tests showed that larvae raised on the parental strain (Ind41_18) 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). Cry4Ba is known to have low toxicity against *Culex spp.* (Angsuthanasombat et al. 1992; Delécluse et al. 1993), and since initial tests of this transformant also showed low toxicity against *A. aegypti*, it was not pursued further. However, as Fig. 7 shows, both the *cry4Aa700* (4A) and *cry11Aa* (11A) transformants were lethal to *C. quinquefasciatus* (Fig. 7a) and *A. aegypti* (Fig. 7b) larvae, with *cry11Aa* exhibiting ~3.5-fold greater toxicity against *C. quinquefasciatus* and ~6-fold greater toxicity than *cry4Aa700* against *A. aegypti* at 1×10^6 cells mL⁻¹ (or 1×). The effect of induced *cry4Aa700* cells on *C. quinquefasciatus* also showed clear evidence of toxicity inhibition at high algal cell numbers (see 4A-I series in Fig. 7a), an effect that was not seen with *A. aegypti* larvae over the same range (compare to the 4A-I series in Fig. 7b). Although not clearly understood, the decreased toxicity of *cry4Aa700* against *C. quinquefasciatus* at high cell numbers may be analogous to the reduced toxicity of Bti when there is excess food or non-food particles (Ben-Dov et al. 2003). The relatively low lethality of the uninduced *cry11Aa* cells (11A-U) at the 10× cell number against *A. aegypti* (Fig. 7b) may result from the same effect, but it seems to be overcome if there is sufficient Cry11Aa protoxin in the cells, because the 10× number of induced cells (11A-I) was highly lethal. Finally, the high lethality of *C. quinquefasciatus* in the dH₂O-alone treatment, compared to *A. aegypti* (Water in Fig. 7a, b), is consistent with the former's known susceptibility to starvation.

To estimate LC₅₀ 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 LC₅₀ was found to be 3.3×10^5 cells mL⁻¹. Given that the Cry11Aa content of the cells is ~65 ng per 10⁶ cells we can infer an LC₅₀ for *in vivo* Cry11Aa as ~22 ng mL⁻¹.

Discussion

We have demonstrated that it is possible to produce, individually, the three Cry proteins from Bti (Cry4Aa, Cry4Ba and Cry11Aa) in the chloroplast of *Chlamydomonas*, and for Cry11Aa and Cry4Aa₇₀₀, at high enough levels to kill mosquito larvae. Cry11Aa was produced at the highest level among the three, which is fortuitous because it is also the most toxic to *Aedes* and *Culex* larvae (Frankenhuyzen, 2009). Using purified Cry11Aa and quantitative westerns, the Cry11Aa level in the algal cells was determined to be 0.35% of total cell protein, or 90 $\mu\text{g g}^{-1}$ wet weight of cells. That level compares favorably to the Cry protein levels in insect-resistant transgenic plants (e.g., Wang et al. 2014). Moreover, the toxicity of this (*cry11Aa*) transformant to *A. aegypti* larvae (LC_{50} was 3.3×10^{-5} cells mL^{-1}) was nearly as high (in cells mL^{-1}) as transgenic *Anabaena* expressing *cry11Aa* and *cry4Aa* (Wu et al. 1999), further supporting the conclusion of high-level expression of *cry11Aa* in *Chlamydomonas*. Although these are not the strains that one would actually use for mosquito control, they did seem to be stable genetically (based on DNA and protein analysis) for at least 1 year.

It should be noted that this is the first report of successful expression of Cry proteins from Bti in a eukaryotic alga or organelle (chloroplast). A previously published attempt to express a Cry4Ba variant in *Chlamydomonas* that was not codon-adapted resulted in mRNA but no detectable protein (Juntadech et al. 2012). Even with the codon-adapted gene, however, we obtained low levels of Cry4Ba protein and low toxicity against *A. aegypti*; we did not evaluate mRNA levels for the Cry4Ba transformant. Low levels of Cry4Ba protein were also observed in transgenic cyanobacteria, even with strong promoters (Angsuthanasombat and Panyim, 1989; Soltés-Rak et al., 1995; Khasdan et al., 2003). Hence, further work will be necessary to achieve high-level expression of cry4Ba, which would be useful in controlling *Anopheles spp* mosquitoes.

Cry4Aa, like Cry4Ba, is a very large protein (135 kDa), and given the negative correlation between size and protein expression levels (Raghava and Han 2005), we truncated the *cry4Aa* gene to create *cry4Aa*₇₀₀. Although *cry4Aa*₇₀₀ expression was lower than *cry11Aa*, it was at least 0.1% of total protein, and the cells were toxic to *A. aegypti*. Since we did not try expressing full-length *cry4Aa*, we cannot be certain that truncating the protein to 74 kDa increased protein levels, but it seems likely that it did. Truncating Cry1 proteins, which are also quite large, increased their expression and toxicity in higher plants (reviewed in Deist et al. 2014). Hence, truncating *cry4Ba* may improve its expression, and an attempt to do that is underway in the laboratory.

The RT-PCR analysis of the *cry4Aa*₇₀₀ and *cry11Aa* strains suggests that mRNA levels play a role in the corresponding protein levels, but the relationship is certainly not simple; this was evident in the case of *cry11Aa*, whose mRNA seemed to be translated more efficiently at lower levels prior to full induction. Nonetheless, the results suggest that it may be possible to increase the expression of these proteins further by using a stronger promoter than *psbD*, such as that from 16S rRNA. The problem that one can run into, however, is toxicity to *E. coli* if the expression level is too high in the bacterium, which it can be with some chloroplast gene expression signals. Thus, it will be important to minimize translation in *E. coli* in order to make constructs with the 16S rRNA promoter.

The growth rates of the cry transformants under induced and uninduced conditions were very similar, indicating that the Cry proteins, including Cry11Aa, were not toxic to the Ind41_18 host strain. This result was somewhat surprising, since our initial attempt to engineer very high level expression by replacing the *psbA* gene (Rasala et al. 2010) was indicative of toxicity problems. Perhaps there is a threshold for toxic effects of cry genes in the chloroplast that we did not reach with these expression levels. Alternatively, there are differences among *Chlamydomonas* host strains in their sensitivity to these proteins. Further work will be needed to sort out that particular aspect of Cry gene expression in the chloroplast of *Chlamydomonas*.

Finally, we would like to make a cautionary statement about quantitating tagged proteins using an antibody against the tag. In a number of papers describing transgenic tagged-protein expression, researchers have used a heterologous tagged protein as a quantitation standard for western blots (e.g., Surzycki et al. 2009; Zedler et al. 2014; Braun-Galleani et al. 2015). We tried that approach also, at first, by using a FLAG-tagged BAP (bacterial alkaline phosphatase) from a commercial source as the standard and obtained values for Cry11Aa that were 10-fold higher, ~3% of total protein (unpublished results). However, that would be an extraordinary level of Cry protoxin for a growing organism. Moreover, it probably should have produced a visible change in the Coomassie stained gel pattern, but that was not observed. Thus, we believe that our quantitation using the homologous tagged protein prepared by us, is the more accurate estimate. Although it may be fine in some, or even most cases it is possible that the binding of the antibody could be affected by the protein that is fused to the tag. Thus, we suggest that caution is warranted, especially when the values obtained using the heterologous standard are very high.

Acknowledgments

We are grateful to Dr. Jean-David Rochaix for providing the Ind41_18 strain, and BEI Resources (NIAID) for *Culex quinquefasciatus*. This work was supported by the Bill and Melinda Gates Foundation (grant OPP1024635 to DLH) and the National Institutes of Health (grant R41-AI115854A to DLH).

References

- Angsuthanasombat C, Panyim S. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl Environ Microbiol.* 1989; 55:2428–2430. [PubMed: 2552921]
- Angsuthanasombat C, Crickmore N, Ellar DJ. Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. *FEMS Microbiol Lett.* 1992; 94:63–68.
- de Barjac, H., Sutherland, DJ. Bacterial control of mosquitoes and black flies: Biochemistry, genetics, & applications of *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. Rutgers University Press; New Brunswick, NJ: 1990.
- Ben-Dov E, Saxena D, Wang Q, Manasherob R, Boussiba S, Zaritsky A. Ingested particles reduce susceptibility of insect larvae to *Bacillus thuringiensis*. *J Appl Entomol.* 2003; 127:146–152.
- Ben-Dov E. *Bacillus thuringiensis* subsp. *israelensis* and its Dipteran-specific toxins. *Toxins.* 2014; 6:1222–1243. [PubMed: 24686769]
- Borovsky D, Sterner A, Powell CA. Cloning and expressing trypsin modulating oostatic factor in *Chlorella desiccata* to control mosquito larvae. *Arch Insect Biochem Physiol.* 2016; 91:17–36. [PubMed: 26440910]

- Boynton, JE., Gillham, NW. Chloroplast transformation in *Chlamydomonas*. In: Ray, W., editor. Meth Enzymol. Vol. 217. Academic Press; New York: 1993. p. 510-536.
- Bravo A, Likitvivanavong S, Gill SS, Soberon M. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem Mol Biol. 2011; 41:423–431. [PubMed: 21376122]
- Braun-Galleani S, Baganz F, Purton S. Improving recombinant protein production in the *Chlamydomonas reinhardtii* chloroplast using vivid Verde Fluorescent Protein as a reporter. Biotechnol J. 2015; 10:1289–1297. [PubMed: 26098300]
- Chen HC, Melis A. Marker-free genetic engineering of the chloroplast in the green microalga *Chlamydomonas reinhardtii*. Plant Biotechnol J. 2013; 11:818–828. [PubMed: 23647698]
- Deist B, Rausch M, Fernandez-Luna M, Adang M, Bonning B. Bt toxin modification for enhanced efficacy. Toxins. 2014; 6:3005. [PubMed: 25340556]
- Delécluse A, Poncet S, Klier A, Rapoport G. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl Environ Microbiol. 1993; 59:3922–3927. [PubMed: 16349095]
- Einhauer A, Jungbauer A. The FLAG™ peptide, a versatile fusion tag for the purification of recombinant proteins. J Biochem Biophys Meth. 2001; 49:455–465. [PubMed: 11694294]
- Finney, DJ. Probit analysis. Cambridge University Press; Cambridge, U.K: 1977.
- Fischer N, Stampacchia O, Redding K, Rochaix JD. Selectable marker recycling in the chloroplast. Mol Gen Genet. 1996; 251:373–380. [PubMed: 8676881]
- Frankenhuyzen KV. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invert Pathol. 2009; 101:1–16.
- Franklin S, Ngo B, Efué E, Mayfield SP. Development of a GFP reporter gene for the *Chlamydomonas reinhardtii* chloroplast. Plant J. 2002; 30:733–744. [PubMed: 12061904]
- Glare, TR., O'Callaghan, M. *Bacillus thuringiensis*: Biology, Ecology and Safety. Wiley; West Sussex: 2000.
- Gorman DS, Levine RP. Cytochrome f and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA. 1965; 54(6):1665–1669. [PubMed: 4379719]
- Harris, EH. The *Chlamydomonas* sourcebook: A comprehensive guide to biology and laboratory use. Academic Press; San Diego, CA: 1989.
- Ibrahim MA, Griko NB, Bulla LA. The Cry4B toxin of *Bacillus thuringiensis* subsp. *israelensis* kills permethrin-resistant *Anopheles gambiae*, the principal vector of malaria. Exp Biol Med. 2013; 238:350–359.
- Juntadech T, Yokthon K, Tangphatsornruang S, Yap YK, Katzenmeier G, Angsuthanasombat C. Efficient transcription of the larvicidal *cry4Ba* gene from *Bacillus thuringiensis* in transgenic chloroplasts of the green alga *Chlamydomonas reinhardtii*. Adv Biosci Biotech. 2012; 3:362–369.
- Kamel F. Paths from pesticides to Parkinson's. Science. 2013; 341:722–723. [PubMed: 23950519]
- Kaufman MG, Wanja E, Maknoja S, Bayoh MN, Vulule JM, Walker ED. Importance of algal biomass to growth and development of *Anopheles gambiae* larvae. J Med Entomol. 2006; 43:669–676. [PubMed: 16892623]
- Khasdan V, Ben-Dov E, Manasherob R, Boussiba S, Zaritsky A. Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing toxin genes from *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol Lett. 2003; 227:189–195. [PubMed: 14592708]
- Kioulos I, Kampouraki A, Morou E, Skavdis G, Vontas J. Insecticide resistance status in the major West Nile virus vector *Culex pipiens* from Greece. Pest Manag Sci. 2014; 70:623–627. [PubMed: 23788440]
- Kouo SY, Chong CS, Vythilingam I, Ng LC, Lee CY. Pyrethroid resistance in *Aedes aegypti* larvae (Diptera: Culicidae) from Singapore. J Med Entomol. 2014; 51:170–181. [PubMed: 24605467]
- Kwon, T., Odom, OW., Qiu, W., Herrin, DL. PCR Analysis of chloroplast double-strand break (DSB) repair products induced by I-CreII in *Chlamydomonas* and *Arabidopsis*. In: Edgell, DR., editor. Homing Endonucleases. Vol. 1123. Humana Press; 2014. p. 77-86. Meth Mol Biol
- Kumar A, Wang S, Ou R, Samrakandi M, Beerntsen BT, Sayre RT. Development of an RNAi based microalgal larvicide to control mosquitoes. Malaria World J. 2013; 4(6):1–6.

- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
- Laird, M. The natural history of larval mosquito habitats. Academic Press; London: 1988.
- Liu YT, Sui MJ, Dar-Der JI, Wu IH, Chou CC, Chen CC. Protection from ultraviolet irradiation by melanin of mosquitocidal activity of *Bacillus thuringiensis* var. *israelensis*. *J Invert Pathol*. 1993; 62:131–136.
- Marten GG. Mosquito control by plankton management: the potential of indigestible green algae. *J Trop Med Hyg*. 1986; 89:213–222. [PubMed: 2879045]
- Mayfield S, Franklin S, Lerner R. Expression and assembly of a fully active antibody in algae. *Proc Natl Acad Sci USA*. 2003; 100:438–442. [PubMed: 12522151]
- Memon AR, Herrin DL, Thompson GA Jr. Intracellular translocation of a 28 kDa GTP-binding protein during osmotic shock-induced cell volume regulation in *Dunaliella salina*. *Biochim Biophys Acta*. 1993; 1179:11–22. [PubMed: 8399350]
- Morens DM, Fauci AS. Emerging infectious diseases: Threats to human health and global stability. *PLoS Pathog*. 2013; 9:e1003467. [PubMed: 23853589]
- Newman SM, Boynton JE, Gillham NW, Randolph-Anderson BL, Johnson AM, Harris EH. Transformation of chloroplast ribosomal RNA genes in *Chlamydomonas*: molecular and genetic characterization of integration events. *Genetics*. 1990; 126:875–888. [PubMed: 1981764]
- Newman SM, Harris EH, Johnson AM, Boynton JE, Gillham NW. Nonrandom distribution of chloroplast recombination events in *Chlamydomonas reinhardtii*: evidence for a hotspot and an adjacent cold region. *Genetics*. 1992; 132:413–429. [PubMed: 1358751]
- Nickelsen J, Fleischmann M, Boudreau E, Rahire M, Rochaix JD. Identification of *cis*-acting RNA leader elements required for chloroplast *psbD* gene expression in *Chlamydomonas*. *Plant Cell*. 1999; 11:957–970. [PubMed: 10330479]
- Ohana B, Margalit J, Barak Z. Fate of *Bacillus thuringiensis* subsp. *israelensis* under simulated field conditions. *Appl Environ Microbiol*. 1987; 53:828–831. [PubMed: 16347326]
- Odom OW, Holloway SP, Deshpande NN, Lee J, Herrin DL. Mobile self-splicing group I introns from the *psbA* gene of *Chlamydomonas reinhardtii*: Highly efficient homing of an exogenous intron containing its own promoter. *Mol Cell Biol*. 2001; 21:3472–3481. [PubMed: 11313473]
- Puigbò P, Guzmán E, Romeu A, Garcia-Vallvé S. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. *Nucl Acids Res*. 2007; 35:W126–W131. [PubMed: 17439967]
- Purton S, Szaub JB, Wannathong T, Young R, Economou CK. Genetic engineering of algal chloroplasts: Progress and prospects. *Russ J Plant Physiol*. 2013; 60:491–499.
- Quinn, JM., Merchant, S. Copper-responsive gene expression during adaptation to copper deficiency. In: Lee, M., editor. *Meth Enzymol*. Vol. 297. Academic Press; 1998. p. 263-279.
- Raghava GPS, Han JH. Correlation and prediction of gene expression level from amino acid and dipeptide composition of its protein. *BMC Bioinformatics*. 2005; 6:59. [PubMed: 15773999]
- Rasala BA, Muto M, Lee PA, Jager M, Cardoso RMF, Behnke CA, Kirk P, Hokanson CA, Crea R, Mendez M, Mayfield SP. Production of therapeutic proteins in algae, analysis of expression of seven human proteins in the chloroplast of *Chlamydomonas reinhardtii*. *Plant Biotechnol J*. 2010; 8:719–733. [PubMed: 20230484]
- Soltes-Rak E, Kushner D, Williams DD, Coleman J. Factors regulating *cryIVB* expression in the cyanobacterium *Synechococcus* PCC 7942. *Mol Gen Genet*. 1995; 246:301–308. [PubMed: 7531814]
- Stevens SE, Murphy RC, Lamoreaux WJ, Coons LB. A genetically engineered mosquitocidal cyanobacterium. *J Appl Phycol*. 1994; 6:187–197.
- Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. Use of T7 RNA polymerase to direct expression of cloned genes. *Meth Enzymol*. 1990; 185:60–89. [PubMed: 2199796]
- Surzycki R, Cournac L, Peltier G, Rochaix JD. Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *Proc Natl Acad Sci USA*. 2007; 104:17548–17553. [PubMed: 17951433]
- Surzycki R, Greenham K, Kitayama K, Dibal F, Wagner R, Rochaix JD, Ajam T, Surzycki S. Factors effecting expression of vaccines in microalgae. *Biologicals*. 2009; 37:133–138. [PubMed: 19467445]

- Tetreau G, Stalinski R, Kersusan D, Veyrenc S, David JP, Reynaud S, Després L. Decreased toxicity of *Bacillus thuringiensis* subsp. *israelensis* to mosquito larvae after contact with leaf litter. *Appl Environ Microbiol.* 2012; 78:5189–5195. [PubMed: 22610426]
- Wang Y, Zhang L, Li Y, Liu Y, Han L, Zhu Z, Wang F, Peng Y. Expression of CryIab protein in a marker-free transgenic Bt rice line and its efficacy in controlling a target pest, *Chilo suppressalis* (Lepidoptera: Crambidae). *Environ Entomol.* 2014; 43:528–536. [PubMed: 24495566]
- WHO. Guidelines for laboratory and field testing of mosquito larvicides. World Health Organization; Geneva, Switzerland: 2005.
- Wintermans JFGM, De Mots A. Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. *Biochim Biophys Acta.* 1965; 109:448–453. [PubMed: 5867546]
- Wu X, Vennison SJ, Huirong L, Ben-Dov E, Zaritsky A, Boussiba S. Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol.* 1997; 63:4971–4974. [PubMed: 9406420]
- Yoshida K, Matsushima YK, Sen HS, Komano T. Insecticidal activity of a peptide containing the 30th to 695th amino acid residues of the 130-kDa protein of *Bacillus thuringiensis* var. *israelensis*. *Agricul Biol Chem.* 1989; 53:2121–2127.
- Zaritsky A, Ben-Dov E, Borovsky D, Boussiba S, Einav M, Gindin G, Horowitz AR, Kolot M, Melnikov O, Mendel Z, Yagil E. Transgenic organisms expressing genes from *Bacillus thuringiensis* to combat insect pests. *Bioeng Bugs.* 2010; 1:341–344. [PubMed: 21326834]
- Zedler JA, Gangl D, Hamberger B, Purton S, Robinson C. Stable expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas reinhardtii*. *J Appl Phycol.* 2015; 27:2271–2277.

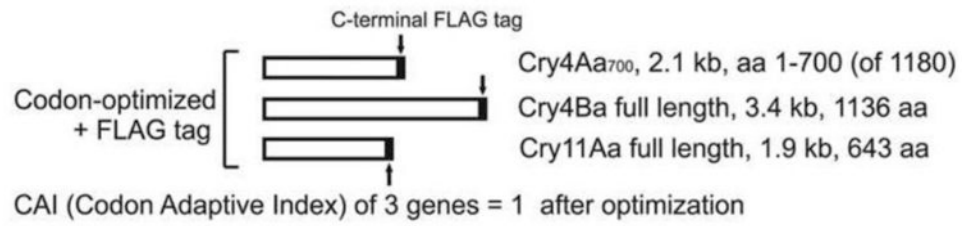


Fig. 1.

Synthetic codon-optimized cry genes, *cry4Aa₇₀₀*, *cry4Ba*, and *cry11Aa* were designed using the native 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. *Cry4Aa₇₀₀* is a truncated version of the native gene, containing only the first 700 amino acids of the 1180 total residues in the *Cry4Aa* protein, whereas *Cry4Ba* and *Cry11Aa* are full-length proteins. The 8-residue FLAG epitope was added to the C-terminus of all three genes for antibody detection

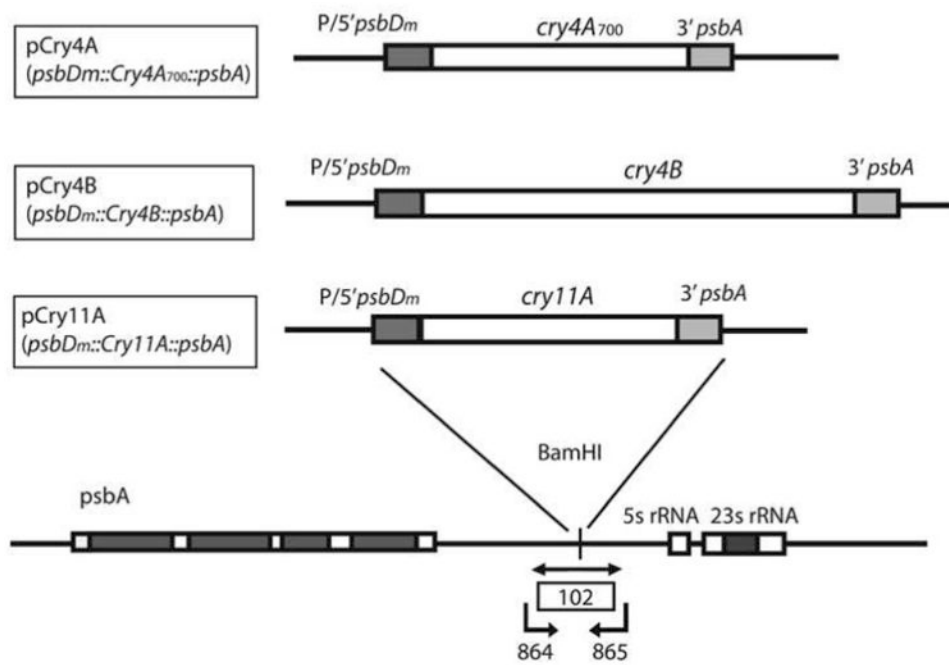


Fig. 2.

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 (see Fig 3) are indicated; note that primers 864 and 865, which give a product of 102 bp, are located upstream and downstream, respectively, of the integration site in CpDNA. Some parts of the diagram are not drawn to scale

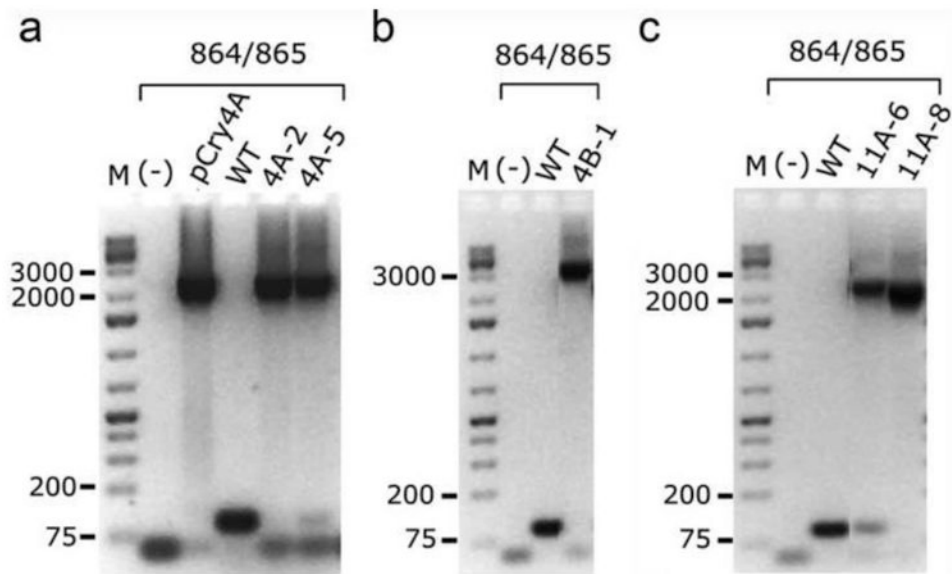


Fig. 3.

PCR analysis of chloroplast transformants. The locations of the primer pair 864/865 are shown in Fig. 2, and the fluorescence gel images were inverted. All of the lanes in the panels are PCR reactions except the size markers (M); these are given in bp. Wild-type (WT) DNA was used to visualize the size of the PCR product (with 864/865) from untransformed CpDNA as it is identical to Ind41_18 in this region. The reactions labeled (-) had no DNA added. **a** Analysis of two *cry4Aa*₇₀₀ transformants. pCry4A is the plasmid that was shot into the chloroplast, and serves as a product size marker. **b** Analysis of a *cry4Ba* transformant. **c** Analysis of two *cry11Aa* transformants. pCry11A is the plasmid that was shot into the cells, and provides a product size marker

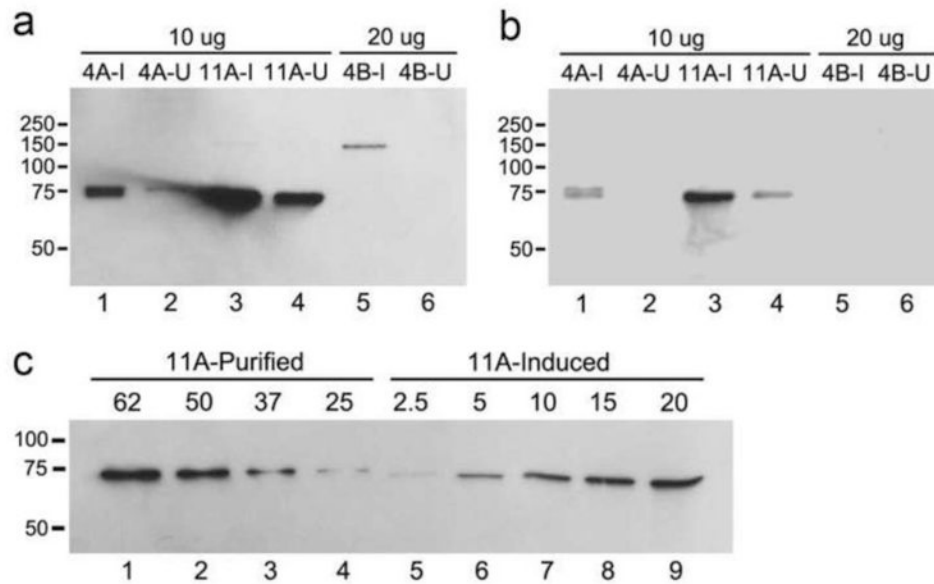


Fig. 4. Western blot analysis of Cry proteins in the transformants. The blots were probed with an anti-FLAG monoclonal antibody coupled to alkaline phosphatase. **a** and **b**, Analysis of Cry proteins in total cell protein, either 10 or 20 μg as indicated, from transformants for *cry4Aa*₇₀₀ (4A), *cry4Ba* (4B), and *cry11Aa* (11A) grown under uninducing (U) and inducing (I) conditions. **a** The blot was exposed to X-Ray film. **b** The same blot was imaged with a Fluorochem imager. **c** Quantitation of Cry11Aa in the *cry11Aa* transformant grown under inducing conditions. Lanes 1-4 contained the indicated amounts (in nanograms) of purified Cry11Aa expressed in *E. coli*, and lanes 5-9 contained the indicated amounts (in μg) of total cell protein from the *cry11Aa* transformant grown under inducing conditions (11A-Induced)

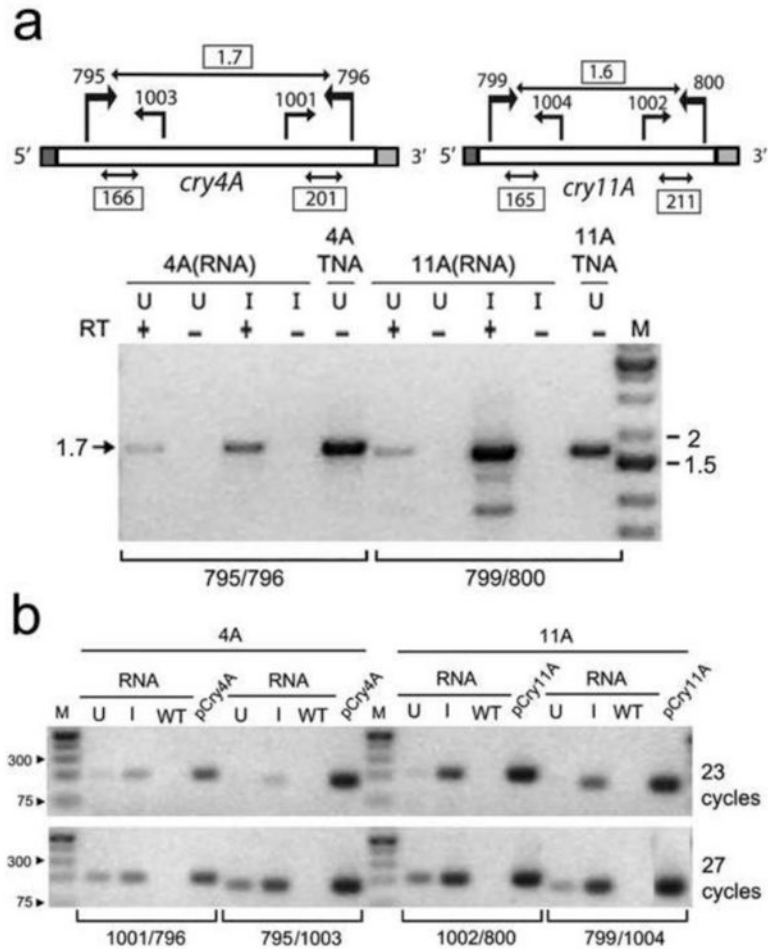


Fig. 5. RT-PCR analysis of the *cry4Aa*₇₀₀ (4A) and *cry11Aa* (11A) transformants. An equal amount of RNA from cultures grown for 72 hrs under uninduced (U) and induced (I) conditions was used for reverse transcription with gene-specific primers; 796 for *cry4Aa*₇₀₀ and 799 for *cry11Aa*. The resulting cDNAs were amplified using the indicated primer sets; the locations of the primers are shown in the diagrams at top. **a** RT-PCR with primers near the ends of the mRNAs. RT-PCR reactions without reverse transcriptase (RT) served as negative controls, and reactions with total nucleic acids (TNA), in place of RNA, served as a positive control for the PCR step. Lane M contained size markers, and the gel image was inverted. **b** RT-PCR with primers that give short products. The primer sets are indicated below the gel, whose image was inverted. The same RNAs from uninduced (U) and induced (I) cultures used in (a) were employed and the PCR products were visualized on the gel after 23 and 27 cycles, when the yield was still increasing. RNA from wild-type cells (WT) was used as a negative control, and a PCR reaction with the plasmid that was shot into the cells provided a size marker for the expected RT-PCR products

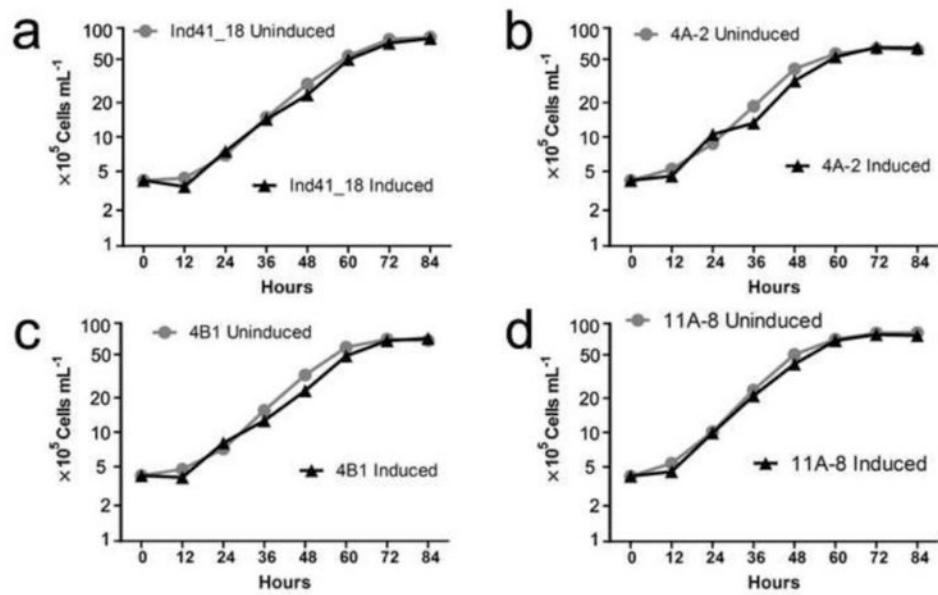


Fig. 6. Effect of inducing the Cry4A₇₀₀, Cry4B, and Cry11Aa proteins on the growth rate of the algal cells. The cells were grown under Uninduced (TAP + Cu²⁺) and Induced (TAP – Cu²⁺) conditions. **a** Ind41_18 parental strain. **b** *cry4Aa700* transformant 4A-2. **c** *cry4Ba* transformant 4B-1. **d** *cry11Aa* transformant 11A-8

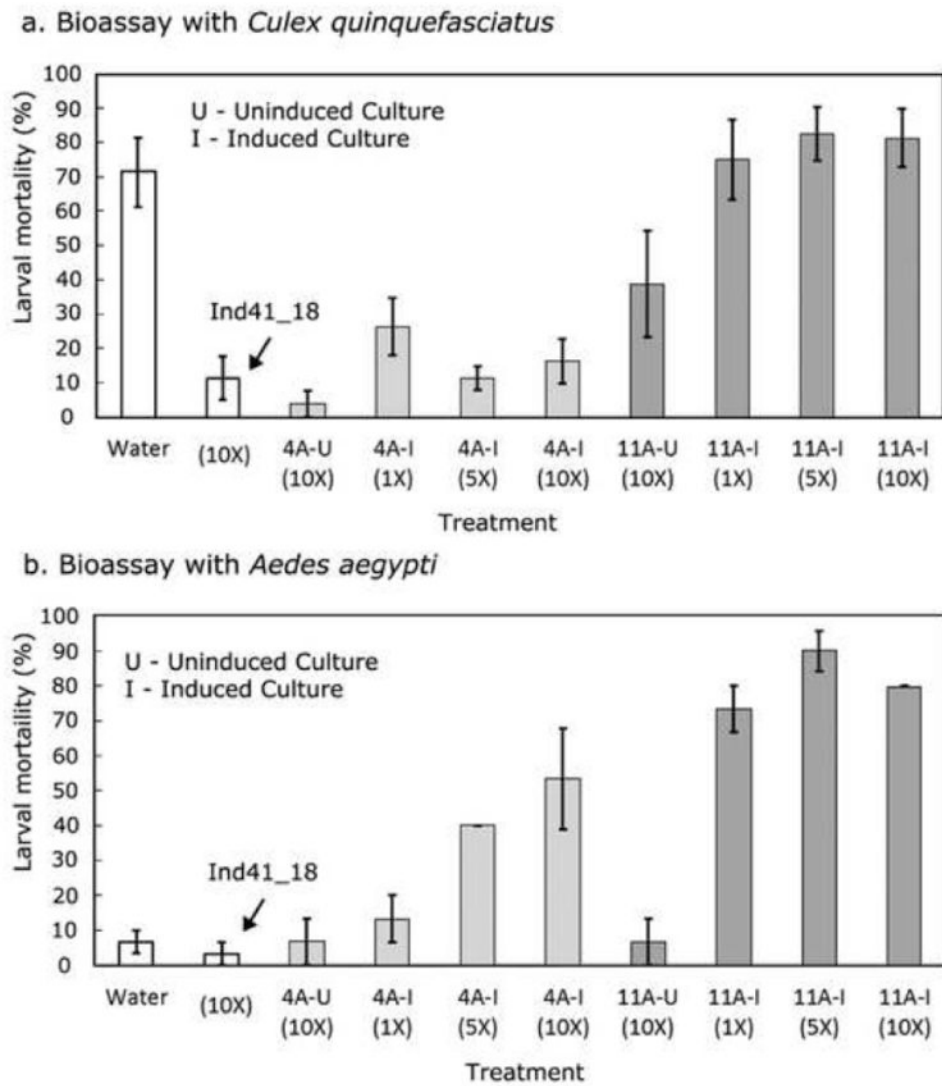


Fig. 7. Lethality of the *cry4Aa*₇₀₀ and *cry11Aa* transformants to *Culex quinquefasciatus* and *Aedes aegypti* larvae. The *cry4Aa*₇₀₀ (4A-2) and *cry11Aa* (11A-8) transformants were grown under uninduced (U) and induced (I) conditions, whereas the parental strain (Ind41_18) was grown only under inducing conditions, $1 \times 1 \times 10^6$ algal cells mL^{-1} . The assays were performed in dH_2O to prevent the algae from growing during the assay, and a dH_2O -only control (Water) was included. Larval mortality was checked every 24 hrs; the data are from 48 hrs. The assays were performed in triplicate with 10 larvae per test. **a** Bioassay with *Culex quinquefasciatus*. **b** Bioassay with *Aedes aegypti*

Table 1
Oligonucleotide sequences

ID no.	Name ^a	Sequence ^b
795	<i>cry4A</i> F	GTCAACAAAACCAACAATACG
796	<i>cry4A</i> R	TTAGTGTAGTCAGTACCTGAG
797	<i>cry4B</i> F	AACGACTTACAAGGTTCAATG
798	<i>cry4B</i> R	TGTCTGGGAATACGTCTACAG
799	<i>cry11A</i> F	GGAAGACTCATCATTAGACAC
800	<i>cry11A</i> R	AGTAGCAGTGTGAAACCAGT
847	<i>psbD</i> 5' F	<u>gctccatggatc</u> TCATAATAATAAAACCTTTATTCAT <i>NcoI BamHI</i>
850	<i>psbD</i> 5' R	<u>ccggcatatg</u> GTGTATCTTTAAAATAAAAAACAACCTCATCGTTACG <i>NdeI</i>
860	<i>psbA</i> 3' F	<u>cggggctg</u> AGCTCAAACAACCTAATTTTTTTTAAAC <i>BlpI</i>
861	<i>psbA</i> 3' R	<u>cagtgtctcagcggg</u> TCCTGCCAACTGCCTATGGTAGC <i>BlpI BamHI</i>
864	Integration site F	TGGAATTGGATATGGACTAG
865	Integration site R	GGTACTTGCATTTTATAAGT
1001	<i>cry4A</i> RT F	TGGTGGTGACTTAATTGACTTC
1002	<i>cry11A</i> RT F	AGACTTCTACTCAAAAAATCACACTAC
1003	<i>cry4A</i> RT R	GGGAATAATACTGGAATTAAGTACCGA
1004	<i>cry11A</i> RT R	GAGAAAGCAGCTTTAGCAGCCCATTT

^aF, forward; R, reverse

^bUpper case nucleotides, cry or chloroplast gene nucleotides; lower case, additional nucleotides; underlined nucleotides, restriction sites; 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.