Synergy Between *Aedes aegypti* Trypsin Modulating Oostatic Factor and δ-Endotoxins

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Abstract: Starved first instar *Aedes aegypti* larvae were 35-fold more sensitive to *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) toxins than fed larvae. Feeding larvae *Pichia pastoris* yeast cells expressing *tmfA* (synthetic gene coding for the Trypsin Modulating Oostatic Factor of *Ae. aegypti*) together with *Escherichia coli* cells expressing *Bti* toxin genes (*cry4Aa, cry11Aa, cyt1Aa* and *p20*) indicate that TMOF and Cry toxins are synergisitic. *tmfA* was cloned and expressed in the cyanobacterium *Anabaena* PCC 7120 and the hormone was purified by HPLC and identified by ELISA. The amount of TMOF synthesized by *Anabaena* was low (0.5 - 1 μg in 10^8 cells). *P. pastoris*, which synthesizes high amounts of heterologous proteins in the presence of methanol and is readily consumed by mosquito larvae, was genetically engineered to produce more TMOF. Codon-optimized synthetic genes, *cry11Aa- tmfA* and *gst-cry11Aa- tmfA*, that were cloned into *P. pastoris* and fed to *Ae. aegypti* larvae caused 87.5% mortality in 5 days. GST (glutathione-S-transferase) enhanced the activity of Cry11A-TMOF and protected it from heat denaturation. Cell free extracts of recombinant *P. pastoris* cells killed 40% of tested 4th instar larvae within 24 h, and mass spectra analysis confirmed that the recombinants synthesize Cry11Aa. This report shows for the first time that Cry toxins and TMOF are synergists to *Ae. aegypti* larvae when jointly fed or expressed in recombinant *P. pastoris*.

Keywords: TMOF, Cry toxins, mosquito larvae, *Anabaena, Pichia pastoris,* genetic engineering, bio-control.

INTRODUCTION

Mosquitoes are vectors of major diseases such as malaria, dengue and encephalitis, and are nuisance in temperate zones. Mosquito control programs that solely rely on chemical insecticides often cause ecological problems and select for resistance due to frequent applications [1]. Thus, mosquito control relying on environmentally friendly insecticides is desired.

Trypsin Modulating Oostatic Factor (TMOF), a deca-peptide hormone originally purified from the ovaries of female *Aedes aegypti* [2], inhibits the biosynthesis of trypsin- and chymotrypsin-like enzymes in the midgut epithelial cells of female and larval mosquitoes by a translational control mechanism [3, 4] stopping food digestion. The lack of free amino acids liberated from the blood meal in adult females or from digested proteins liberated in the larval gut causes inhibition of egg development (sterility) to adults and anorexia and death to larval mosquitoes, respectively [4, 5]. TMOF traverses the gut, enters the hemolymph, binds a TMOF specific receptor(s) on the epithelial cells, and stops trypsin biosynthesis and egg development [4-8].

Mosquito larvae synthesize trypsin-like enzyme(s) in their gut as major digestive protease(s). TMOF expressed on the coat protein of Tobacco Mosaic Virus that was fed to mosquito larvae caused anorexia and death at a low dose (1 ng per larva) [4]. Thus, orally fed TMOF can be used as an effective larvicide against many mosquito species [4, 9]. Using a synthetic TMOF to control mosquito larvae is, however, expensive and inefficient because it is readily soluble in water, whereas mosquito larvae are filter feeders [10]. On the other hand, expressing TMOF in bacterial, algal and yeast cells that are readily eaten by larvae is a simple, efficient and safe way to control mosquitoes.

Bacillus thuringiensis subsp. *israelensis* (*Bti*) is the first subspecies of *B. thurengiensis* (*Bt*) that was found and used as an effective biological control agent against larvae of many mosquito and black fly species [11]. Its Cry toxins are *per os* poisons; they bind to larval midgut causing rapid death, and hence are considered safe larvicides that do not damage the environment [12]. Low probability of developing resistance has been observed in field populations of mosquitoes despite 25 years of extensive field usage [13, 14].

Field persistence of current preparations of *Bti* is unfortunately low [12], but could be improved by expressing

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the toxin-encoding genes in heterologous organisms that are readily eaten by mosquito larvae and grow in larval breeding habitats. Alternatively, genetically modified organisms can be killed, formulated, and then released into the environment. Encapsulating potential larvicides inside live or dead cells would protect the toxins from degradation by sunlight. Proper formulation will keep these cells afloat in water allowing the larvae to feed on them [15, 16]. Genetic engineering of cyanobacteria can overcome the low efficacy and short half-life in nature of current formulations [17]. Indeed, transgenic strains of the nitrogen-fixing filamentous cyanobacterium *Anabaena* PCC 7120 expressing combinations of *Bti* genes (*cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20*) are highly toxic to mosquito larvae [18-20].

Synergy has been described for bacterial endochitinase and δ-endotoxins by several subspecies of *Bt* against the Egyptian cotton leafworm S*podoptera littoralis* larvae [21, 22] and against *Ae. aegypti* larvae [23]. The diverse physiological effects of Cry toxins and TMOF prompted us to investigate whether TMOF and the δ-endotoxins of *Bti* can synergistically kill mosquito larvae.

This study reports the cloning and biological activities of *Ae. aegypti* TMOF in *Anabaena* and Cry11Aa-TMOF and GST-Cry11Aa-TMOF in *Pichia pastoris*. In addition, we tested the toxicities of *cry4Aa, cry11Aa* and *cyt1Aa* expressed in *Escherichia coli* together with TMOF produced in recombinant *P. pastoris*. The newly engineered transgenic strains are presumed to be environmentally friendly biological-control agents because Cry toxins have been used for three decades with no reported damage to the environment and TMOF has been approved by the EPI. The use of TMOF and Cry toxins in concert will effectively prevent future resistance of these insecticides.

MATERIALS AND METHODS

Cloning and Expression in Bacteria

The sub-cloning vector pBS-SK/N and the shuttle vector pRL488 [18] were used to clone *tmfA* with and without IEGR (trypsin cleavage site), yielding pRVE4-TE and pRVE4-T, respectively (Fig. **1**). The *E. coli* donor strain DH10B (kindly provided by Dr. X. Wu), carrying helper plasmid pRL623 (derivative of pRL528), conjugal plasmid

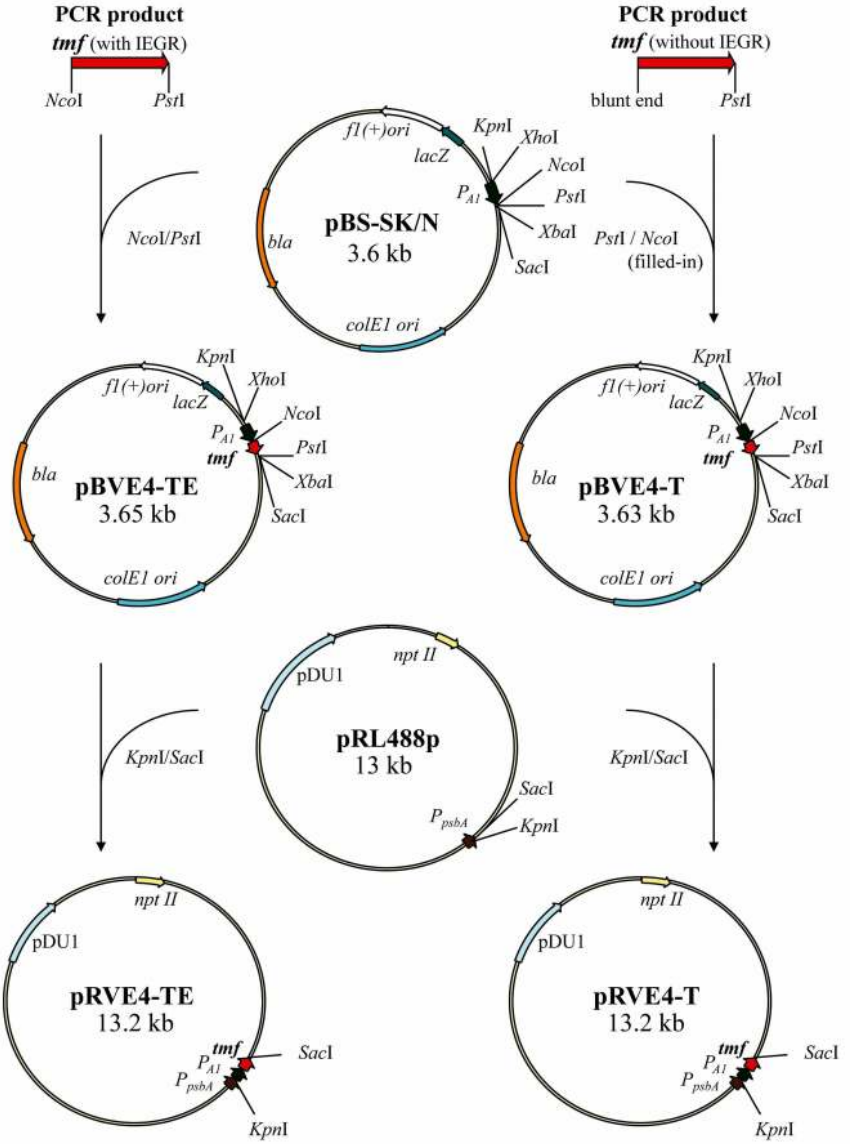

Fig. (1). Cloning of *tmfA* with and without IEGR (trypsin cleavage site) into the *E.coli-Anabeana* shuttle vector pRL488p.

pRL443 (derivative of RP4) and incoming plasmid, were used for biparental conjugation into *Anabaena* PCC 7120 [24]. The constructed plasmids were transformed into *E. coli* strain XL-Blue MRF' (Stratagene, La Jolla, CA) and *Anabaena* PCC 7120.

E. coli DH10B cells harboring helper, conjugal and either pRVE4-TE or pRVE4-T were mixed with *Anabaena* PCC 7120, pre-grown in BG11 liquid medium at 28°C under cool white fluorescent light illumination and the mixed suspensions were spread onto nitrocellulose membranes over BG11 agar plates and incubated for 24 h. Membranes were then transferred to selective plates containing neomycin (25 μg ml^{-1}). Colonies that appeared after 10 days were transferred into 1 ml BG11 containing neomycin and grown for two weeks until true ex-conjugants were detected; they were then purified by repeated streaking on agar plates in the presence of neomycin, each was diluted with BG11 containing neomycin and grown as described above. Growth was followed by measuring chlorophyll concentrations in methanol extracts [18]. Existence of inserted genes was confirmed by PCR using appropriate primers.

Cloning and Expression in *P. pastoris*

A Zeocin-selectable plasmid pPICZB (Invitrogen, Carlsbad, CA) that was used for cloning and expressing heterologous proteins in *P. pastoris* contains an alcohol oxidase 1 promoter (P_{AOX1}) from *P. pastoris* fused to a multiple cloning site and an *AOX1* termination sequence. Fermentation in the presence of methanol (0.5%) induces P_{AOX1} to initiate transcription in KM71H yeast cells (Invitrogen, Carlsbad, CA).

The *tmfA*, *cry11Aa-tmfA* and *gst-cry11Aa-tmfA* sequences were optimized using *Pichia* codons (www.kazusa.or.jp) (Fig. **2c**). *Ae. aegypti tmfA* was synthesized by Gemini Biotech (Gainesville, FL) and cloned directly into pPICZB, and the combined genes *cry11Aa-tmfA* and *gst-cry11Aa-tmfA* were synthesized by GenScript (Piscataway, NJ). The synthetic genes were cut with *Xho*I and *Xba*I, cloned into pUC57 and subsequently ligated into pPICZB *Xho*I and *Xba*I sites (Fig. **2a** and **b**). The cloned genes were sequenced (Applied Biosystems, Foster City, CA) and analyzed at the University of Florida Biotech Center (http://www.biotech.-ufl.edu/about.html). Plasmids carrying full length genes without mutations were used.

E. coli INVαF cells (Invitrogen, Carlsbad, CA) were transformed and clones were selected in the presence of 25 μg ml^{-1} zeocin (Invitrogen, Carlsbad, CA). Positive clones were grown in LB medium in the presence of zeocin, and plasmids were harvested, purified using Qiagen plasmid miniprep kit and screened by PCR. Positive plasmids with inserts were linearized with *Sac*I and used to transform *P. pastoris* KM71H with Easy Comp kit (Invitrogen, Carlsbad, CA). The transformed cells were spread on YPD plates containing sorbitol (1 M) and zeocin (100 μg ml^{-1}). Positive colonies were screened for zeocin resistance (100 to 3,000 μg ml^{-1}). Cells that showed high resistance to zeocin (2-3 mg ml^{-1}; harboring 10 to 20 inserts) and grew slowly in the presence of methanol (Mut^{S}) were selected. The cells were stored at -80ºC in glycerol until used. For protein studies, single colonies were isolated from YPD plates containing zeocin and grown in minimal glycerol medium (1%)

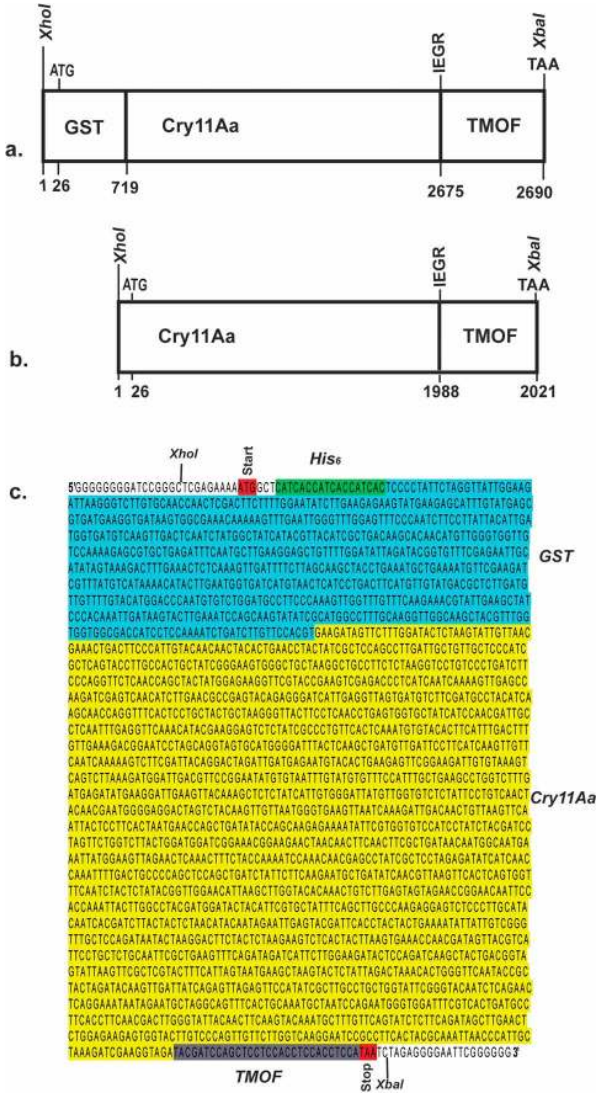

Fig. (2). Genomic organization of *cry11Aa-tmf* synthetic gene. a. *gst-cry11Aa-tmfA* (2,690 nt) genetic map with restriction enzymes cleavage sites for cloning, the ATG start signal, the IEGR trypsin cleavage site and the TAA stop signal. b. *cry11Aa-tmfA* (2,021 nt) genetic map with restriction enzymes cleavage sites for cloning, IEGR trypsin cleavage site and start (ATG) and stop (TAA) signals. c. *gst-cry11Aa-tmf* optimized synthetic gene sequence: His_6-*gst* (green-blue), *cry11Aa* (yellow) and *tmfA* (grey), with the stop and start signals (red).

containing yeast nitrogen base in the presence of zeocin (both at 100 μg ml^{-1}) at 30ºC in a shaking (250 rpm) incubator for 2-3 days. The cells were then centrifuged, and after supernatant removal induced with a fresh minimal medium (yeast nitrogen base with ammonium sulfate without amino acids) containing biotin and zeocin (100 μg ml^{-1}) in the presence of 0.5% methanol for 24-96 hours.

Protein Purification and Mass Spectrometry

Yeast cells were collected by centrifugation, broken with Y-PER (Pierce, Rockford, IL) or with 8 M Urea and glass beads in a DNA FastPrep (BIO 101, Inc., Vista, CA), centrifuged and stored frozen at -20ºC until use. The extracted proteins were adsorbed onto Ni-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Valencia, CA), washed with phos-

phate buffer pH 8.0, and eluted with 20 mM and 250 mM imidazole. Samples from the eluted peaks were removed, dried by Speed Vac, rehydrated in 3% SDS and 5% β-meracptoethanol, 50 mM Tris pH 8.8, heated for 5 min at 95ºC and analyzed by 10% SDS PAGE [25]. The gels were fixed and stained with coomassie brilliant blue and bands that migrated at the same molecular weight of the recombinant proteins were cut, digested with trypsin and analyzed by mass spectrometry (http://www.biotech.ufl.edu/about.-html).

Bioassays for Larvicidal Activity

Recombinant *E. coli* were grown at 37ºC in LB medium, induced with IPTG and harvested after 4 h [26]. Recombinant *Anabaena* were grown at 30°C in BG11 medium containing 25 μg ml^{-1} neomycin in 250 ml flasks for 12 days, as previously described [19]. *Bti* and *P. pastoris* cells were grown in the appropriate liquid media and the latter induced with methanol (0.5%). Yeast recombinant cells were collected by centrifugation, washed and resuspended in distilled water, and used without or with heat inactivation at 50°C for 3 h.

Autoclaved brewer's yeast and heat inactivated (75°C or 50°C for 3 h) *Pichia* cells without *tmfA* were used as controls. Recombinant cells producing TMOF, GST-Cry11Aa-TMOF or Cry11Aa-TMOF were washed 3-times in sterile distilled water and added to 48 well plates, each containing a single 1st-instar *Ae. aegypti* larva in 1 ml sterile water. For large scale feedings, 20 such larvae were added into glass dishes containing 160 ml sterile water and different concentrations of toxins (Cry4Aa, Cry11Aa, Cyt1Aa and P20) produced in *E. coli* [27] or TMOF produced in *P. pastoris* [28]. Each bioassay was independently performed in triplicates. Mortality was followed for 12 days at 28ºC. Values of LC_{50} and LC_{90} (concentration of cells that kill 50% and 90% of the exposed populations, respectively) were determined using probit analysis (EPA Probit analysis program) for 6-12 doses.

ELISA (Enzyme Linked Immunoadsorbant Assay)

Recombinant bacteria were dried in a Speed Vac or lyophilized. TMOF-producing *P. pastoris* KM71H cells were grown in 4 L flasks and stimulated for 120 h with 0.5% methanol. Cells were washed with sterile water to remove medium and external proteins, and collected by centrifugation. To the washed cells 400 μl of Y-PER (Pierce, Rockford, IL) was added and the cultures were gently shaken for 20 min at room temperature. The cells were disrupted by ultrasonic disintegration or with glass beads by shaking for 40 sec at speed 6 with Lysing Matrix C in a DNA Fast Prep Instrument (BIO 101, Vista, CA).

The amounts of TMOF in samples were determined by ELISA [29]. Briefly, to each well of reacti-bind maleic anhydride activated polystyrene plates (Pierce, Rockford, IL) 100 μl of PBS buffer (0.1 M Phosphate, 0.15 M NaCl, pH 7.2) and TMOF of known concentrations (10 ng to 1.2 mg in 10 μl PBS), pH 7.2 were added to construct a calibration curve. Similarly, proteins that were extracted from recombinant cells were also tested. The maleic anhydride well plates were incubated by gentle shaking at room temperature for 1 h and at 4ºC overnight without shaking. The solutions were then decanted from the wells, and to each well 300 μl of a blocking solution TTBS (50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, 3% BSA, pH 7.2) was added and incubated for 1 h. The solutions were then decanted from the wells, and to each well 100 μl of TMOF antiserum that was diluted 1:100 in 0.05% Tween-20, PBS, 0.1% BSA (dilution buffer) was added and incubated for 1 h. The wells were washed 3-times with TTBS by gentle shaking for 5 minutes each. To each well 100 μl of Goat anti-rabbit antibody linked to alkaline phosphatase diluted in a dilution buffer (1:15,000) was added, and incubated for 1 h. The wells were washed 3-times with TTBS and to each well 200 μl of alkaline phosphatase liquid substrate (Sigma, St. Louis, MS) was added and incubated for 30-60 min until a yellow color developed and the absorbance was read at 405 nm in an ELISA plate reader Model EL 308 (Bio-Tek Instruments, Burlington, Vermont).

The absorbency at 405 nm was plotted against the log concentration (ng) of the TMOF standard to construct a calibration curve. The amount of TMOF in the tested recombinant cells was read directly from the linear part of the calibration curve.

HPLC (High Pressure Liquid Chromatography)

Recombinant *Anabaena* cells were grown, harvested, lyophilized, washed and sonicated. The broken cells were centrifuged and the supernatants collected and acidified with 50 μl TFA (trifluoroacetic acid). The acidified solution was vortexed and centrifuged at 14,000 rpm for 5 min. Supernatants were adsorbed onto Sep-Pak C_{18} reversed-phase cartridges (Millipore, Milford, Mass), the column washed with 4 ml water, 0.1% TFA and TMOF was eluted with 100% acetonitrile, 0.1% TFA (4 ml), dried by Speed Vac at 40ºC, and taken up in 100 μl water. To the rehydrated samples (90 μl), TFA (10 μl) was added and the samples chromatographed by a high-performance liquid chromatography (HPLC) system with a Microsorb 5 μm C_{18} reversed-phase column (4.6 mm ID by 25 cm), a 5-μm guard cartridge, and a gradient of acetonitrile (0-100%) in 0.1% TFA. Absorbance was followed at 220 nm at 1 absorbance unit at full scale (AUFS). Fractions (1 ml) were collected, dried by Speed Vac and rehydrated in 100 μl PBS. Samples were analyzed by ELISA for the presence of TMOF using reacti-bind maleic anhydride activated polystyrene plates (Pierce, Rockford, IL).

RESULTS

Cloning and Expression of *tmfA* in *Anabaena*

TMOF-coding region (bases 25-54, underlined; *tmfA*) was optimized using *Anabaena* PCC 7120 codons. The sequence of 12 bases coding for a trypsin cleavage site (IEGR) was introduced upstream (bases 13-24), to release in the larval midgut free TMOF that inhibits trypsin biosynthesis:

```
1         10        20        30        40        50        60  65
GCATCCATGCGGATTGAAGGTCGTTATGATCCAGCTCCACCTCCCCCACCTCCTTAACTGCAGGAAC
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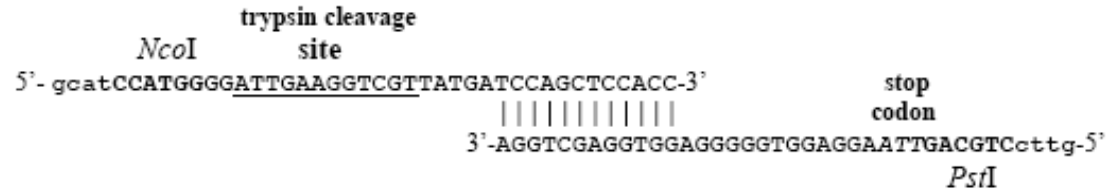
To construct pBVE4-TE (Fig. **1**), two overlapping oligonucleotides of 41 (*Nco*I) and 38 (*Pst*I) bases with overlap of 12 nucleotides were synthesized, annealed and amplified by PCR:



The 67 bp *Nco*I-*Pst*I amplicon was purified by PAGE, cut and ligated into pBS-SK/N at the *Nco*I-*Pst*I site upstream P_{A1} [18], and the resultant pBVE4-TE was transformed into *E. coli* XL1-Blue MRF'. pBVE4-TE was cut with *Kpn*I/*Sac*I and the P_{A1}-*tmfA* fragment ligated into pRL488p, containing P_{psbA} downstream of the *Kpn*I-*Sac*I site, yielding pRVE4-TE (Fig. **1**). The recombinant plasmid was transformed into and amplified in *E. coli* XL1-Blue MRF'. The plasmid with the dual promoters P_{psbA} and P_{A1} was introduced into *Anabaena* PCC 7120 by biparental conjugation [24].

Another sequence encoding TMOF without IEGR was also designed for expression in *E. coli* and in *Anabaena* PCC 7120:

```
                                    stop
   Y  D  P  A  P  P  P  P  P  P codon PstI
5'-TATGATCCAGCTCCACCTCCCCCACCTCCTTAACTGCAGgaac-3'
   |||||||||||||||||||||||||||||||||||||||||||
3'-ATACTAGGTCGAGGTGGAGGGGGTGGAGGAATTGACGTCcttg-5'
```

Two oligonucleotides, 43 bases each, were synthesized and annealed and the gene was ligated into the *Pst*I/*Nco*I cloning site of pBS-SK/N (*Nco*I site of the vector was filled-in by Klenow fragment), yielding pBVE4-T that was transformed into *E. coli* XL1-Blue MRF'. pBVE4-TE and pBVE4-T were digested with *Kpn*I/*Sac*I and the P_{A1}-*tmf*A fragments ligated into pRL488p, containing P_{psbA} downstream the *Kpn*I-*Sac*I site, yielding pRVE4-TE and pRVE4-T, respectively (Fig. **1**). The recombinant plasmids were transformed and amplified in *E. coli* XL1-Blue MRF'. The plasmids with the dual promoters P_{psbA} and P_{A1} were introduced into *Anabaena* PCC 7120 through DH10B (carrying conjugal and helper plasmids) by biparental conjugation [24].

To find out the expression level of *tmfA* alone (without *Bti* toxin genes) in *Anabaena*, it was fused in frame to the C-terminal segment of *p20* (encoding an accessory, non toxic *Bti* protein) and the gene was expressed with and without a protease cleavage site (IEGR). Recombinant cells were grown, and protein extracted and analyzed by HPLC and ELISA for TMOF. Transgenic clones of *Anabaena* PCC 7120 synthesized 0.5-1 μg of TMOF in 10^8 cells, higher than in *E. coli* (35-650 ng in 10^8-10^9 cells). However, when *tmfA* was expressed in *Bti* the synthesis of the hormone was even lower (67-267 ng in 10^8-10^9 cells). These results indicate that the expression level of *tmfA* in *Anabaena, E. coli* and *Bti* is low. Perhaps the decapeptide precipitates in inclusion bodies or it needs to be fused to a larger protein in order to efficiently be synthesized in bacteria.

Bti δ-Endotoxins and TMOF

It is anticipated that starved mosquito larvae are more sensitive to δ-endotoxins than well nourished larvae because they are weaker, swim slower in water and may consume more toxin than non starved larvae (Borovsky, unpublished observations). Indeed, 1st instar larvae starved from emergence were 35-fold more sensitive than well nourished larvae fed with Brewer's yeast (80 mg ml^{-1}), whereas 3rd instar larvae that were starved of Brewer's yeast for a day were only 6-fold more sensitive to δ-endotoxins than well nourished larvae (Table **1**).

Table 1. Toxicity of *Bti* Against *Ae.aegypti* Larvae

Larval Instar	LC_{50} (ng ml^{-1})	
	Starved	**Fed**
First	1.6	55.6
Third	17.7	105.5

Aedes aegypti larvae (2 groups of 20 larvae per group) were fed a dry preparation of *B. thurengiensis* subsp. *israelensis* δ-endotoxins and mortality was determined after 24 h. Results are average of 2 determinations with no more than 5-10% differences between groups.

These results encouraged us to express a synthetic *tmfA* in *P. pastoris* KM71H. The yeast cells were heat inactivated (50°C for 3 h) and 3 different concentrations of the hormone were fed together with 10^5 *E. coli* cells expressing *cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20* [27], to groups of twenty 1st instar *Ae. aegypti* larvae, and larval survival was monitored daily for 6 days (Table **2**). All the larvae that were fed by the transgenic *E. coli* alone died, but none died upon addition of *P. pastoris* cells transformed with empty pPICZB, as in the control. Thus, *P. pastoris* not only serves as a good food source for the larvae—it protects them from the *Bti* toxins as well. Feeding recombinant *P. pastoris* cells with high TMOF concentration alone (298 nM) did not cause appreciable larval mortality in 6 days. After 12 days of feeding, however, 90% of the larvae died from starvation and lack of trypsin biosynthesis (data not shown). Lower TMOF concentrations (75 nM) in the transgenic *P. pastoris* cells restored the toxicity of the δ-endotoxins produced by *E. coli* (10^5 cells); mortality reached 95% after 6 days of feeding (Table **2**). Feeding *E. coli* expressing *Bti* toxin genes in the presence of *P. pastoris* cells or *P. pastoris* expressing *tmfA* alone did not affect mosquito larvae (Table **2**). These results indicate that TMOF enhances the effect of *Bti* toxins in organic rich environments that are abundant in nature.

Table 2. Feeding of Recombinant *E. coli* (pVE4-ADRC) and *P. pastoris-tmfA* to *Ae. aegypti* Larvae

Larvae fed with:	TMOF (nM)	*E. coli* (cells ml^{-1})	Surviving larvae ± S.E.M.
Pichia (control)	0	0	16.7 ± 0.3[a,c]
E. coli (pVE4-ADRC)	0	10^5	0 ± 0[a,d]
Pichia + *E. coli* (pVE4-ADRC)	0	10^5	17 ± 0.6[c]
Pichia –*tmfA* + *E. coli* (pVE4-ADRC)	298	10^5	0.7± 0.7[a,b,d]
Pichia -*tmfA* + *E. coli* (pVE4-ADRC)	149	10^5	1.3 ± 0.7[a,b]
Pichia - *tmfA* + *E. coli* (pVE4-ADRC)	75	10^5	1.3 ± 0.9[a,b]
Pichia –*tmfA*	298	0	18.7 ± 0.7[c,e]

E. coli cells transformed with pVE4-ADRC expressing *cry4Aa*, *cry11Aa*, *cyt1Aa* and *p20* [27] were fed for 6 days to 3 groups of 1st instar larval *Ae.aegypti* (20 larvae per group in 160 ml water) in the presence of different concentrations of recombinant *P. pastoris* cells expressing TMOF that were heat inactivated at 75°C for 3 h. Larval survival was followed in triplicates and is expressed as mean ± S.E.M. The amount of TMOF in recombinant *P. pastoris* cells was determined by ELISA.
[a]Significant difference from control by student's *t*-test $p<0.008$,
[b,c,d]not significant difference $p<0.6$,
[e]Pichia-*tmfA* (75-298 nM) kill 90% of mosquito larvae in 12 days.

Larvicidal Activities of Recombinant *P. pastoris* Cells and Cell-Free Proteins

a. Cry11Aa-TMOF

KM71H Cells of *P. pastoris-cry11Aa-tmfA* were grown for 96 h in the presence of 0.5% methanol. At 24 h intervals, the cells were harvested, washed and aliquots (10^8 cells) were fed to individual *Ae. aegypti* larvae in 48 well-plates. Cells that were induced for 24 and 48 h killed few larvae at 5-6 days and 82% at day 12 (Fig. **3**). A longer induction period of 96 h caused rapid mortality; 63% of the larvae died within 2 days. Only 12.5% of the larvae died when fed *P. pastoris* cells transformed with an empty pPICZB plasmid (control) or brewer's yeast cells (Fig. **3**). These results are consistent with the conclusion that TMOF enhances the activity of *Bti* toxins (Table **2**).

b. GST-Cry11Aa-TMOF

To find out if some of the newly synthesized Cry11Aa-TMOF precipitated in the cytoplasm or was shunted to the peroxisome and thus was less toxic to the larvae, we synthesized a glutathione-S-transferase (GST) synthetic gene and fused it in frame to *cry11Aa-tmfA* yielding *gst-cry11Aa-tmfA* with 10-20 copies per cell. *Ae. aegypti* larvae that fed on these cells, induced with methanol, died faster than larvae that fed on cells producing Cry11Aa-TMOF (compare Fig. **3** with Fig. **4**). At day 5, 88%, 75% and 73% of the larvae died after eating cells that were induced for 96 h, 72 h and 48 h,

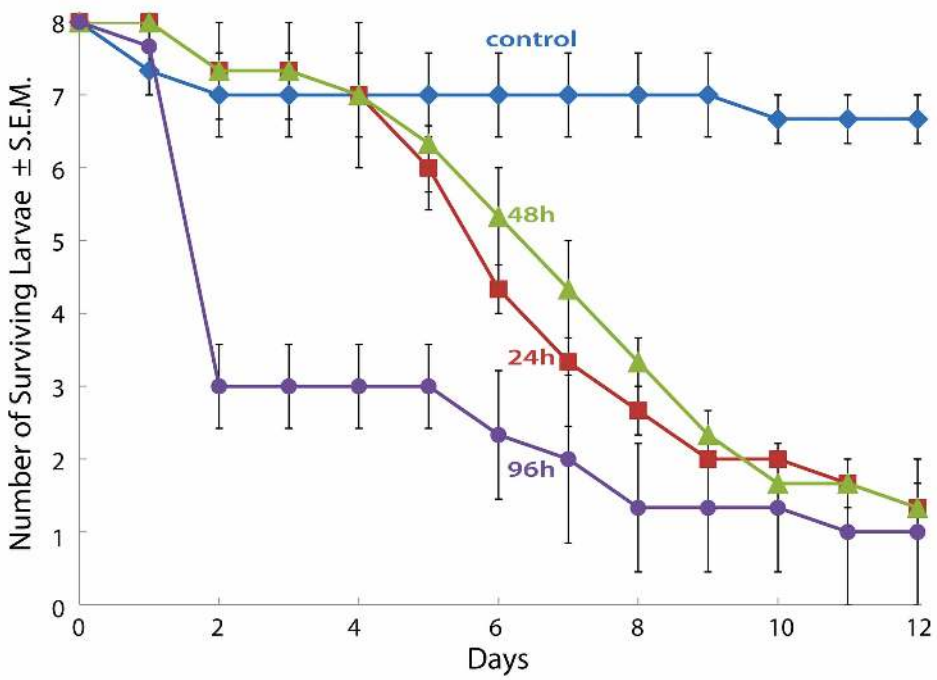


Fig. (3). Feeding *P. pastors-cry11Aa-tmfA* cells to *Ae. aegypti* larvae. *P. pastoris* cells expressing *cry11Aa-tmfA* were fermented for 24, 48 and 96 h in the presence of methanol (0.5%). Three groups of eight 1st instar larvae were fed for 12 days 10^8 cells/larva in 48 well-plates. Larval survival was followed daily. Results are expressed as means of 3 determination ± S.E.M. Controls were fed *P. pastoris* cells without *cry11Aa-tmfA*.

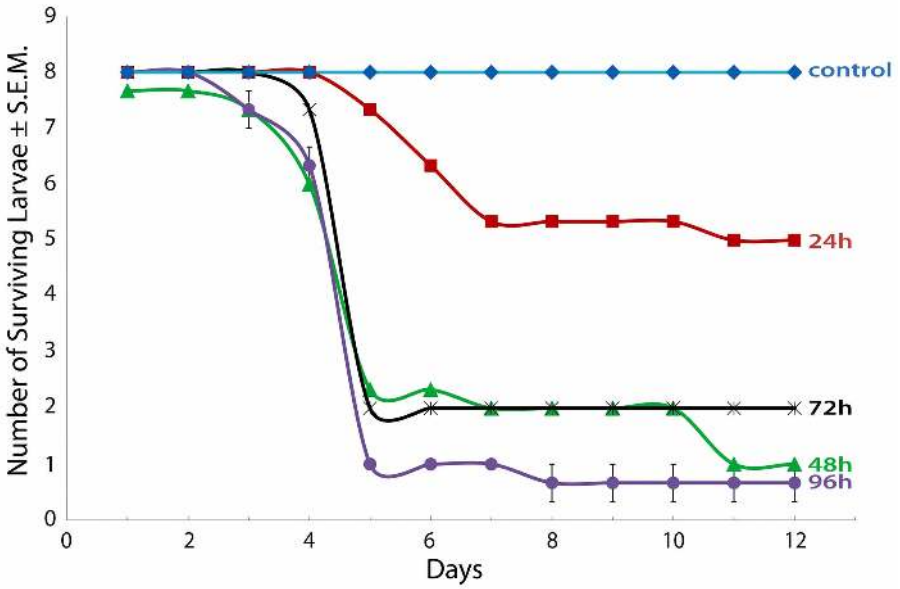


Fig. (4). Feeding *P. pastoris-gst-cry11Aa-tmfA* to *Ae. aegypti* larvae. Cells were fermented in the presence of methanol and fed to *Ae. aegypti* larvae as in Fig. (**3**). Survival was recorded daily, and results are expressed as means of 3 determinations ± S.E.M. Controls were fed *P. pastoris* cells without *gst-cry11Aa-tmfA*. No effect was found when *Pichia-gst* cells were fed to larvae (results not shown).

respectively (Fig. **4**). Cells that were induced for 24 h were not as effective; 12.5% of the larvae died at day 5 and 37.5% at day 12 (Fig. **4**), whereas cells expressing *gst* alone did not kill larvae (data not shown). These results indicate that fusing GST to Cry11Aa-TMOF enhances its solubility in the cytoplasm and its larvicidal activity.

c. Cell-Free Extracted Proteins

Early 4th instar *Ae. aegypti* larvae were fed protein extract (5.69 μg) isolated from *P. pastoris* cells that were transformed with *gst-cry11Aa-tmfA*. After 24 h of feeding, 40% of the larvae died. Mortality in a control group that were fed non-transformed *P. pastoris* extract was 6.5-fold lower (Table **3**). These results indicate that the transformed *P. pastoris* cells synthesize soluble GST-Cry11Aa-TMOF in the cell cytoplasm that is toxic to *Ae. aegypti* early 4th instar larvae.

d. Heat Stability of GST-Cry11Aa-TMOF

Exposure of *gst-cry11Aa-tmfA*-expressing *P. pastoris* cells to 50ºC for 3 h increased LT_{50} and LT_{90} (time required to kill 50 and 90% of the larval population) by 1.2 and 3.7 days respectively, as compared with untreated proteins (Table **4**). These results indicate that for future field testing, it is possible to heat inactivate our recombinant yeast cells synthesizing GST-Cry11Aa-TMOF with little effect on the toxicity of the cells towards mosquito larvae.

Purification and Mass Spectrometry Analysis of Cry11Aa

To find out whether the biological activity of the recombinant *P. pastoris* cells is due to Cry11Aa-TMOF, *P. pastoris* cells (*AOX1:cry11Aa-tmfA*) were induced for 96 h and broken with Y-PER and glass beads, followed by extraction with 8 M urea, and the combined cell extract was purified by Ni-NTA column chromatography. A protein peak (235 μg) eluted at 250 mM imidazole was collected and analyzed by PAGE SDS [25]. After electrophoresis, the gel was stained, a band at M_r 72 kDa isolated and analyzed by mass spectrometry (Fig. **5**). Nine unique peptides at different locations on the protein were found that cover 14% of the entire protein identifying Cry11Aa with 95% confidence.

DISCUSSION

Insect resistance to insecticides poses a serious agricultural and public health problem. Resistance has been reported against *Bti* in field populations of *Culex pipiens* [30] and in laboratory populations of *Cx. quinquefasciatus* [31]. Co-expressing several toxin genes having different modes of action might enhance toxicity and delay or prevent resistance. Since TMOF and δ-endotoxins kill mosquito larvae in different mechanisms, it is possible that they are synergistic, just as δ-endotoxins from other *Bt* subsps and bacterial endochitinase against *S. littoralis* [21, 22] and *Ae. aegypti* larvae [23].

TMOF inhibits the biosynthesis of trypsin [2], which activates δ-endotoxins in the larval midgut. If both, δ-endotoxins and TMOF are fed together to mosquito larvae in recombinant cells expressing their genes, inhibition of trypsin biosynthesis by TMOF may interfere with the activation of the δ-endotoxins. Therefore, genes encoding the active portion of these polypeptides [32] should be used in concert with TMOF. Since larval killing by δ-endotoxins is fast [12], whereas that of TMOF is slow and transmitted through signal transduction pathway, translational control and secondary messengers [2, 4, 7, 33-35]. The activation of the δ-endotoxins may be accomplished by pre-existing endogenous larval gut trypsin before its level in the midgut is reduced by TMOF. In addition, larval starvation, which occurs later on, will slow down repair of sub-lethal damaged gut epithelium by δ-endotoxins [36], enhancing gut absorption of TMOF into the hemolymph. Starved larvae may also eat more recombinant yeast cells bearing toxins thus enhancing larval demise. Toxicity of *cry1C* against advanced larval stages (3rd- and 5th-instars) of *S. littoralis* is, indeed, reduced due to high levels of protease activity in the gut which

Table 3. Feeding Larvae Cell Free Extract of *P. pastoris* Cells Expressing *gst-cry11Aa-tmfA*

Cells Extract	N	Dead larvae ± S.E.M.	Mortality (%)
a. *Pichia gst-cry11Aa-tmfA* 96 h fermentation	8	6.38 ± 0.86	40
b. Pichia control	4	1.0 ± 0	6

P.pastoris cells were fermented for 96 h and the cells were broken with glass beads and Y-PER, the extract was centrifuged, the supernatant collected and aliquots of (5.69 μg) fed to individual early 4th instar *Ae. aegypti* larvae (16 larvae per group) in 48 well plates in 1.0 ml water. Larval mortality was recorded 24 h later. Controls were fed protein extract from cells that were not transformed. The results are expressed as averages of 4 or 8 determinations ± S.E.M.

Table 4. Feeding of Heat Treated and Untreated *P. pastoris* Cells Expressing *gst-cry11Aa-tmf*A

P. pastories KM71H *AOX1:gst-cry11Aa-tmfA* cells were induced for 96 h and fed to mosquito larvae	LT_{50} (days)	Confidence Limit (95%) (days)	LT_{90} (days)	Confidence Limit (95%) (days)
a. Untreated	4.62	3.655-5.477	8.27	6.853-11.461
b. Heat inactivated at 50° C for 3 h	5.88	4.822-6.896	12.07	9.705-18.095

Cells were induced with methanol for 96 h, and 3 groups of 8 larvae were fed individually in 48 well plates for 12 days. The recombinant cells were untreated or heat inactivated at 50°C for 3 h. The mortality of each group was recorded at 24 h intervals for 12 days, and the results were subjected to probit analysis. Each experiment was repeated 3 times. LT_{50}=Lethal time required to kill 50% of the larvae. LT_{90}= Lethal time required to kill 90% of the larvae.

rapidly degrades the ingested toxin [37]. The proteases that are responsible for the inactivation of Cry1C are inhibited by trypsin-specific inhibitor such as PMSF. Thus, lowering the level of trypsin-like enzymes in the larval gut by TMOF might protect extensive degradation and inactivation of *Bti* toxins. Synergy is thus anticipated between TMOF and *Bti* enhancing larval killing at lower δ-endotoxin concentrations. Thus, constructing genes encoding chimeric proteins that use trypsin to release TMOF and simultaneously activate the δ-endotoxins is an advantage, and our report confirms this hypothesis.

We report here that starved *Ae. aegypti* larvae are 6-35 fold more sensitive to *Bti* toxins than well-fed larvae (Table **1**), and starved 1st instar larvae are 10-fold more sensitive than 3rd instars. Since TMOF is more effective against 1st and 2nd instar larvae [9], the use of both toxins in early developmental stages is expected to enhance the effect of low toxin doses and reduce the amount of *Bti* required in sewage treatment ponds in which it is not effective alone.

The high toxicity of recombinant *E. coli* expressing *cry4Aa*, *cry11Aa* and *cyt1Aa* [27] to 1st instar *Ae. aegypti* larvae was dramatically reduced in the presence of *P. pastoris* as a nutritious food source (Table **2**). Mortality was restored (about 95%) upon replacing it with recombinant *P. pastoris* producing TMOF (75-249 nM), which was not toxic alone (Table **2**). These results confirm our hypothesis that TMOF and *Bti* are synergisitic against larvae of *Ae. aegypti* in organic rich environment. TMOF enhances the toxicity of δ-endotoxins by inhibiting trypsin biosynthesis, causing anorexia, starvation and death. These results confirm earlier observation that ingested nutritious particles reduce the susceptibility of insect larvae to δ-endotoxins [38], indicating that other mechanisms (e.g. blocking of toxins receptor sites) may also play an important role. This observation explains why *Bti* is not effective against mosquito larvae in sewage treatment ponds. TMOF, on the other hand, traverses the gut epithelial cells and binds to its gut receptors at the hemolymph side [6, 7, 34, 35]. Since TMOF and *Bti* have different mechanisms of action, increased resistance in exposed mosquito population against their combination is not likely. Although *Bti* and TMOF act like synergists in the laboratory, they must be tested under stringent field conditions for future larval control applications.

When *cry4Aa-tmfA* was cloned and expressed in *P. pastoris* KM71 cells without codon-optimization [28], the activity of the construct was poor. Codon optimized chimeric *cry11Aa-tmfA* and *gst-cry11Aa-tmfA* (Fig. **2**) expressed in *P. pastoris* cells was effective against *Ae. aegypti* larvae (Figs. **3** and **4**). Fusing GST and SUMO (small ubiquitin-related modifier) to proteins enhances their activity in bacteria and yeasts by increasing solubility in the cytoplasm [39]. Indeed, GST enhanced the heat stability of Cry11Aa-TMOF (Table **4**), allowing heat inactivation of the yeast cells in future field applications without endangering the environment.

The GST-Cry11Aa recombinant protein produced in *Saccharomyses cerevisiae* without using codon optimized *gst-cry11Aa* was purified by affinity chormatogarphy on glutathione Sepharose column [40]; the purified protein and the cell free crude extract were biologically active against 3rd instar *Ae. aegypti* larvae, but it was not reported whether the intact cells were also active. The cell free extract (5.69 μg) of our *P. pastoris* clone expressing the chimeric *gst-cry11Aa-tmfA* was also biologically active, killing 40% of the early 4th instar *Ae. aegypti* larvae in 24 h (Table **3**).

Ogunjimi *et al*. [41] cloned and expressed *cry2* in *P. pastoris* using pPICZαB but did not report if the secreted protein was biologically active. A recombinant protein of 70 kDa was identified by SDS PAGE, but no mass spectrometry or N terminus analyses were reported that identified the putative protein as Cry2. Mass spectrometry analysis of Cry11Aa-TMOF that was purified from *P. pastoris* identified Cry11Aa but not TMOF that is fused to it with a trypsin cleavage site (IEGR) (Figs. **2** and **5**). PCR-analysis of the recombinant cell DNA prior to the fermentation found a

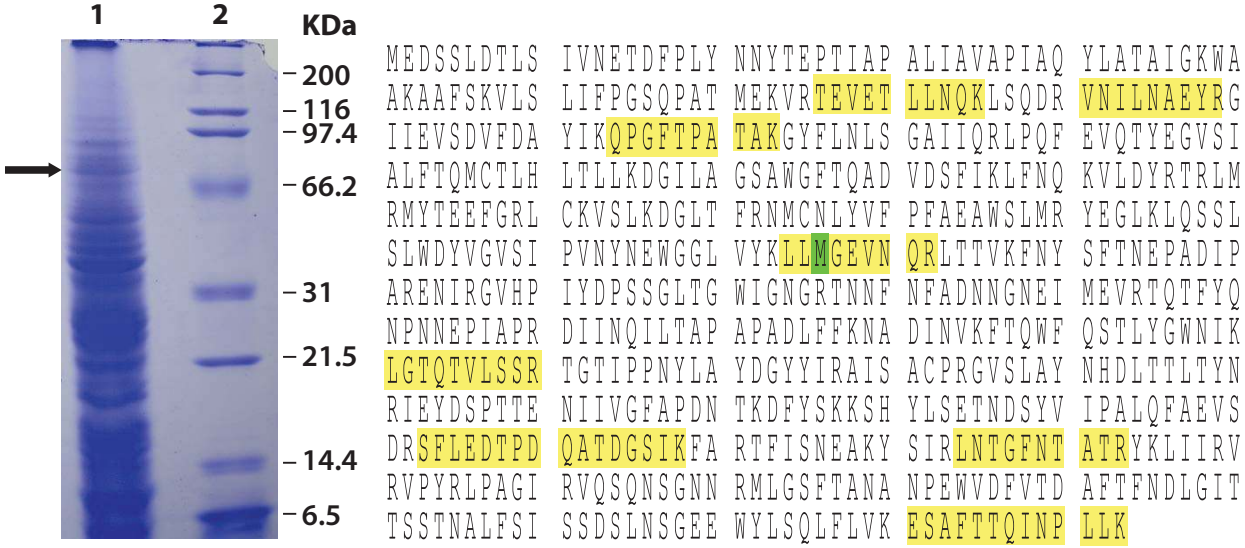


Fig. (5). SDS-PAGE of Ni-NTA eluted proteins and mass spectra identification of Cry11Aa. *P. pastoris cry11Aa-tmfA* cells were fermented in the presence of methanol (0.5%) for 96 h, cells extracted with Y-PER and 8 M urea and proteins purified by affinity chromatography. Eluted proteins concentrated, separated by SDS-PAGE and stained with coomassie blue. A band at M_r 72 kDa (lane 1, arrow) was identified, cut and incubated with trypsin and the released peptides were assayed by mass spectrometry. Sequences that were identified by mass spectrometry are stained yellow, except for M which is stained green. Lane 2, molecular weight standards.

full length gene (*cry11Aa-tmfA*). There are two ways to explain why TMOF was not detected by mass spectrometry: (a) the peptide was not cleaved in sufficient quantities during the tryptic digestion step, and (b) TMOF had been cleaved during the fermentation by yeast proteases releasing the hormone from the fusion protein into the cell cytoplasm. Because the His_6 tag is fused to Cry11Aa, the cleaved TMOF lacks it and thus did not bind to the Ni-NTA column and could not be detected. Our recombinant proteins that are produced inside *P. pastoris* cells are protected by the yeast's cell wall from sunlight and bacterial degradation, and are more palatable to mosquito larvae [10].

Photosynthetic cyanobacterial species are excellent candidates for toxin delivery [16, 20]. They are ubiquitous, float in the upper water layer and resist adverse environmental conditions. In addition, they are used as natural food sources for mosquito larvae [42, 43] and can be cultured on a large scale [44] and genetically altered [17-20, 45-48]. Therefore, co-expressing *cry*, *cyt* and *tmfA* in *Anabaena* PCC 7120 should have a potential advantage.

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