6 Trypsin Modulating Oostatic Factor for Developing Resistant Crops

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1 Introduction

Crop protection against agricultural pest insects is a major problem in growing and producing cultivated plants. Traditional control measures of breeding, chemical spraying, and integrated pest management have alleviated some of these problems. However, the rapid increase in insect resistance to chemical spraying and the slow pace of traditional plant breeding are posing a big challenge to agriculture in the 21st century. It can take 7–10 years and over US$50 million to develop and register a new insecticide (Zaim and Guillet 2002). Although biotechnological approaches offer an alternative to traditional agricultural control of pest insects, the effective genes that were expressed in agricultural crops (e.g., cotton and rice) to protect them against pest insects are mainly bacterial toxins of *Bacillus thuringiensis* (*Bt*). These toxins are effective against lepidopteran, dipteran, and coleopteran insects and most are still used as a microbial formulation (Metz 2003). While the use of *Bt* sprays and genetically modified (GMO) crops, especially cotton, seems to control agricultural pest insects, we cannot ignore the fact that insects are capable of rapidly developing resistance against *Bt* toxins (e.g., the diamondback moth *Plutella xylostella* and the tobacco budworm *Heliothis virescens*) and new approaches should continuously be developed to control insects effectively.

One approach is to utilize insect-specific peptide hormones to selectively control different insects; these hormones control diverse functions in insects such as digestion, reproduction, water balance, feeding behavior, metamorphosis, and sex attraction (Gäde and Goldworthy 2003; Menn et al. 1991). The advantage of this approach is that these hormones are insect-specific and they control vital functions in the life cycle of insects. Disruption of these processes causes irreversible damage and, eventually, death. Because these hormones are peptides, and are found naturally in insects, they are not xenobiotic and would not cause harm to the environment. One of the problems of using insect peptide hormones was that until recently not too many of them were fully characterized. Because of the peptidic nature of these compounds, they cannot be sprayed on an insect's cuticle unless they are attached to lipolitic moieties that will allow their transport through the cuticle into the insect's hemolymph. They also exhibit short residual activity, photolability of certain amino acids (e.g., tyrosine), pH sensitivity, and rapid degradation in

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the environment unless they are protected. Since many of these hormones are blocked either at the amino terminus as pyroglutamic acid derivatives or are amidated at the carboxylic terminus, the peptides cannot be expressed in baculovirus, bacterial, yeast, or plant cells, because molecular engineering of these cells to enable them to amidate or to block the amino terminus with pyroglutamic acid derivative has not been developed yet. However, several of these peptide hormones in the absence of the blocked carboxylic and amino termini have reduced activities that are still effective (e.g., diuretic hormone and ovarian ecdysiotropic hormone). Many of these peptide hormones have a high degree of sequence conservation among agricultural pest insects and economically may be good candidates for future plant protection. Most of these peptide hormones have not been cloned and expressed in plants or tested on agricultural pest insects except for trypsin modulating oostatic factor (TMOF) and pheromone biosynthesis activating neuropeptide (PBAN) from *Helicoverpa zea*, which was cloned and expressed in baculovirus and reduced neonate and 3rd instar survival time of *Trichoplusia ni* larvae by 26 and 19%, respectively (Ma et al. 1998). Thus, TMOF may offer an alternative approach in controlling agricultural pest insects.

2 Biochemical and Physiological Studies

2.1 The Discovery of Mosquito TMOF

Diverse antigonadotrophins or factors that inhibit egg development (oostatic hormones) have been demonstrated in the cockroach, *Blattela germanica* (Iwanov and Mescherskaya 1935), decapod crustaceans (Carlise and Knowles 1959), and the housefly, *Musca domestica*, (Adams et al. 1968; Kelly et al. 1984). In mosquitoes, Meola and Lea (1972) and Else and Judson (1972) similarly demonstrated an ovary-produced humoral factor secreted during oogenesis that inhibited yolk deposition in less developed follicles. In *Rhodnius prolixus*, oostatic hormone produced by the abdominal neurosecretory organs is a small peptide of M_r 1,411 that inhibits the action of Juvenile Hormone (JH) on vitellogenic follicle cells and prevents the ovary from accumulating vitellogenin from the hemolymph (Liu and Davey 1974; Davey 1978; Davey and Kunster 1981). In the house fly *M. domestica,* oostatic hormone seems to inhibit the release or synthesis of egg developmental neurosecretory hormone (EDNH) (Adams 1981), but in mosquito it was proposed that the hormone acts directly on the ovary (Meola and Lea 1972). Kelly et al. (1984) injected a crude extract of oostatic hormone from *M. domestica* into the autogenous mosquito *Aedes atropalpus*, and demonstrated inhibition of both egg development and ecdysteroid biosynthesis.

Borovsky (1985) reported that the mosquito ovary is a rich source for "oostatic hormone". Injections of the hormone into female mosquitoes inhibited yolk deposition and vitellogenin biosynthesis (Borovsky 1985).

However, when partially purified "oostatic hormone" was injected into female *A. aegypti*, both egg development and blood digestion were inhibited (Borovsky 1988). Injections of the hormone into decapitated and ovariectomized females (these females do not synthesize ecdysteroids and do not develop eggs but synthesize protease in their gut) inhibited trypsin-like enzyme biosynthesis and blood digestion in their midgut. These results suggested that "oostatic hormone" inhibits trypsin biosynthesis in cells of the midgut, and not the ovary or the endocrine system as was earlier suggested (Borovsky 1988). The hormone is not species specific, as injection of the hormone caused inhibition of egg development and trypsin biosynthesis in *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* (Borovsky 1988), and feeding of the hormone adsorbed to yeast particles to larval *Anopheles quadrimaculatus, Culex quiquefasciatus, Culex nigripalpus, Aedes aegypti* and *Aedes taeniorhynchus* caused trypsin inhibition, larval starvation and mortality (Table 1, Borovsky and Meola 2004). The hormone was named "trypsin modulating oostatic factor" (TMOF), and Borovsky and co-workers purified, sequenced and, using mass spectrometry, characterized the hormone as an unblocked decapeptide (NH_2-YDPAPPPPPP-COOH) (Borovsky et al. 1990). Several peptide analogues were synthesized and shown to possess TMOF activity (Borovsky et al. 1990, 1991, 1993, Borovsky and Meola 2004) (Table 2). The solution structure of the hormone was determined by NMR studies showed that the TMOF formed a rod-shaped left-handed helix about 30 angstrom long in solution due to the six-proline residues (Curto et al. 1993; Borovsky et al. 1990, 1993) (Fig. 1a).

2.2 Biological Activity and Mode of Action of TMOF

Because female mosquitoes take several blood meals, TMOF is rapidly metabolized in the thorax (half-life of 1.6 h) of intact mosquitoes after the blood meal has been digested, and trypsin biosynthesis has been terminated (Borovsky et al.

Table 1. Effect of *Aea*-TMOF on different mosquito species

Mosquito species	N	LC_{50} (mM ± S.E.M.)
Anopheles quadrimaculatus	3	0.383 ± 0.005[a]
Culex quinquefasciatus	3	0.458 ± 0.02[a]
Culex nigripalpus	3	1.056 ± 0.097[a]
Aedes aegypti	3	0.2 ± 0.015[b]
Aedes taeneorhynchus	3	0.483 ± 0.049[a]

Three groups of larvae (12 larvae per group) of first instar larvae were individually grown in microtiter plates containing different concentrations of TMOF (1.46–376 µg). Larval mortality was followed for 5–7 days. Mortality in control wells lacking TMOF was 5% or less. Larval mortality at lethal concentrations (LC_{50}) were determined using probit and two tailed Student's *t*-test.
[a]Significant difference from b, $p < 0.0148$. (With permission from Borovsky and Meola 2004)

Table 2. Activity profile of TMOF and its analogues on mosquito larvae

TMOF Analogues	LC_{50} (mM ± S.E.M.)	Activity (%)
YDPAPPPPPP	0.2 ± 0.015[a,b]	100
YDPAPPPPPPR	>71.6	0
$(H)_6$IEGRYDPAPPPPPP	0.34 ± 0.032[b]	59
YDPAPPPP	0.44 ± 0.05[b]	45
YDPAPP	0.64 ± 0.032[b]	31
YDPAP	0.64 ± 0.028[b]	31
YDPAPR	0.24 ± 0.01[a]	80
YDPAPK	>>2.9	0
YDPA	0.21 ± 0.01[a]	95
YDPAR	0.12 ± 0.017[a]	166
$(YDPAR)_4$	0.095 ± 0.007[b]	210
YDP	2.3 ± 0.36[b]	9
YDPR	0.24 ± 0.02[a]	80
YD	1.24 ± 0.05[b]	16
DPA	0.4 ± 0.03[b]	50
DPAR	0.46 ± 0.011[b]	43
$(DPAR)_4$	0.048 ± 0.002[b]	417
DPAP	0.98 ± 0.017[b]	20
DPAPPPPPP	0.44 ± 0.015[b]	45
PAPPPPPP	0.58 ± 0.029[b]	34
APPPPPP	1.18 ± 0.065[b]	17
PPPPPP	1.1 ± 0.025[b]	18
PPPP	1.5 ± 0.085[b]	13
PP	1.83 ± 0.07[b]	11
PAP	6.4 ± 0.23[b]	3
(d)YD	1.4 ± 0.017[b]	14
(d)YDP	0.51 ± 0.05[b]	39
Y(d)DP	0.28 ± 0.015[b]	71
(d)Y(d)DP	1.7 ± 0.029[b]	12
Y(d)DPAP	1.2 ± 0.26[b]	16

TMOF and its analogues were fed to first instar mosquito larvae (three groups of 12 larvae per group) in microtiter plates. Larval mortality was followed for 6 days. Lethal Concentrations (LC_{50}) at 50% mortality were obtained by Probit analyses. Statistical analyses were done using two tailed Student's *t*-test of paired samples. Mortality in control wells containing Brewer's yeast and without TMOF and its analogues was 0–5%.

[a]No significant difference from TMOF (LC_{50}) $p > 0.05$

[b]Significant difference from TMOF (LC_{50}) $0.001 < p < 0.05$. (With permission, adapted from Borovsky and Meola 2004)

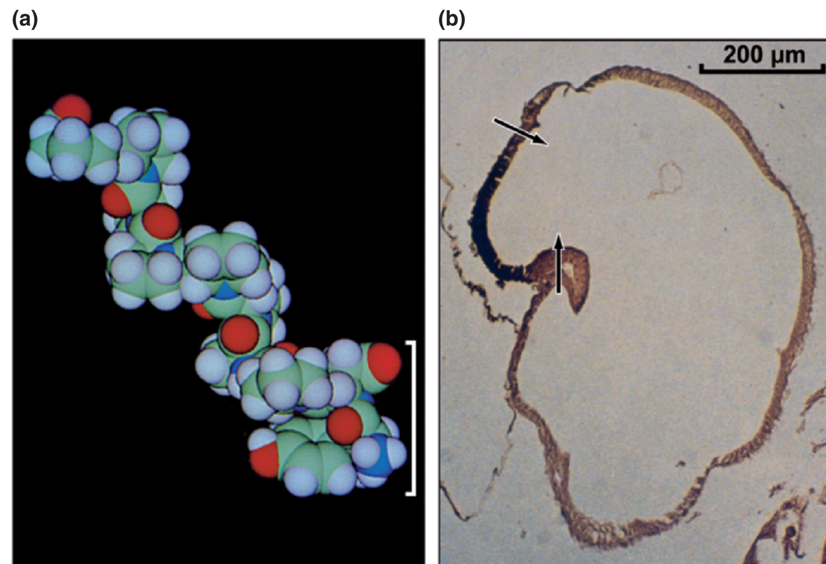

Fig. 1. **(a)** A 3-dimensional nuclear magnetic resonance (NMR) model of *Aea*-TMOF. A left-handed helix of six prolines can be observed at the C-terminus. The N-terminus with the first 4 amino acid sequence (YDPA), important in binding to the TMOF gut receptor is underlined. **(b)** Immunolocalization of TMOF binding to mosquito midgut receptor 72 h after the blood meal. Distinct binding of TMOF to its receptor was observed (*dark area between arrows*). With permission from Borovsky (2003) and Borovsky et al. (1994)

1993). Thus, inhibition of trypsin biosynthesis in the midgut was followed in ligated abdomens that synthesize trypsin but do not metabolize TMOF. At concentrations of 3×10^{-9} M and 6.8×10^{-6} M, TMOF inhibited 50 and 90% of trypsin-like enzyme biosynthesis in the midgut of *A. aegypti*, respectively (Borovsky et al. 1993). The amount of TMOF present in the hemolymph of control, untreated mosquitoes at 30 h and 38 h after the blood meal was determined by ELISA (Borovsky et al. 1992) to be between 33 and 37 ng, which is at least 30 fold higher than the amount that was found to cause 90% inhibition in the TMOF-treated mosquitoes (Borovsky et al. 1993). Similar results were obtained with hemolymph of female *C. quinquefasciatus* (Borovsky unpublished observations). TMOF does not act as a classical trypsin inhibitor (TLCK, TPCK and Soybean trypsin inhibitor) that binds to the active site of serine proteases and prevents protein hydrolysis. TMOF binds to a specific gut epithelial cell receptor (Fig. 1b) and then stops trypsin biosynthesis (Borovsky et al. 1990, 1994a).

2.3 Inhibition of Trypsin Biosynthesis by TMOF in Other Insects

Mosquito TMOF or its analogues inhibit trypsin biosynthesis in the cat flea, *Ctenocephalides felis*, in the stable fly, *Stomoxys calcitrans*, in the house fly, *Musca domestica*, in the sand fly, *Lutzomyia anthophora* and, the midge,

Culicoides variipennis (Borovsky et al. 1990, 1993). TMOF from the grey flesh fly *Neobellieria bullata* has been sequenced and characterized. The hormone is an unblocked hexapeptide (NH_2-NPTNLH-COOH), that like *Aedes* TMOF, stops trypsin biosynthesis and egg development in the flesh fly (Bylemans et al. 1994). The mosquito hormone did not affect trypsin biosynthesis in the flesh fly and the flesh fly's hormone did not affect trypsin biosynthesis in the mosquito. Both hormones specifically terminate trypsin biosynthesis in the gut of the mosquito or flesh fly, respectively, after the protein meal has been digested (Borovsky et al. 1990, 1992, 1993; De Loof et al. 1995; Bylemans et al. 1994).

TMOF also affects agricultural pest insects. Topical treatment of the citrus weevil *Diaprepes abbreviatus* larvae with TMOF that was dissolved in dimethyl sulfoxide (DMSO) caused 75 and 40% inhibition of weight gain and trypsin biosynthesis, respectively. Feeding TMOF (0.04%) in the diet to *D. abbreviatus* larvae caused significant decrease in the growth rate and trypsin biosynthesis (Yan et al. 1999). *Heliothis virescens* larvae synthesize both trypsin- and chymotrypsin-like enzymes in their guts (80 and 20% of gut proteases activity, respectively). Feeding or injections of *Aea*-TMOF into fourth instar *H. virescens* larvae caused 30 and 70% inhibition of trypsin biosynthesis in the larval gut, respectively (Nauen et al. 2001). Highly purified hemolymph fraction, crossreacted with *A. aegypti* TMOF antibodies indicating that *H. virescens* hemolymph contains a TMOF like peptide. Injecting *Aea*-TMOF and several analogues into second instar *H. virescens* stopped trypsin biosynthesis in the larvae (Table 3), and injecting a highly purified hemolymph fraction into *H. virescens* larvae caused 54% inhibition of trypsin biosynthesis in the larvae. *H. virescens* hemolymph when injected into *A. aegypti* females also inhibited trypsin biosynthesis in the female guts (45.6 to 100%) (Nauen et al. 2001). These results indicate that mosquitoes and *Lepidoptera* have close related TMOF like factor(s) that control trypsin biosynthesis.

Table 3. Effect of TMOF and its analogues on trypsin biosynthesis in *H. virescens*

Analogue	N	Inhibition of 50% trypsin biosynthesis (In_{50})
		(µM ± S.E.M.)
$YDPA(P)_6$	3	0.42 ± 0.026
YDPAPP	3	46 ± 2.8
FDPAP	3	145 ± 8
DPAP	3	190 ± 46
DPA	3	50 ± 2

Twelve groups of *H. virescens* (ten larvae per group) were injected with 0.5 µl of water containing TMOF and its analogues (1 µg to 1 ng). Twenty-four hours later, guts were removed and analyzed with BApNA for trypsin biosynthesis. Controls were injected with water and compared with non-injected controls. Results are expressed as 50% inhibition of trypsin biosynthesis and are average of three determinations ± S.E.M. (with permission, adapted from Nauen et al. 2001)

2.4 Genetic Characterization and Expression of TMOF

The effect of TMOF on the trypsin gene was first studied in *Neobellieria*. After injecting TMOF into these flies, the biosynthesis of trypsin mRNA was followed using Northern analysis (Borovsky et al. 1996). Feeding these flies a liver meal caused degradation of the endogenous trypsin early mRNA and synthesis of a new mRNA that corresponded with late trypsin biosynthesis associated with post meal digestion. In flesh flies that were injected with *Neobellieria* TMOF (10^{-9} M) the early mRNA did not disappear and the late mRNA that was synthesized was not translated. These results indicate that TMOF controls the translation of the late trypsin mRNA as would be expected for a hormone that is released after trypsin mRNA has already been transcribed (Borovsky et al. 1996). Injecting TMOF into female *A. aegypti* and *C. quinquefasciatus* following the late trypsin mRNA by RT-PCR and Northern blot analysis confirmed the observations that were reported for *Neobellieria* (Borovsky, unpublished observations). Similar results were obtained when 1–10 μg of TMOF and several analogues (DPA, FDPAP, YDPAP, YDPAPR) were injected into third instar *H. virescens* larvae (Borovsky and Butaye, unpublished observations); TMOF did not affect trypsin mRNA transcription but its translation, i.e., inhibition of trypsin biosynthesis, as was shown for *Neobellieria* (Borovsky et al. 1996).

2.5 The Effect of TMOF and Its Analogues on Insect Larvae

Feeding of [^{3}H]TMOF mixed with the blood meal to female *A. aegypti* stopped trypsin biosynthesis and inhibited egg development in the ovaries (Borovsky and Mahmood 1995). The [^{3}H]TMOF was also found circulating in the hemolymph indicating that TMOF traversed the mosquito gut into the hemolymph and bound a gut receptor on the hemolymph side of the gut (Borovsky et al. 1994, Fig. 1b). When *A. aegypti* and *C. quinquefasciatus* larvae were fed TMOF that was adsorbed onto yeast cells (188 μg TMOF/200 μg yeast cells), the larvae stopped synthesizing trypsin (88 and 91.7% inhibition, respectively, Table 4) (Borovsky and Meola 2004) and stopped growing. These results indicate that shutting off trypsin biosynthesis with TMOF can be used as a new approach to control larval growth and development, possibly leading to new biorational insecticides, which are desperately needed (Zaim and Guillet 2002). Nauen et al. (2001) have reported that *H. virescens* (Lepidoptera) larvae control their trypsin biosynthesis with a hormone that is similar to *Aea*-TMOF. Injections of *Aea*-TMOF or feeding it to fourth instar *H. virescens* larvae caused inhibition of trypsin biosynthesis and larval growth. TMOF also showed enhanced activity against the cotton boll weevil *Anthonomus grandis* by retarding its growth rate and enhanced its mortality and the black cutworm *Agrotis segetum* by retarding its growth rate. The hormone also affected ecdysteroid production in the prothoracic glands of the gypsy moth *Lymantria dispar*

Table 4. Effect of TMOF on trypsin biosynthesis in *Aedes aegypti* and *Culex quinquefasciatus* larvae

Species	Treatment	Trypsin (ng ± S.E.M./larva)	Inhibition (%)
A. aegypti	TMOF	1.17 ± 0.13[a]	88.0
	Yeast	9.87 ± 1.63[a]	
C. quiquefasciatus	TMOF	1.45 ± 0.36[b]	91.7
	Yeast	17.54 ± 2.68[b]	

Four groups of *Aedes aegypti* and *Culex quinquefasciatus* (24 larvae per group) 24 h after emergence were fed *Aea*-TMOF (188 µg) in microtiter plates containing 188 µl of water and 20 µg of Brewer's yeast. Forty-eight hours later, larvae were removed and analyzed for trypsin biosynthesis (Borovsky and Schlein 1988). Controls were fed only Brewer's yeast.
[a,b]Significant difference by two tailed Student's *t*-test $p < 0.01$ and $p < 0.0066$, respectively (adapted with permission from Borovsky and Meola 2004)

(Gelman and Borovsky, 2000). No effect was observed on *Spodoptera litura*, *Plutella xylostella* and *Cydia pomonella* indicating that these insects may have a different TMOF that regulates their digestion.

3 Molecular Biology Studies

3.1 Cloning and expression of *Aea*-TMOF by TMV, Chlorella, *Saccharomyces cerevisia,* Tobacco and Alfalfa plants

Although TMOF by itself is useful for establishing activity against insect larvae, the cost of chemical synthesis limits its usefulness as a commercial product. Consequently, several biological methods for producing TMOF were tried. TMOF was fused to the coat protein of tobacco mosaic virus (TMV) and the recombinant protein was fed to mosquito larvae causing inhibition of trypsin biosynthesis and larval mortality (Borovsky et al. 1998). When *H. virescens* larvae (fourth instars) were fed tobacco leaf discs infected with TMV-TMOF for 4 days a 2.3-fold decrease in weight, 2.2-fold decrease in trypsin and 2.6 decrease in chymotrypsin biosynthesis was observed (Borovsky D. results to be published elsewhere). These observations confirm that TMOF expressed on the coat protein of TMV infected tobacco plants can cause starvation of larval *H. virescens* after the larvae ate the recombinant tobacco leaves.

TMOF was also expressed in *Chlorella* sp. and the recombinant cells caused larval mortality within 72 h after feeding (Borovsky et al. 1998). Because *Chlorella* is a slow growing organism and the expression of the hormone was transient, TMOF and GFP-TMOF genes were cloned into a haploid strain of *S. cerevisiae* using homologous recombination and free plasmid expression (Nauwelaers and Borovsky 2002). Synthesis of TMOF and GFP-TMOF was followed by ELISA (Borovsky et al. 1992) and by fluorescence microscopy.

Mosquito larvae that were fed recombinant yeast cells that synthesized TMOF or GFP-TMOF in ng quantities did not digest the yeast cells and stopped growing and 38–83% of the larvae that were fed the recombinant yeast cells died. On the other hand, only 4–8% of larvae that were fed cells that were transformed with plasmids that were not carrying TMOF, or larvae that were fed Brewer's yeast died (Nauwelaers and Borovsky 2002).

Tortiglione et al. (2002) cloned and expressed six *Aea*-TMOF genes in transgenic tobacco plants and reported an increase in mortality of 20 to 33% in *H. virescens* that were fed on the transgenic plants. When TMOF was expressed in tobacco plants as a fusion with tomato prosystemin about 0.004% of the total soluble proteins was attributed to TMOF with low inhibition (4%) of *Heliothis virescens* larval growth (Tortiglione et al. 2003).

In collaboration with Professor Charles Powell (University of Florida, Indian River Research and Education Laboratory, Ft. Pierce, FL) and Dr. Robert Shatters (USDA, Ft. Pierce, FL) TMOF was cloned and expressed in alfalfa plants (results to be published elsewhere). To compare the potency of the transformed alfalfa plants, non-transformed alfalfa leaf discs were coated with recombinant yeast cells (*Pichia pastoris*) synthesizing TMOF and compared with non-transformed untreated wild type alfalfa. Larvae that fed on leaf discs that were treated with *Pichia*-TMOF didn't cause damage to the leaf discs (Table 5), and trypsin biosynthesis was inhibited by 84% (Table 6). From five recombinant plants that were tested only two plants C9 and B23 showed moderate leaf damage of 56 and 36%, respectively and inhibition of trypsin biosynthesis of 59 and 41.5%, respectively (Tables 5 and 6). Comparison between C9 that inhibited trypsin biosynthesis by 59% (Table 4) and C13 that inhibited the biosynthesis by 19.7% (results not shown) indicate that larvae cause less damage to C9 than to C13 leaf discs. These results indicate that for TMOF to be an effective insecticide and to fully protect plants the expression level will have to be increased.

Table 5. Leaf damage to recombinant alfalfa-TMOF by *H. virescens*

Larvae were fed:	N	Leaf damage (%)
TMOF (on leaf)	12	0
Recombinant plants		
C2	11	68
C9	12	56
C13	11	72
B5	12	78
B23	12	36

H. virescens larvae 24 h after emergence (first instar larvae) were fed for 3 days on alfalfa leaf discs that were transformed with pKylx *Aea*-TMOF plasmid using agrobacterium. Following the feeding, leaf discs were assessed for leaf damage. Control leaf discs with non-recombinant *E. coli* or yeast cells did not prevent leaf damage (results not shown) (unpublished observations from Borovsky et al.)

Table 6. The effect of feeding first instar *H. virescens* larvae on alfalfa TMOF

H. virescens larvae were fed	N	Trypsin (nmol/min/gut ± S.E.M.)	Inhibition (%)
TMOF (on leaf)	3	0.11 ± 0.001[a]	84
Recombinant plants			
C-2	8	0.72 ± 0.11	0
C-9	5	0.29 ± 0.05[b]	59
B-5	6	1.13 ± 0.07	0
B-23	4	0.34 ± 0.09[c]	41.5
Control (wild type)	6	0.79 ± 0.08[a,b,c]	0

H. virescens larvae 24 h after emergence (first-instar larvae) were fed for 3 days on alfalfa leaf discs that were transformed with pKylx *Aea*-TMOF plasmid using agrobacterium. Following the feeding, larvae were assayed for trypsin activity in their guts using BApNA (Borovsky and Schlein 1988). Control leaf discs with yeast cells without TMOF did not inhibit trypsin biosynthesis (results not shown). Same letters results indicate significant difference from control by Student's *t*-test
[a]$p < 0.0002$
[b]$p < 0.0003$
[c]$p < 0.003$ (Unpublished observations from Borovsky et al.)

4 Insect Resistance and Safety Issues

4.1 Potential Resistance Development to TMOF

A potential problem facing all insecticides is the development of resistance by the target insects by spontaneous mutation of the targeted gene and inactivation of the insecticide. The regulation of digestive proteases in the gut requires the synthesis and secretion of TMOF into the hemolymph, and binding to a midgut receptor. Without this mechanism, the insect's synthesis of digestive proteases will be unregulated, causing deleterious metabolic consequences for the insect. Because the regulation of trypsin requires both TMOF and its receptor to function, a point mutation in either of the TMOF or its receptor gene would also inactivate protease down regulation. For genetic mutations to effectively lead to insect resistance to TMOF, a simultaneous, complementary double mutation in both the TMOF and TMOF-receptor genes is required. The probability of a point mutation is estimated to occur at a frequency of 1 in 10^6 events. However, a simultaneous double mutation would occur with a frequency of 1 in 10^{12} events ($10^6 \times 10^6$). To maintain protease regulation the mutation to the TMOF gene and the TMOF receptor gene must be complementary with a frequency of 1 in 10^{14} based on a 1 in 400 probability that the mutations will yield complimentary amino acids in TMOF and the TMOF receptor (20×20 amino acids). Thus, resistance is unlikely to occur, even with the high reproductive capacity of insects and the selective pressure by exposure to TMOF.

4.2 Safety of TMOF

Safety, and the effect of TMOF and its analogues on non-target organisms, is the major concern of every new insecticide that is introduced to the environment. Thus, extensive testing has been initiated by the U.S. Environmental Protection Agency (Table 7) to determine if TMOF is suitable to be used in the environment (see Thompson et al. 2004 for details). The results of this testing indicates that TMOF can be degraded in vitro by leucine amino

Table 7. EPA Tier I safety tests requirements for the registration of TMOF in the USA

Test	OPPTS Protocol	Result
Health effects		
Acute oral toxicity	870.1100	LD_{50} > 9 g/kg
Acute dermal toxicity	870.1200	LD_{50} > 5 g/kg
Acute inhalation toxicity	870–1300	LC_{50} > 2.4 mg/L
Mutagenicity (1)		
Ames test	870–5100	NM to limit (5 mg/plate)
Mouse lymphoma	870.5300	NM to limit (5.36 mg/ml)
Chromosomal aberration	870.5375	NM to limit (5 mg/ml)
TMOF protease digestion (1)	IBI/NCSU	Degraded
Killed organism (yeast) testing	IBI/UF	100% killed
Ecological effects		
Acute avian oral toxicity (ducks)	885–4050	LD_{50} > 1.25gr/kg
Fish toxicity, feathered minnow	850–1400	LD_{50} > 1×10^6 dead cells/ml
Fish toxicity, sheephead minnow	850–1400	LD_{50} > 1×10^6 dead cells/ml
Mysid chronic toxicity	850–1350	LD_{50} > 1×10^6 dead cells/ml
Daphnid chronic toxicity	850–1300	LD_{50} > 1×10^6 dead cells/ml
Product performance testing:		
Mosquito, black fly and biting midge treatments	885.4050	Kills early instars of mosquito larvae
Additional Studies:		
Live TMOF-*Pichia* effect on *Daphnia magna*	850–1300	NNE > 10^6 cells/ml
Acute oral toxicity of live TMOF-*Pichia*	885–3050	LD_{50} > 2×10^8 live cells/ml
Survival of TMOF-*Pichia* in a simulated environment	IBI/UF	No growth of cells

Pichia pastoris cells expressing TMOF were assayed using protocols developed by the Office of Prevention, Pesticide and Toxic Substances (OPPTS) to find out the safety of using recombinant *Pichia*-TMOF or TMOF alone (1). *NM* No mutagenic, *LD* Lethal dose, *LC* Lethal concentration, *NNE* No negative effect, *IBI* Insect BioTechnology Inc., *NCSU* North Carolina State University, *UF* University of Florida, Florida Medical Entomology Laboratory. Several tests, procedures and results have been described by Thompson et al. (2004)

peptidase, a pancreatic enzyme found in the pancrease of vertebrates into $DPAP_6$, PAP_6 and eventually AP_6 (Thompson et al. 2004), which have a 6-fold lower TMOF activity (Table 2). TMOF when administered by gavage to male and female mice at 2.0 g dry weight/kg body weight produced no negative effects, and when male and female mallard ducks were treated by gavage with 1.25 g dry weight of TMOF/kg body weight/day for 5 days no toxic effects were noted 35 days after the last treatment. Application of TMOF to the shaved skin of male and female rabbits at 2.0 g/kg for 1–2 days did not affect the rabbits. Additional studies using *Daphnia magna* with recombinant *Pichia*

Table 8. Effect of *Aedes aegypti* TMOF on different insects

Insect	Effect
Mosquito	
Aedes aegypti, Aedes taeniorhynchus, Culex quinquefasciatus, Culex nigripalpus, Anopheles albimanus, Aedes atropalpus·	Larval starvation and death, inhibition of blood digestion and egg development in adults[b, c, d, e, f, g, h, i]
Stable fly, *Stomoxys calcitrans*	Inhibition of blood digestion and egg development in adults[e,h]
House Fly, *Musca domestica*	Inhibition of trypsin biosynthesis in adults[e]
Fleshfly, *Neobelliera bullata*	No effect on tryspin biosynthesis, the flesh fly has a unique TMOF[j]
Cat flea, *Ctenocehalides felis*	Inhibition of trypsin biosynthesis and blood digestion[e,h]
Midge, *Culicoides variipennis*	Inhibition of blood digestion in adults[e,h]
Sand fly, *Lutzomyia anthophora*	Inhibition of blood digestion[h]
Tobacco Budworm, *Heliothis virescens*	Larval starvation, inhibition of trypsin biosynthesis and food digestion[m]
Black Cutworm, *Agrotis segetum*	Larval growth retardation[a]
Cotton Boll Weevil, *Anthonomus grandis*	Larval growth retardation and enhanced[a] mortality
Citrus Weevil, *Diaprepes abbreviatus*	Larval growth retardation and inhibition of trypsin biosynthesis and food digestion[r]
Gypsy Moth, *Lymantria dispar*	Effect on ecdysteroid production in the prothoracic glands[k]
Diamondback Moth, *Plutella xylostella*	No apparent effect on larval growth[a]
Codling Moth, *Cydia pomonella*	No apparent effect on larval growth[a]
Colorado Potato Beetle, *Leptinotarsa decemlineata*	No apparent effect on growth[a]
Tobacco hornworm, *Manduca sexta*	No apparent effect on growth[q]

Based on unpublished observations from the authors's laboratory[a], and published observations: Borovsky 1988[b], Borovsky and Mahmood 1995[c], Borovsky and Meola 2004[d], Borovsky et al. 1990[e], 1991[f], 1992[g], 1993[h], 1998[i], Bylemans et al. 1994[j], Gelman and Borovsky 2000[k], Kelly et al. 1994[l], Nauen et al. 2001[m], Nauwelaers and Borovsky 2002[n], Tortiglione et al. 2002[o], 2003[p] Vanderherchen et al. 2005[q], Yan et al. 1999[r]

pastoris cells producing TMOF at a level of 10^6 yeast cells/ml (10 mg/ml) equivalent to TMOF (0.104 mg/ml) did not affect the mortality, growth, molting, time to first brood and production of viable neonates (Thompson et al. 2004) (Table 7). From these studies it appears that TMOF can be degraded by vertebrate digestive proteases and TMOF is not toxic to non-target organisms examined. Following these studies, the EPA issued a registration number 74411–1 for TMOF allowing it to be formulated into insecticides for the control of mosquito larvae. Since TMOF was shown to affect *H. virescens*, *Anthonomus grandis* and *Agrotis segetum* and not *Spodoptera litura*, *Plutella xylostella* and *Cydia pomonella*, we can assume that the hormone probably has a narrow species specificity (Table 8) and is safe to be used in the environment in recombinant plants against several agricultural pest insects.

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