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# Title: A viral chitinase enhances oral activity of TMOF

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Keywords: bioinsecticide; gut absorption; peptide; peritrophic membrane; transgenic plants.

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Abstract: In this study we investigate the combined effect on Heliothis virescens (Lepidoptera, Noctuidae) larvae of Aedes aegypti-Trypsin Modulating Oostatic Factor (Aea-TMOF), a peptide that inhibits trypsin synthesis by the gut, impairing insect digestive function, and Autographa californica nucleopolyhedrovirus Chitinase A (AcMNPV ChiA), an enzyme that is able to alter the permeability of the peritrophic membrane (PM). Aea-TMOF and AcMNPV ChiA were provided to the larvae by administering transgenic tobacco plants, co-expressing both molecules. Experimental larvae feeding on these plants, compared to those alimented on plants expressing only one of the two molecules considered, showed significantly stronger negative effects on growth rate, developmental time and mortality. The impact of AcMNPV ChiA on the PM of H. virescens larvae, measured as increased permeability to molecules, was evident after five days of feeding on transgenic plants expressing ChiA. This result was confirmed by in vitro treatment of PM with recombinant ChiA, extracted from the transgenic plants used for the feeding experiments. Collectively, these data indicate the occurrence of a positive interaction between the two transgenes concurrently expressed in the same plant. The hydrolytic activity of ChiA on the PM of tobacco budworm larvae enhances the permeation of TMOF molecules to the ectoperitrophic space, and its subsequent absorption. The permeation through the paracellular route of Aea-TMOF resulted in a spotted accumulation on the basolateral domain of enterocytes, which suggests the occurrence of a receptor on the gut side facing the haemocoel. The binding of the peptide, permeating at increased rates due to the ChiA activity, is considered responsible for the enhanced insecticide activity of the transgenic plants expressing both molecules. These data corroborate the idea that ChiA can be effectively used as gut permeation enhancer in oral delivery strategies of bioinsecticides targeting haemocoelic receptors.

Dear Rene,

We have revised the manuscript as requested. Below are the reviewers comments and for each issue raised our remedial action or an explanation to address their concerns is reported in bold.

I think that this version is certainly improved, and we will be glad to consider any further suggestion you may have.

All the best,
Franco

Reviewer #1: Fiandra et al.

In this manuscript Fiandra et al present some interesting data on apparent enhancement of TMOF action by chitinase. Overall, this is a sound study that will be improved with the following modifications:

1. What is the mechanism of action of TMOF? This should be introduced upfront. The action of TMOF in inhibiting trypsin synthesis and impeding larval digestive processes is key to the logic behind these experiments. Not until page 16 is this information provided for the reader! This information should actually be included in the abstract. Clearly introduce TMOF.

   **The information requested has been added in the manuscript (Abstract and Introduction).**

2. Page 4 What is the size of TMOF? Why is it restricted by the peritrophic matrix? What is the size exclusion limit of the PM? Provide some specific details here - this is important to justify the use of chitinase.

   **All the details about the size of TMOF and its permeability through the peritrophic membrane have been provided (Introduction).**

3. Chi-HDEL - the original paper should be cited for this, and the KDEL-HDEL mutation should be explained in the context of the mutated chitinase not being retained in the ER. This is important information that the reader should know.

   **The ChiA coding sequence was fused to the sequence coding for the HDEL, the ER retrieval signal peptide (**Napier et al., **Journal of Cell Science, 102: 261-271). The ER localization of recombinant proteins via the addition of KDEL or HDEL increases by one or two orders of magnitude the accumulation of foreign protein in transgenic plants. We think that this piece of information is not relevant in the context of the present paper. In addition, all the details related to the Chi A engineering and subsequent cloning are described in Corrado et al., 2008, as indicated in the text.**

4. There are several other examples where chitinase has been shown to enhance the insecticidal action of a toxin (e.g. Bt toxins, protease - see W. Fang et al 2009 J Invertebr Pathol.), this is the not the first example. These other examples should be discussed, rather than creating the impression that this is the first case of such potentiation.

**The discussion now includes a sentence and a couple of references that address the issue raised by the reviewer on the presence in the literature of studies reporting the combined expression of chitinase and other insecticidal molecules.**

5. Table 1 could be provided as supplementary information (should read "detection" rather than "diction").

   **We corrected the caption, but we have maintained the table in the text, as we would consider supplementary material large pieces of information, not easy to incorporate in the manuscript but useful.**

6. Data presented in Figures 3 and 4 should be combined and presented in a Table. (There is no reason to use different colored bars if only one parameter is being measured here).

   **We followed the reviewer's suggestion.**

7. Figure 7 - images of control treatments, and labels should be added (e.g. gut, lumen, columnar cell, microvilli etc).

   **Figure 7 has been corrected on the basis of reviewer's suggestion. We have specified in figure 7 caption that control midgut (incubated in the absence of FITC-TMOF) did not emit fluorescence when excited at the wavelength used.**

8. Figure legend: Fig 2 legend needs statistical analysis. All legends lack a concluding statement.

   **The statistical analysis has been added.**

9. Table 2 title "fourth instars"

   **We have corrected in the title of table 2 "fourth instars"**

Editorial corrections

1. Abstract, provide abbreviations for TMOF and AcMNPV ChiA AFTER the full name, not before.

   **We have provided the abbreviations for TMOF and AcMNPV ChiA after the full name in the Abstract.**

2. P3 line 8 "the insecticide molecules in the environment"

   **We have corrected the sentence on the basis of reviewer's suggestion.**

3. P3 "predator derived toxins"? Be more specific here.

   **We feel that the terms used are informative and we do not see a real need for change, unless a better word is suggested.**

4. P4 line 1 worthy of further

**Done.**

5. P4 line 4. Cite the following review on uptake of macromolecules into the hemocoel from the gut: Jeffers, L. A.; Michael Roe, R., The movement of proteins across the insect and tick digestive system. J Insect Physiol 2008, 54, 319-332.

   **We have added the citation suggested by the reviewer**.

6. P4 Cite the original work on the AcMNPV chitinase (R. Hawtin et al) rather than a review paper.

   **We preferred not to change the reference and maintain a review, because we refer not only to AcMNPV chitinase, but to a number of studies addressing the impact of different viral enzymes on PM structure.**

7. Page 5 and page 12, 15 replace "evidenced" by "showed"

8. Northern and Western should not have capital letters.
9. P7 Waring Blender
10. P7 three lines from end "surviving larvae"
11. P9 l3 known amounts
12. P9 line8 Explain here that the TMOF is synthesized.
13. P10 first sentence should be combined with previous sentence.

**7-13: all corrections done.**

14. P11. Was TMOF (GenScript) mentioned on p9 also FITC-labeled? Be consistent

**FITC-TMOF was only used for the analysis of whole mount midguts by confocal microscopy (P11), while the TMOF mentioned on P9 was not labelled and it was detected by Zonal Capillary Electrophoresis, as indicated in Material and Methods . Both compounds were purchased by GenScript Coorporation.**

15. P11. "with A coverslip.scanNING microscope"
    **Corrected as requested**.

16. P 11 last line "and excluded events of post-transcriptional gene silencing". You cannot claim this, given that transgenic expression can out-express the plant RNAi response (see J Baum et al, Nature Biotechnology paper on rootworm control by dsRNA)

**We agree with the reviewer and modify the text accordingly.**

17. P12 "immunodetection of TMOF was not performed due to its small molecular mass". Too small to detect on a gel? Doubtful if an appropriate % is used.

**The *polyTMOF* synthetic gene expressed in the tobacco parental plants encodes for a precursor which is likely to be processed in tobacco cell (Tortiglione et al., 2002, quoted in the manuscript). As a consequence of the processing, several products of different sizes may be produced. Western analyses of peptides extracted from transformed plants detected no peptide corresponding to any of the expected sizes. This may be due to the small size of the peptides, their presence in very low amount, or to the unsuitability of the antibodies raised**

**against the synthetic TMOF to recognize both poly-TMOF or its partial cleavage products. For these reasons we preferred to monitor the expression of** the *polyTMOF* synthetic gene **only at transcriptional level.**

18. P12 line 8 "included the weight of the pupae"
19. P12 two lines from end "showed significantly"
20. P13 line 1 "Was affected by"
21. P13 line 7 "resulted in fragility"; line 15 "performed with larvae.because the PM was too small".
22. P14 Was the lack of TMOF detection in hemolymph discussed? Seems you could say more here.
23. P14 line 13 "prevented from reaching" ; line 16 "TMOF promoted by"; line 17 "were located nearby"; line 19 "occurrence of discrete signals on"
24. P15 line 5 "suggesting binding.sites on the basolateral"
25. Discussion line 1, "In recent"; line 3 "from public opinion for new"; line 4 "agents and their possible"
26. P17 line 3 "regulate physiological", "suggested a long"; line 8 "and much experimental evidence indicates that"; line 13 "worthy of further"
27. P18, 5 lines from end "as in B. mori"
28. Fig 3 legend Replace "alimented" with "fed"

**18-28: all corrections done**

Reviewer #2: IB-D-10-00052

Very interesting paper that I recommend for publication in IBMB. But before, there are two points that need the attention of the authors.

I would encourage the authors to cite also the cloning and expression of TMOF on the coat protein of TMV as published by Borovsky et al. in PNAS 2006. The latter innovative paper showed that Heliothis virescens trypsin activity and growth were effectively affected by TMOF, and that high amounts of TMOF alone as produced by the TMV in the leaves of the tobacco plant can produce high insecticide effects. The current paper reported that the transgenic plants poorly affected the insects, but can this be due to the fact that these plants poorly synthesized the hormone peptide and thus were not very effective. I suggest that the authors discuss their interesting results in relation to the PNAS paper.

**This has been done.**

Another thing that is somewhat bothersome to me was the fact that chitinase by itself causes mortality and growth inhibition. I suggest the authors comment and explain this. I think that the chitinase may also disrupt the epithelial cells causing perhaps holes that affect the insect even without TMOF.

**It is has been widely demonstrated how chitinase alone is able to induce an alteration of larval fitness and mortality in Lepidoptera, by affecting midgut physiology (Rao et al., 2004; Corrado et al., 2008). The degradative action of chitinase on the chitin mesh of peritrophic**

**membrane results in an alteration of the compartmentalization of the digestive enzymes between the endo- and ectoperitrophic spaces, which is known to be critical for the efficiency of digestion in lepidopteran larvae (Terra, W.R., 2001. Arch. Insect Biochem. Physiol. 47, 47–61). We have added in the manuscript (Discussion) this information.**

**On the other hand, an effect of chitinase on the epithelial cells is not feasible, since cell apical brush border membranes are not a target for this enzyme.**

Insect Biochemistry and

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# A viral chitinase enhances oral activity of TMOF

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Running title: ChiA-TMOF oral activity

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**Abstract**

In this study we investigate the combined effect on *Heliothis virescens* (Lepidoptera, Noctuidae) larvae of *Aedes aegypti*-Trypsin Modulating Oostatic Factor (*Aea*-TMOF), a peptide that inhibits trypsin synthesis by the gut, impairing insect digestive function, and *Autographa californica* nucleopolyhedrovirus Chitinase A (*Ac*MNPV ChiA), an enzyme that is able to alter the permeability of the peritrophic membrane (PM). *Aea*-TMOF and *Ac*MNPV ChiA were provided to the larvae by administering transgenic tobacco plants, co-expressing both molecules. Experimental larvae feeding on these plants, compared to those alimented on plants expressing only one of the two molecules considered, showed significantly stronger negative effects on growth rate, developmental time and mortality. The impact of *Ac*MNPV ChiA on the PM of *H. virescens* larvae, measured as increased permeability to molecules, was evident after five days of feeding on transgenic plants expressing ChiA. This result was confirmed by *in vitro* treatment of PM with recombinant ChiA, extracted from the transgenic plants used for the feeding experiments. Collectively, these data indicate the occurrence of a positive interaction between the two transgenes concurrently expressed in the same plant. The hydrolytic activity of ChiA on the PM of tobacco budworm larvae enhances the permeation of TMOF molecules to the ectoperitrophic space, and its subsequent absorption. The permeation through the paracellular route of *Aea*-TMOF resulted in a spotted accumulation on the basolateral domain of enterocytes, which suggests the occurrence of a receptor on the gut side facing the haemocoel. The binding of the peptide, permeating at increased rates due to the ChiA activity, is considered responsible for the enhanced insecticide activity of the transgenic plants expressing both molecules. These data corroborate the idea that ChiA can be effectively used as gut permeation enhancer in oral delivery strategies of bioinsecticides targeting haemocoelic receptors.

*Key-words*: bioinsecticide, gut absorption, peptide, peritrophic membrane, transgenic plants.

## Introduction

The reduction of chemical insecticide use is one of the major issues for safe food production. The importance of this objective in modern agriculture has fostered significant research efforts towards the development of innovative technologies based on the use of biological control agents (Bale et al., 2008), natural insecticides, which include small organic molecules (Dayan et al., 2009) and peptide or protein toxins, deriving from plants and insect natural antagonists (Whetstone and Hammock, 2007).

The success and safety of pest management technologies largely depends on the efficacy of the delivery methods used to distribute the insecticide molecules in the environment. When dealing with peptide/protein toxins, the choice of the most appropriate delivery vector is directed by the localization of the receptor to be targeted, which can be in the gut or behind the gut wall. The delivery of biopesticides through oral ingestion, for example by transgenic plant expression, is considered more appropriate for molecules exerting their activity in the gut, while those targeting haemocoelic receptors are more efficiently delivered via insect-specific symbionts and pathogens (Inceoglu et al., 2006; Whetstone and Hammock, 2007). This conceptual dichotomy is largely motivated by the assumption that most macromolecules are unable to pass across the gut barrier in significant amounts, but can easily cross it if expressed in recombinant baculoviruses (Liu et al., 2006). However, a growing number of exceptions to this assumption can be found in the literature, with cases of parasitoid (Maiti et al., 2003) and predator derived toxins (Liu et al., 2006), which have conferred a significant protection level when expressed in transgenic plants. However, none of these studies provided direct evidence that the toxins passed from the gut lumen to the haemocoel of the target insects (reviewed in Liu et al., 2006), even though they indirectly indicated that gut absorption of macromolecules in insects is likely possible and of practical value for pest control. The possibility of delivering intrahaemocoelic toxins with food opens very interesting new

perspectives in the field of biotechnology for insect control, and is certainly worthy of further research efforts.

In spite of these promising perspectives, the study of the physiological mechanisms mediating the absorption of macromolecules in the insect gut has received limited attention (Jeffers and Roe, 2008). Over the last few years, we have contributed to this research area, by focusing our interest on the absorption pathways of peptides and proteins in the midgut of lepidopteran larvae, demonstrating that the paracellular route (Fiandra et al., 2006) is mostly exploited by small peptides (Fiandra et al., 2009), while transcytosis is the main route of entrance for proteins (Casartelli et al., 2005; 2007). The absorption pathway of peptides can be modulated by manipulating the intracellular concentration of cAMP and $Ca^{++}$ (Fiandra et al., 2006); the ligand specificity of the receptor involved in the internalization of albumin can be exploited for promoting the uptake of fusion proteins, bearing toxic domains along with domains which are involved in the receptor-mediated endocytosis (Casartelli et al., 2008). This information provides the background on which new strategies for enhancing the rate of gut absorption can be developed.

However, the gut epithelium is only one of the two major intestinal barriers to be crossed by ingested macromolecules, and the peritrophic membrane (PM) represents the first physical layer with pores that discriminates the passage of large molecules (Lehane, 1997; Barbehenn, 2001). In *Bombyx mori* larvae, for instance, the PM was largely permeable to methylene blue, a molecule with a molecular mass of 320 Da, and almost impermeable to PEG 4000, while the Trypsin Modulating Oostatic Factor from *Aedes eagypti* (*Aea*-TMOF) had an intermediate permeability coefficient, in line with its molecular mass (1005 Da) (Fiandra et al., 2009). Therefore, the structural disruption of the PM can facilitate the passage of molecules, as naturally occurs in the case of infection by baculoviruses, which use specific metalloproteases for disrupting the peritrophic membrane, to allow the contact of viral particles with midgut epithelial cells (Slavicek and Popham, 2005; Liu et al., 2006).

In the framework of a coordinated effort towards the development of new delivery strategies and combinations of bionsecticides, we discovered that the Chitinase A (ChiA) of *Autographa californica* nucleopolyhedrovirus (*Ac*MNPV), which has a key-role in the post-mortem liquefaction of the infected larval host cadaver (Bonning, 2005), determined structural alterations on lepidopteran larvae PM (Rao et al., 2004), and had a significant negative effect on insect biological performance and survival when the recombinant protein was delivered either with artificial diet or with transgenic plants (Rao et al., 2004; Corrado et al., 2008). The same studies also clearly showed a strong increase of the permeability to molecules of the PMs treated *in vitro* with ChiA. These results stimulated the idea of using ChiA in combination with *Aea*-TMOF, which targets receptors expressed in the basolateral membrane of epithelial midgut cells and causes the inhibition of trypsin synthesis, thus impairing the insect digestive processes (Borovsky et al., 1994; Nauen et al., 2001; Borovsky and Meola, 2004). *Aea*-TMOF exerts mild insecticide activity on *Heliothis virescens* larvae when expressed in transgenic tobacco plants (Tortiglione et al., 2002; 2003), and negatively interferes with larval growth of the tobacco budworm (*Heliothis virescens*), when fused to Tobacco Mosaic Virus coat protein (Borovsky et al., 2006).

In this study we demonstrate that tobacco plants co-expressing both *Aea*-TMOF and *Ac*MNPV ChiA show a significantly stronger impact than parental lines, expressing only one of the two genes, on the development and survival of the tobacco budworm larvae, which is associated with a higher permeability to *Aea*-TMOF of the peritrophic membrane of larvae fed on transgenic plants. This corroborates the hypothesis that the use in tandem of gut permeating agents and insecticide molecules targeting haemocoelic receptors can result in a more efficient insect control activity, as a consequence of functional complementation of the molecules used and reduced risk of resistance in the target population.

## Material and Methods

*Production of hybrid tobacco plants co-expressing AcMNPV ChiA and polyTMOF*

Transgenic tobacco plants constitutively expressing the *polyTMOF* synthetic gene (line R1-2) and *ChiA* gene (ChiA HDEL line 9) were obtained as reported in Tortiglione et al. (2002) and Corrado et al. (2008), respectively. Both transgenic lines were screened for resistance to kanamycin on Murashige Skooge medium, supplemented with 100 mg/l kanamycin, and then transferred to soil and grown under containment glass house conditions. Crosses of the two transgenic lines originated tobacco genotypes co-espressing both genes, *ChiA* and *polyTMOF*, which, for simplicity reasons, are hereafter denoted as hybrids.

*Molecular characterization of tobacco hybrid genotypes*

The presence of both polyTMOF and ChiA mRNA in the tobacco hybrids was detected by Northern blot, with the appropriate cDNA probes, as previously described (Tortiglione et al., 2002; Corrado et al., 2008).

Furthermore, the expression of the ChiA protein was monitored by Western blot. Total proteins were isolated from leaves, quantified and resolved by SDS-PAGE (Sambrook et al., 1989). Western analysis was carried out on 40 µg of water soluble proteins, using as primary antibody the anti-myc (Santa Cruz Biotechnology, CA), diluted 1:500, and anti-rabbit IgG conjugated with horseradish peroxidase, diluited 1:2000, as secondary antibody, according to the procedures already published (Corrado et al., 2008).

*ChiA purification from tobacco transgenic plants*

The recombinant ChiA protein was purified from transgenic tobacco leaves as described in Di Maro et al. (2010). The enzymatic activity of the isolated protein was assayed using the substrate 4-methyl-umbelliferyl-β-D-N-N'-N''-acetyl-chitotriose [4MU-$(GlcNAe)_3$, Sigma–Aldrich, Italy] for the detection of endo-chitinolytic activity, as reported elsewhere (McCreath and Gooday, 1992;

Rao et al., 2004). Briefly, for the ChiA protein purification procedure, leaves were homogenized in 1x PBS, in presence of EDTA 5 mM, PMSF 1 mM and PVP-40 1.5%, by 20-s bursts at full power using a Waring Blender (Waring Products, CT, USA). The proteins were subjected to ammonium sulfate precipitation, followed by ion exchange and gel filtration chromatography. The purification was monitored by analyzing the chromatography fractions by SDS-PAGE and Western blot.

ChiA, separated by SDS-PAGE, was transferred onto PVDF membrane and directly subjected to Edman degradation on a Procise Model 491 sequencer (Applied Biosystems), for N-terminal sequencing, as previously described (Di Maro et al., 2001).

*Feeding bioassay on transgenic plants*

The insecticidal activity of transformed tobacco plants was assayed *in vivo* on larvae of the tobacco budworm *Heliothis virescens*. Selected transformants expressing either the TMOF peptide (line polyTMOF R1-2) or ChiA (line ChiA HDEL 9) or both of them and control plants (NN) were daily supplied as leaf disks to newly hatched larvae. Experimental larvae were singly maintained at 29 ± 1 °C, in multiwell plastic trays, bottom lined with a thin layer of a 2% agar solution and closed with transparent plastic covers provided by the commercial supplier (CD International). Two different well sizes were used: 4×4×2 cm (for instars 1st–4th) (CD International BIO-RT-32) and 8×8×2 (for 5th instars) (CD International BIOSMRT- 8). Larvae were weighed every other day, starting on day 4 from the beginning of the bioassay. Mortality was daily checked during the whole larval feeding period. In each of the 4 replicate, 16 larvae were assayed for each treatment.

The larval development was compared by combining the larval growth and survival into a single parameter, the total larval biomass, calculated every other day, as the sum of the weight of the surviving larvae in each treatment. The growth curves of the larval biomass of individuals fed on control or transformed plants were compared by Repeated Measures Analysis of Variance (Sokal and Rohlf, 1995). The interaction of diet and the within-factor time was tested using linear, quadratic and cubic order polynomial contrasts, in order to assess differences in the slope of the

growth curves. Compound symmetry was checked by Huynh-Feldt statistics (Systat 12, Systat Software Inc.).

Developmental times and survival rates were analyzed by One-Way Analysis of Variance, and mean comparison (Tukey's test) was performed when statistical significance ($\alpha$=0.05) occurred. Percentages were arcsine transformed before analysis (Zar, 2009). Mean percentages presented in figures were transformed back into proportions after analysis. Because the confidence limits are not symmetrical about the means when expressed again in proportions, in the result section we report the mean values and the mean values plus and minus the SE.

All data analyses were performed with the statistical package Systat 12 (Systat Software Inc.).

*Evaluation of the peritrophic membrane permeability*

To assess the impact of feeding on transgenic plant lines, experimental larvae of *H. virescens,* fed with artificial diet until the end of the third instar, were divided into four groups of 16 larvae each and then reared from the first day of the fourth instar on the following tobacco genotypes: NN, polyTMOF R1-2, ChiA HDEL 9 and hybrids. Their survival and body weight were monitored during the fifth instar at 120, 132 and 144 hours since the beginning of treatment. After 132 h, randomly selected larvae from each experimental group were used to study *in vitro* the permeability of their peritrophic membrane. The PM was isolated as described in detail in Rao *et al*. (2004). Briefly, the PM was carefully extracted from the dissected midgut, and cut longitudinally on a thin cotton gauze, which maintained the PM extended, avoiding its fluttering once mounted in the experimental apparatus. A portion of the PM was mounted as a flat sheet in Ussing chambers (World Precision Instruments, Berlin, Germany), with an exposed surface area of 12,6 $mm^2$. Thus, the PM separated the endoperitrophic and ectoperitrophic compartments, both filled with 500 µl of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$ and 1.4 mM $KH_2PO_4$, pH 7). The PMs explanted from experimental larvae fed on different plant lines were incubated for 90 minutes, in

the presence of 1 mg/ml methylene blue in the endoperitrophic compartment. The total amount of dye diffused to the ectoperitrophic compartment was collected and determined spectrophotometrically (Ultrospec 3000 Pharmacia Biotech, Cambridge, UK), at the wavelength of 661 nm. A calibration curve was carried out with known amounts of the molecule dissolved in the incubation buffer.

To demonstrate that the increased permeability was due to *Ac*MNPV ChiA produced by transgenic plants, this enzyme, extracted and purified from ChiA HDEL 9 genotypes, was used in PM permeability assays to TMOF. PMs were explanted from larvae continuously reared on artificial diet. The flux of TMOF (synthesized by GenScript Coorporation, USA) was measured by adding the peptide (1 mg/ml) to the endoperitrophic compartment in the absence (control) or in the presence of 40 µg/ml ChiA and by recovering the solution in the ectoperitrophic compartment after 90 min of incubation. The amount of permeated TMOF, detected by Zonal Capillary Electrophoresis (Beckman Coulter P/ACE MDQ Capillary System), was determined using a suitable calibration curve.

The calculated methylene blue and TMOF flux values were expressed as nmol/cm$^2$/h. Mean values were compared by Student's *t* test.

*Detection of TMOF in the haemolymph of experimental larvae*

Experimental larvae fed for 132 h on polyTMOF R1-2 and hybrid tobacco genotypes, as described in the previous section, were used for haemolymph collection. Fifty µl of haemolymph, collected from the cut proleg of 5 larvae using capillary glass tubes, were diluted 1:10 in methanol and stored at -20°C. Samples to be analysed were centrifuged at 4,000 rpm for 10 min and cleaned up on a reversed phase Strata C18-E 500 mg cartridge (Phenomenex, Torrance, CA, USA). A volume of 500 µl was loaded on the cartridge, previously conditioned with methanol (3 ml) and water (3 ml). The column was then washed with water (3 ml), and eluted with 3 ml of pure methanol. The eluate was dried under a gentle nitrogen stream, dissolved in 50 µl of methanol,

centrifuged at 12,000 rpm for 3 min and used for liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) analyses. Chromatographic separation was obtained using an HPLC apparatus, equipped with two Micropumps Series 200 (Perkin Elmer, Shellton, CT, USA), a UV/VIS detector series 200 set at 220 nm and an Aquapore RP300 C8, 7 μm 220 × 2.1 mm column (Brownlee, CT). The eluents were: A: $H_2O$, 0.1% formic acid; B: $CH_3CN$, 0.1% formic acid. The gradient program was as follows: 0–50 % B (13 min), 50–100% B (3 min), 100 % B (4 min), 100-0 % B (5 min) at a constant flow of 0.2 ml/min. Injection volume was 20 μl and all samples were centrifuged, before the analysis, at 12,000 rpm for 3' using a centrifuge 5415 R (Eppendorf, Germany).

MS and MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada), equipped with a TurboIonSpray. Acquisition was in positive ion mode, in MRM (Multiple Reaction Monitoring). The analyses were performed using the following settings: drying gas (air) was heated to 350 °C, capillary voltage (IS) was set at 5500 V. The declustering potential (DP), focus potential (FP) and the collision energy (CE) were optimized infusing directly into the mass spectrometer a TMOF peptide standard solution (10 μg/ml) at a constant flow rate of 6 μl/min using a model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). The detection limit (LOD with a signal to noise ratio of 3) was 2 ng/ml. TMOF peptide showed an $[M+H]^+$ ion at *m/z* 1047.6 and a $[M+2H]^{2+}$ ion at *m/z* 524.6. The LC/MS/MS characteristics of TMOF are reported in Table 1.

The recovery of TMOF was about 100% and was assessed by spiking a sample of haemolymph with a solution of standard TMOF, at a final TMOF concentration of 22 ng/ml.

*Incubation of larval midguts in Ussing chambers and fluorescence analysis of FITC-TMOF in whole mount tissues*

Larvae reared on the artificial diet were sacrificed on the second day of the last instar, and the midgut, deprived of the peritrophic membrane, was mounted as a sheet in the Ussing chambers, as

previously described (Fiandra *et al*., 2006). Tissues were perfused with 2.5 ml of the following physiological solution (in mM): 5 $CaCl_2$, 24 $MgSO_4$, 20 Kgluconate, 190 sucrose and 5 Tris adjusted to pH 7 in the haemolymph compartment, or 5 CAPS adjusted to pH 10 in the luminal one. The solutions, connected *via* Ag-AgCl voltage electrodes in series with agar bridges (3 M KCl, 5.5% Agar) to a voltmeter for the measurement of the transepithelial voltage ($V_t$), were circulated by gas influx (100% $O_2$) and maintained at 25°C in water-jacketed reservoirs.

One hundred thirty μM of FITC-TMOF (GenScript Coorporation, USA) was added to the luminal solution, which contained a cocktail with the following peptidase inhibitors: 1 mM 1-10 phenanthroline, 10 μM bestatine and 10 μM amastatine (Sigma-Aldrich, Italy). After 2 hours of incubation, the midgut was removed from the Ussing chambers, washed five times with the physiological solution and fixed for 30 minutes in 4% paraformaldehyde. After further five rinsing in PBS, the samples were mounted in DABCO (Sigma)-Mowiol (Calbiochem). The tissues covered with a coverslip were examined with a confocal laser scanning microscope imaging system (CLSM TCS SP2 AOBS- Leica Microsystems, Heidelberg, GmbH, Germany), equipped with an argon-krypton laser and an UV laser.

**Results**

*Production and characterization of tobacco plants co-expressing ChiA protein and polyTMOF peptide*

Tobacco plants co-expressing ChiA and TMOF peptides, obtained by crossing the two parental transformants, were subjected to Northern blot analysis, to monitor the expression of the two transgenes. The hybridization of the total RNA extracted from the hybrids showed the presence of both transcripts of the expected size, in 5 out of the 10 hybrids analyzed (Fig. 1A). The presence of the two bands, one of 0.4 kb (*polyTMOF* transcript) and the other of 2 kb (*ChiA* transcript), separately present in the parental lines and absent in the control plants, confirmed the success of the hybridization between transgenic lines. The presence of the recombinant ChiA protein in the

hybrids was showed by Western blot (Fig. 1B). A single band, with an estimated molecular mass of 60 kDa, was detected in all the lines where the ChiA gene was actively transcribed. The immunodetection of TMOF was not performed, for the technical reasons already discussed elsewhere (Tortiglione et al., 2002).

*Biological performance and survival of H. virescens larvae fed on transformed tobacco plants*

We compared the larval development by combining the growth and survival into a single parameter, the total experimental biomass, which, after the maximum larval weight was attained, included also the weight of the pupae. The effect of the experimental conditions considered on this parameter is shown in Figure 2. The mean total experimental biomass obtained on the transformed and control plants was different in a highly significant way (analyzed with Repeated Measures ANOVA until day 10: $F=8.824$; df 3, 12; $p=0.002$). The interaction between diet and time was also highly significant ($F=8.802$; df 9, 36; $p<0.001$), indicating that the pattern of the total biomass growth curves obtained with transgenic and control tobacco plants were significantly different. Polynomial contrasts showed that the linear ($F=10.731$; df 3, 12; $p=0.001$) and quadratic ($F=5.327$; df 3, 12; $p=0.014$) components accounted for the differences among the curves. The lowest value of total biomass growth curve was registered for the experimental larvae fed on the hybrid transformed plants. On day 8, the total biomass of the larvae fed on control plants was significantly higher than the total biomass of both ChiA HDEL 9 and hybrid fed larvae (Tukey's test, $\alpha=0.05$). On day 10, the total biomass of larvae fed on control plants was significantly higher than that registered for all the other three experimental groups (Tukey's test, $\alpha=0.05$).

The mean time to the completion of development, until the adult emergence , also differed significantly ($F=5.949$; df 3, 122; $p=0.001$) among the different experimental treatments, as well as the pre-adult mortality rate (Table 2) ($F=8.175$; df 3, 12; $p=0.003$). Larvae fed on the hybrid transformed lines showed significantly longer development compared to control, and also a significantly lower survival rate (Tukey's test, $\alpha=0.05$).

*Peritrophic membrane permeability affected by ChiA ingestion*

To demonstrate that feeding of tobacco budworm larvae on transgenic plants expressing ChiA enhances the permeability of PM, we determined *in vitro* the flux of methylene blue through the PMs isolated from larvae reared on the different plant genotypes, starting from the first day of the fourth instar. The reduction of the rearing time on transgenic plants was motivated by the need of using tobacco budworm larvae exposed to the ChiA activity for shorter time intervals, as longer exposures, starting from egg hatching to the mature larvae, resulted in fragility of PMs and in poor synchronization of the biological material. The impact of feeding on body weight (Table 3) was evident even for the shorter time intervals considered, and in agreement with the trend obtained in the experiment reported in Figure 2. Moreover, an increase of the mortality rate was observed after 120 h for ChiA HDEL 9 (12.5%) and hybrids (19%).

The permeability of the PMs was measured in fifth instar larvae fed on the experimental leaves for 132 h, because at this time of development the dimensions of the PM were wide enough to avoid any leakage between the two compartments in the Ussing chambers. The experiments could not be performed with larvae fed on the hybrid plants because the PM was too small. The PMs were incubated in the presence of methylene blue in the endoperitophic compartment: this dye is a reliable and easily quantified tracer of PM permeability (Rao et al., 2004; Corrado et al., 2008; Fiandra et al., 2009). Figure 3 shows that the flux of methlylene blue was significantly higher than that of controls in larvae reared on ChiA-expressing tobacco plants, whereas, as expected, the flux across the PMs explanted from larvae fed with the polyTMOF R1-2 line did not differ from that of controls.

To demonstrate that the increased permeability was due to the hydrolytic activity of *Ac*MNPV ChiA on the PM chitin mesh, this enzyme was extracted and purified from ChiA HDEL 9 genotypes and its activity tested on the permeability of PMs of control larvae isolated in Ussing

chambers. Figure 4 shows that PM incubation with 40 µg/ml ChiA in the endoperitrophic compartment caused a significant increase of TMOF flux compared to control.

*Detection of TMOF in the haemolymph of the experimental larvae.*

The analysis of haemolymph, collected 132 h after the onset of feeding on experimental plants, showed that TMOF was undetectable in all samples.

*In vitro distribution of permeating FITC-TMOF within the midgut epithelium.*

The biological effects of TMOF recorded on tobacco budworm larvae (Nauen et al., 2001; Tortiglione et al., 2002), enhanced by ChiA as here reported, and the apparently contradictory absence of a detectable amount of TMOF in the haemolymph of affected larvae, prompted us to further investigate the TMOF fate after ingestion. TMOF receptors in mosquitoes are located on the basolateral plasma membrane of the midgut epithelium (Borovsky et al., 1994): if similar receptors were also present in *H. virescens*, the observed biological effects could be explained by the capture of the low permeating amounts, which are, then, prevented from reaching the haemolymph. We have shown that TMOF permeability across the midgut is very low and most part of its transfer takes place by diffusion through the paracellular route across the septate junction (Fiandra et al., 2009). The enrichment in this microenvironment of TMOF promoted by ChiA could more easily exert a detectable effect if the target receptors were located nearby. To assess this, we observed the distribution of FITC-TMOF molecules permeating *in vitro* a perfused midgut, looking for the possible occurrence of discrete signals on the plasma membrane, which would be indicative of a specific interaction with a putative receptor.

The midguts of last instar larvae, isolated in Ussing chambers, were incubated for two hours in the presence of 130 µM FITC-TMOF in the luminal compartment. The tissues, rapidly rinsed and immediately fixed to preserve the distribution of the peptide in the intercellular spaces (Fiandra et al., 2009), were observed as whole mounts by confocal microscopy. The acquisition of a single

optical section at the junctional level of the epithelium (Figure 5A) revealed that FITC-TMOF was extensively localized in the paracellular space and, therefore, permeated across the epithelium by this route. An optical section acquired at a deeper plane of the tissue (Figure 5B) confirmed the intercellular localization of the peptide and showed intense fluorescent dots around the cells, suggesting binding of the peptide to specific sites in the basolateral membrane of the epithelial cell. Numerous spots of FITC-TMOF molecules associated with the basolateral membranes were even more evident in a more basal optical section (Figure 5C). In this section, the freely dispersed fluorescent TMOF molecules had already left the basal intercellular spaces, so that only the bound peptides were apparent as spots on the cell surface.

**Discussion**

In recent decades, a number of research efforts have been focused on the identification and use of new bioinsecticide molecules (Whetstone and Hammock, 2007; Dayan et al., 2009), in response to the growing demand from public opinion for new production protocols in agriculture less dependent on the use of chemical insecticides. The use of biocontrol agents and their possible integration with transgenic plants appears a very promising alternative (Bale et al., 2008). Moreover, the study of the molecular bases of the interactions between insects and their natural antagonists represents a very attractive possibility for the isolation of new molecules and genes for insect control (Beckage and Gelman, 2004; Pennacchio and Strand, 2006; Ferry et al., 2006; Whetstone and Hammock, 2007). This objective is pursued not only in the perspective of finding alternative genes for engineering new insect-resistant plants, which is highly needed (Gatehouse, 2008), but also to develop new bioinsecticides and effective delivery strategies, not exclusively based on the use of transgenic plants. This approach aiming at a sustainable exploitation of insect natural antagonism, obviously applies to a large variety of insect antagonists, which offer a virtually unlimited reservoir of new molecules with insecticide activity (Wethstone and Hammock, 2007).

The disruption of insect life and reproduction can be achieved not only by using natural antagonists and molecules/genes derived from them, but also by targeted manipulation of key signalling pathways which regulate physiological homeostasis. This was suggested a long time ago, and the idea of manipulating the titre and metabolism of peptides with endocrine functions appeared particularly amenable to develop new insect control technologies, even though quite a few concerns were raised about the effective occurrence of peptide gut absorption (Schneidermann, 1984; Keeley and Hayes, 1987; Menn and Borkovec, 1989). However, the peptide/protein degradation in the gut upon ingestion is not an absolute barrier, and a consistent experimental evidence indicates that absorption of molecules and macromolecules takes place at detectable levels (Bavoso et al., 1995; Kurahashi et al., 2005; Casartelli et al., 2005; 2007; Uchoa et al., 2006; Fiandra et al., 2009). As the background information in these basic field of study continues to grow, the possibility of integrating different tools and approaches to develop new biotechnologies for insect control appears to be particularly promising, and certainly worthy of further research efforts.

Among the numerous peptides which were isolated and characterized in the last three decades, *Aea*-TMOF appeared of particular interest, as it targets the gut of mosquitoes, by inhibiting trypsin synthesis, through the interaction with a receptor localized on the haemolymphatic side of the gut epithelium (Borovsky et al., 1994). Moreover, the same molecule is also active on *H. virescens* larvae (Nauen et al., 2001), even when expressed in transgenic plants (Tortiglione et al., 2002; 2003). The strategy pursued in these latter studies was to deliver in the gut, with different constructs, a TMOF precursor made of multiple peptide units, spaced by dibasic residues, Arg-Arg, as potential post-translational cleavage site. The significant impact on biological performance and survival of *H. virescens* fed on these transgenic plants corroborated the validity of the expression approach proposed, which, in principle, could be applied to any other peptide to be used for insect control. However, the mild effects observed in terms of mortality, further corroborated by the negative impact on the growth of tobacco budworm larvae which ingested TMOF fused with the coat protein of the tobacco mosaic virus (Borovsky et al., 2006), suggested the idea of combining

this transgene with others capable to hit different functional targets, possibly exerting a synergistic interaction.

Among the different alternatives available, we decided to focus our attention on *Ac*MNPV ChiA, which proved to be active, upon ingestion, in lepidopteran larvae, by disrupting the peritrophic membrane and increasing its permeability (Rao et al., 2004). This determined direct negative effects (i.e. developmental alterations, mortality) on the larvae fed on artificial diet containing the recombinant ChiA (Rao et al., 2004) or on transgenic plants (Corrado et al., 2008). These adverse effects on larval development well agreed with the apparent delamination and perforation observed in enzyme-treated PMs (Rao et al., 2004), and corroborated the notion that PM has a primary physiological role in the compartmentalization and recirculation of digestive enzymes, as proposed by Terra (reviewed in: Terra, 2001; Terra and Ferreira, 2005), essential for the full exploitation of dietary compounds.

Thus, the availability of transgenic plants expressing two genes with mild but significant effects, potentially able to interact in a synergistic way, to enhance gut absorption, stimulated the idea of generating hybrids, using them as parental lines. This not only to test experimentally a new combination of bioinsecticide molecules, but also to validate a delivery strategy *per os* of toxic molecules targeting haemocoelic receptors, which, even though already proposed with encouraging results (Wang et al., 2005; Arakane and Muthukrishnan, 2010), has never been thoroughly investigated.

The stronger detrimental effect on growth and survival recorded for larvae reared on the hybrid tobacco line (Fig. 2, Table 2) indicates that a positive interaction takes place between the two transgenes concurrently expressed in the same plant. The data generated by this study show that the hydrolytic activity of ChiA on the PM of tobacco budworm larvae enhances the permeation of TMOF molecules, increasing the hormone concentration in the ectoperitrophic space, in direct contact with the intestinal epithelium. The experiments performed *in vitro* demonstrate that the incubation of larval PM with ChiA extracted from transgenic tobacco does increase the diffusion of

TMOF across the membrane to the ectoperitrophic compartment (Fig. 4). The chitinolytic effect was also observed in the PMs of larvae reared on the ChiA-expressing tobacco line, as demonstrated by the higher flux of the test molecule methylene blue through the isolated membranes (Fig. 3). This latter experiment directly demonstrates that feeding on transgenic plants is effective in disrupting the PM, and the results are comparable to those originally recorded *in vitro* (Rao et al., 2004). The enhanced TMOF concentration at the apical side of the intestinal epithelium due to the PM lesions increased TMOF flux through the epithelium, as corroborated by the more pronounced toxicity of the hybrid plants. Therefore, we looked for the presence of the peptide in the haemolymph. We expected different titres directly associated with the biological effects observed, although quite low, due to the poor permeability of this peptide across the lepidopteran midgut (Fiandra et al., 2009). But this was not the case, as TMOF was undetectable in all haemolymph samples analyzed. However, we cannot rule out that TMOF may be chemically modified after ingestion, attaining a different molecular mass.

The alteration of larval growth upon TMOF ingestion and, more pronouncedly, when hybrid tobacco genotypes were eaten (Table 2), clearly indicates that an increase of the haemolymphatic titre of TMOF is not necessary for the biological effects observed. This apparent contrast can be reasonably reconciled by taking into consideration the specific biological features of this peptide. According to the model described in their review by Borovsky et al. (2003), TMOF produced from *Aedes aegypti* gene (*Aea*-TMOF) and fed to mosquito larvae, affects the normal growth and survival of the insects by inhibiting trypsin synthesis in midgut cells, after its binding to gut receptors on the haemocoel side of the tissue (Borovsky et al., 1994). In a recent study on *Spodoptera littoralis* larvae, the presence of *Aea*-TMOF receptors on the basolateral membrane of midgut cells has been hypothesized (Lemeire et al., 2008). The effect of ingested and injected *Aea*-TMOF on trypsin biosynthesis (Nauen et al., 2001) and on the growth of *H. virescens* larvae (Tortiglione et al., 2002; 2003) also suggests the presence of a TMOF-like hormone in Lepidoptera, and, therefore, of receptors similar to those identified in mosquitoes. In the present study we show

that FITC-TMOF crosses *H. virescens* larval midgut by diffusion through the paracellular pathway (Fig. 5A), as in *B. mori* (Fiandra et al., 2009). A specific binding of TMOF to the basolateral membrane of the enterocytes of lepidopteran larvae has not yet been described. According to our results, we can reasonably surmise that, as soon as FITC-TMOF reaches the intercellular spaces, it binds to well defined sites on the basolateral membrane of the intestinal cells (Fig. 5B, C), as suggested by the numerous fluorescent spots clearly visible in the confocal images. Overall, based on the experimental data we gathered so far, we can assume that ingested TMOF molecules can pass more freely across the damaged PM, reaching a higher concentration in the ectoperitrophic space; this allows a more rapid diffusion along the septate junction, that determines higher concentrations of TMOF, or of TMOF-like bioactive molecules, in the microenvironment where the putative receptors are located, with obvious negative consequences on trypsin biosynthesis.

In conclusion, this study directly demonstrates that chitinases, besides being used as biopesticides, can be profitably exploited to compromise the PM permeability and, when delivered in tandem with other peptide/protein toxins, to enhance their gut absorption rates. This significantly contributes to the development of innovative delivery strategies of bioinsecticides, by enriching the toolkit that biotechnology can use in the continuous effort towards a more effective exploitation of toxin biodiversity for sustainable insect control.

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**Figure Captions**

Figure 1. (A) Northern blot hybridization of ChiA and polyTMOF transcripts. 1-10: ChiA-polyTMOF hybrids; R1-2: tobacco parental line expressing the *polyTMOF* synthetic gene; ChiA: tobacco parental line expressing *ChiA* gene; NN: untransformed tobacco plant.

(B) Western blot analysis of ChiA protein. NN: untransformed tobacco plant, R1-2: transgenic tobacco parental line expressing *polyTMOF* synthetic gene; ChiA: tobacco parental line expressing *ChiA* gene; 1, 2, 6, 8 and 10: ChiA polyTMOF hybrids. The blot was probed with the rabbit anti-c-myc as primary antibody and anti-rabbit IgG conjugated with horseradish peroxidise as a secondary antibody.

Figure 2. Growth curves of the total biomass (Mean ± SEM) of *Heliothis virescens* larvae as affected by feeding with leaf disks obtained from transgenic or control plants (Repeated Measures ANOVA: F=8.82; df 3,12; p=0.002). Total larval biomass combines larval growth and survival and is calculated as the sum of the weight of the surviving larvae in each treatment. For each treatment, n=16 per replicate, for a total of 4 replicates.

Figure 3. Methylene blue flux across the isolated peritrophic membrane of *Heliothis virescens* larvae fed with NN (control), ChiA HDEL 9 or polyTMOF R1-2 tobacco genotypes. Mean ± SEM of at least 3 replicates. Student's *t*-test vs control (*$P < 0.05$).

Figure 4. TMOF flux across the isolated peritrophic membrane of *Heliothis virescens* larvae incubated in the absence (control) or in the presence of 40 µg/ml ChiA. Mean ± SEM of 3 replicates. Student's *t*-test vs control (*$P < 0.05$).

Figure 5. Confocal laser scanning micrographs (single optical sections) of a whole-mount midgut after 2 h of incubation in the presence of 130 µM FITC-TMOF in the luminal compartment. The

optical sections reported on the right side roughly correspond to the scheme of a traversal section of the midgut epithelium on the left side. (A): optical section acquired at the junctional level of the epithelial cells monolayer; (B): optical section acquired at the basal side, immediately under the junction; (C): a more basal optical section. Arrows indicate binding of FITC-TMOF to the basolateral membrane of cells. Midguts incubated in the absence of FITC-TMOF did not emit fluorescence when excited at the wavelength used (not shown). Bars: 20 μm (A), 10 μm (B and C). C: columnar cell; G: goblet cell; mv: microvilli; bi: basal infoldings; sj: septate junction.

**Table(s)**

**Table 1** MS-MS Instrumental parameters optimized for the detection of TMOF (DP Declustering potential, CE Collision Energy)

| Precursor ion $[M+H]^+$ (*m/z*) | Product ions *(m/z)* | DP (V) | CE (V) |
|---|---|---|---|
| 1047,6 | 601,6 | 68 | 46 |
| | 504,4 | | 59 |
| | 407,7 | | 53 |
| Precursor ion $[M+2H]^{+2}$ (*m/z*) | Product ions *(m/z)* | DP (V) | CE (V) |
| 524,6 | 385,4 | 47 | 29 |
| | 213,0 | | 20 |
| | 279,4 | | 25 |

**Table(s)**

**Table 2**. Developmental time, from egg hatching to adult emergence and percent survival rates until adult emergence of *Heliothis virescens* larvae fed with control or transgenic leaf-disks. Means denoted with different letters are significantly different (Tukey's test, $\alpha$=0.05). For each treatment, n=16 per replicate, for a total of 4 replicates. See Materials and Methods for an explanation relative to the SE of the survival rate.

| Leaf-disks fed | Developmental Time (Mean ± SEM) | Survival Mean Percentage (+SEM to –SEM) |
|---|---|---|
| NN | 23.44 ± 0.26 a | 75.27 a (81.48 – 68.47) |
| PolyTMOF R1-2 | 24.19 ± 0.31 ab | 50.08 ab (57.62 – 42.54) |
| ChiA HDEL 9 | 24.58 ± 0.32 b | 48.51 ab (56.07 – 40.99) |
| Hybrids | 25.47 ± 0.46 b | 24.74 b (31.53 – 18.52) |

**Table 3** Larval weight of *H. virescens* larvae 120, 132 and 144 h after they started to feed, as newly moulted fourth instars, on the tobacco genotypes NN (control), ChiA HDEL 9, polyTMOF R1-2, hybrid.

| | Larval weight (g) | | |
|---|---|---|---|
| | **120 h** | **132 h** | **144 h** |
| NN | 0.20 ± 0.01 (16) | 0.22 ± 0.01 (16) | 0.23 ± 0.01 (10) |
| ChiA HDEL 9 | 0.22 ± 0.01 (14) | 0.24 ± 0.02 (14) | 0.23 ± 0.02 (8) |
| PolyTMOF R1-2 | 0.22 ± 0.01 (16) | 0.24 ± 0.01 (16) | 0.20 ± 0.01$^{*}$ (10) |
| Hybrid | 0.16 ± 0.01$^{**}$ (13) | 0.19 ± 0.01$^{*}$ (13) | 0.20 ± 0.03 (7) |

Symbols indicate significant differences by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$ vs. corresponding control for each experimental time.

**Figure(s)**

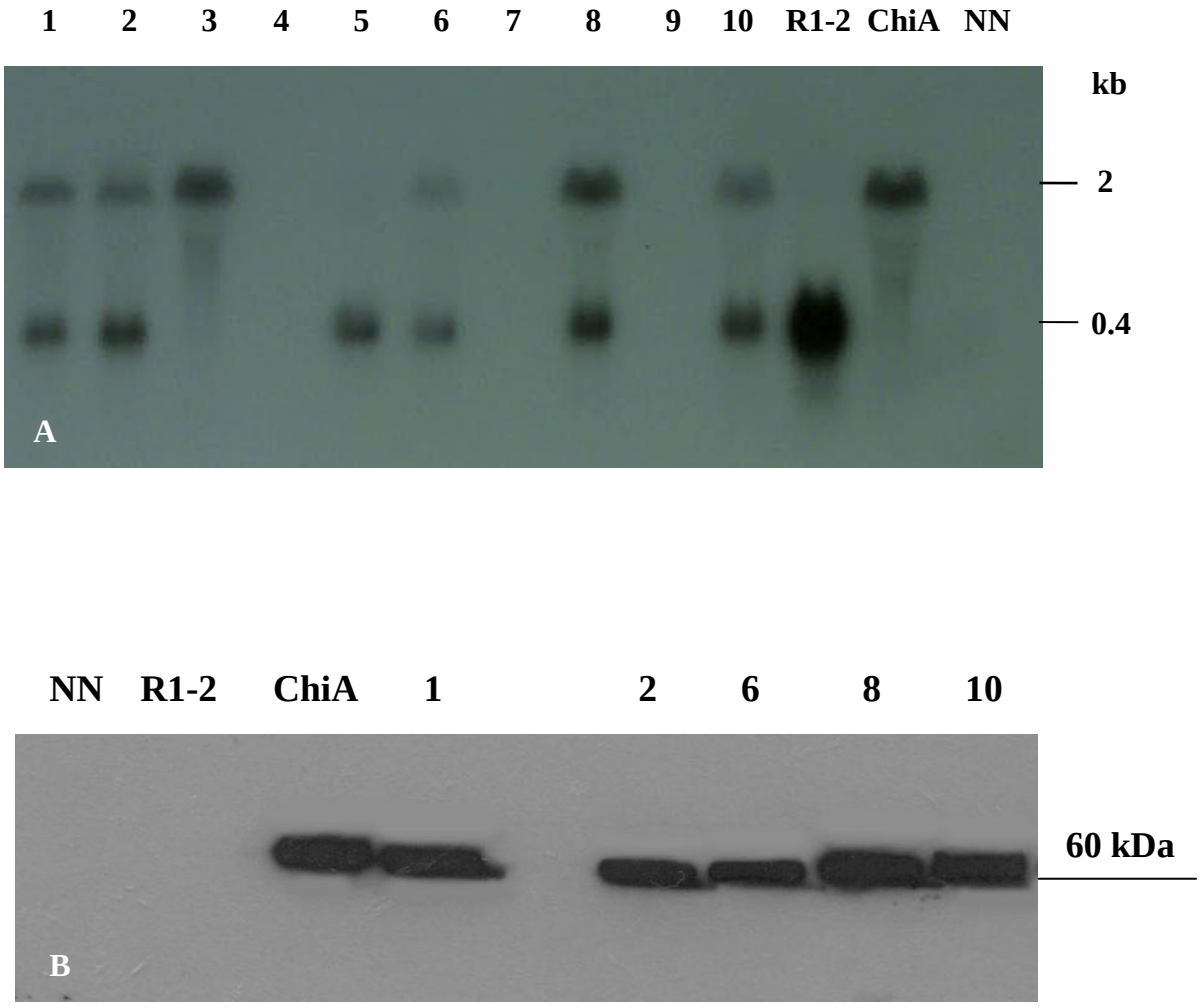


Figure 1

**Figure(s)**

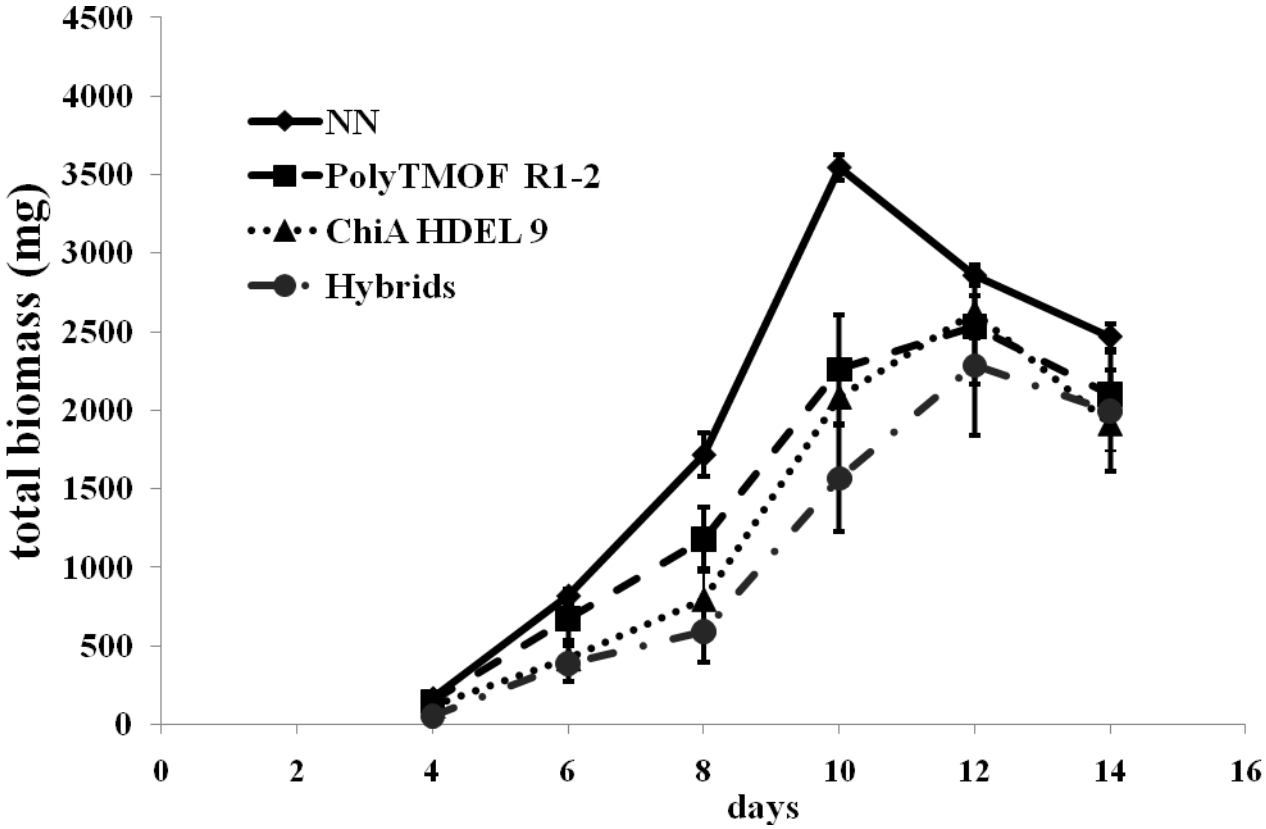


Figure 2

**Figure(s)**

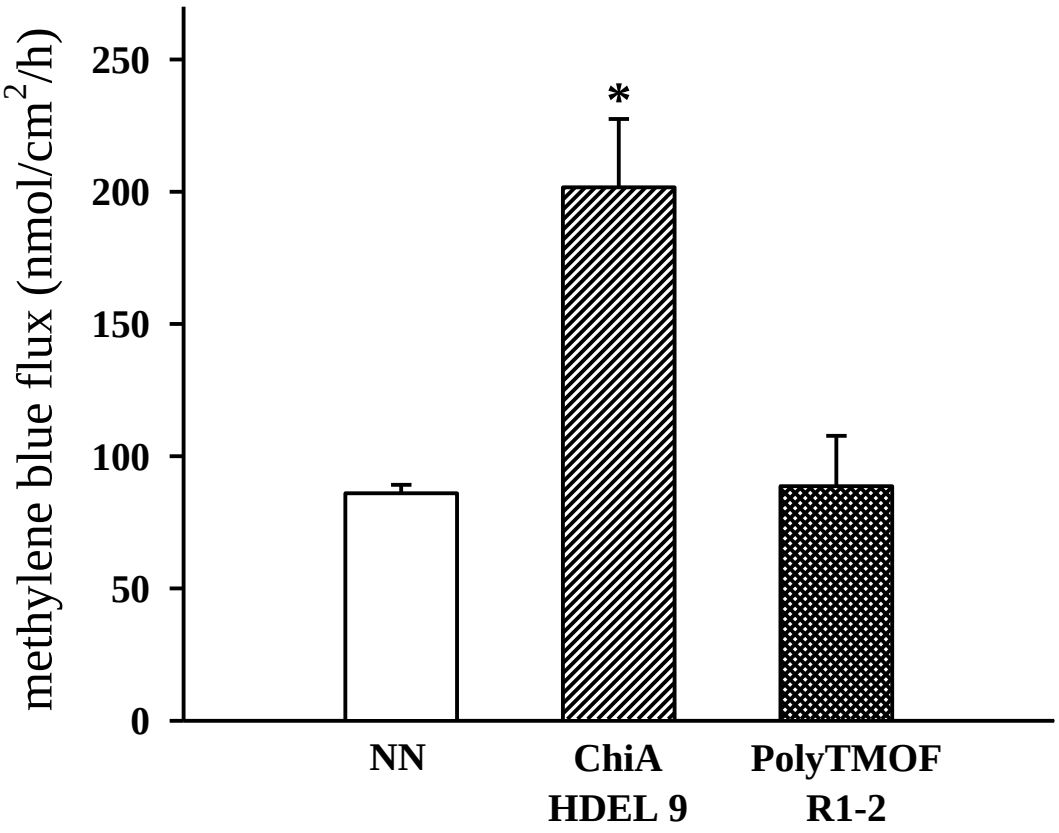


Figure 3

**Figure(s)**

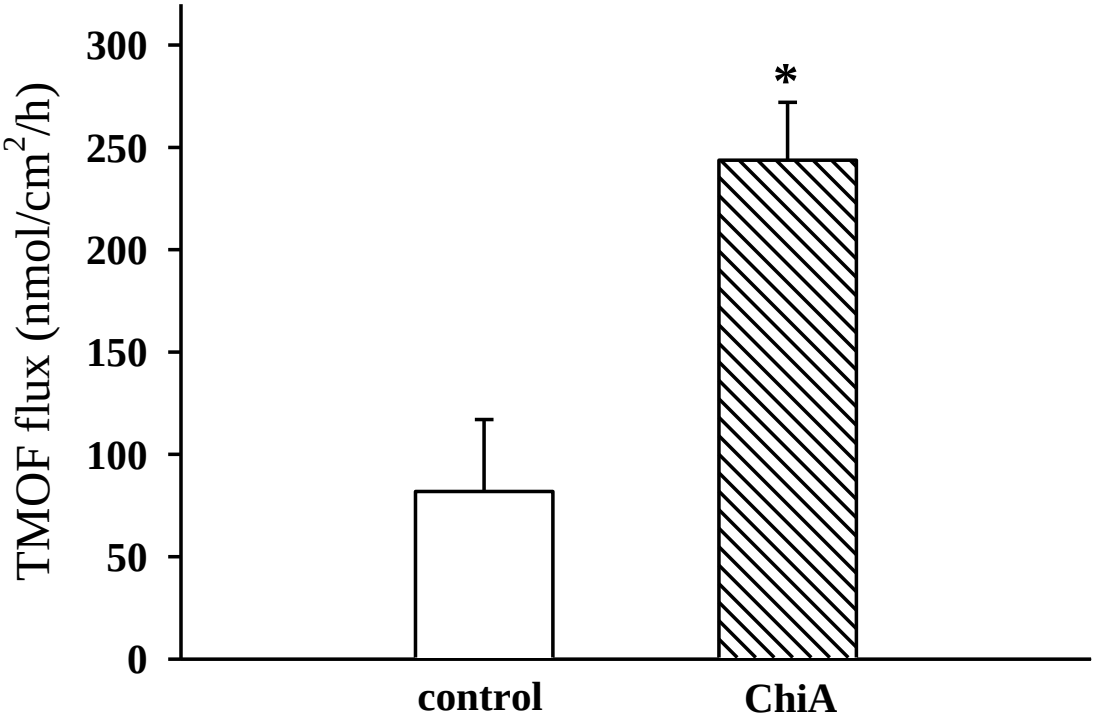


Figure 4

**Figure(s)**

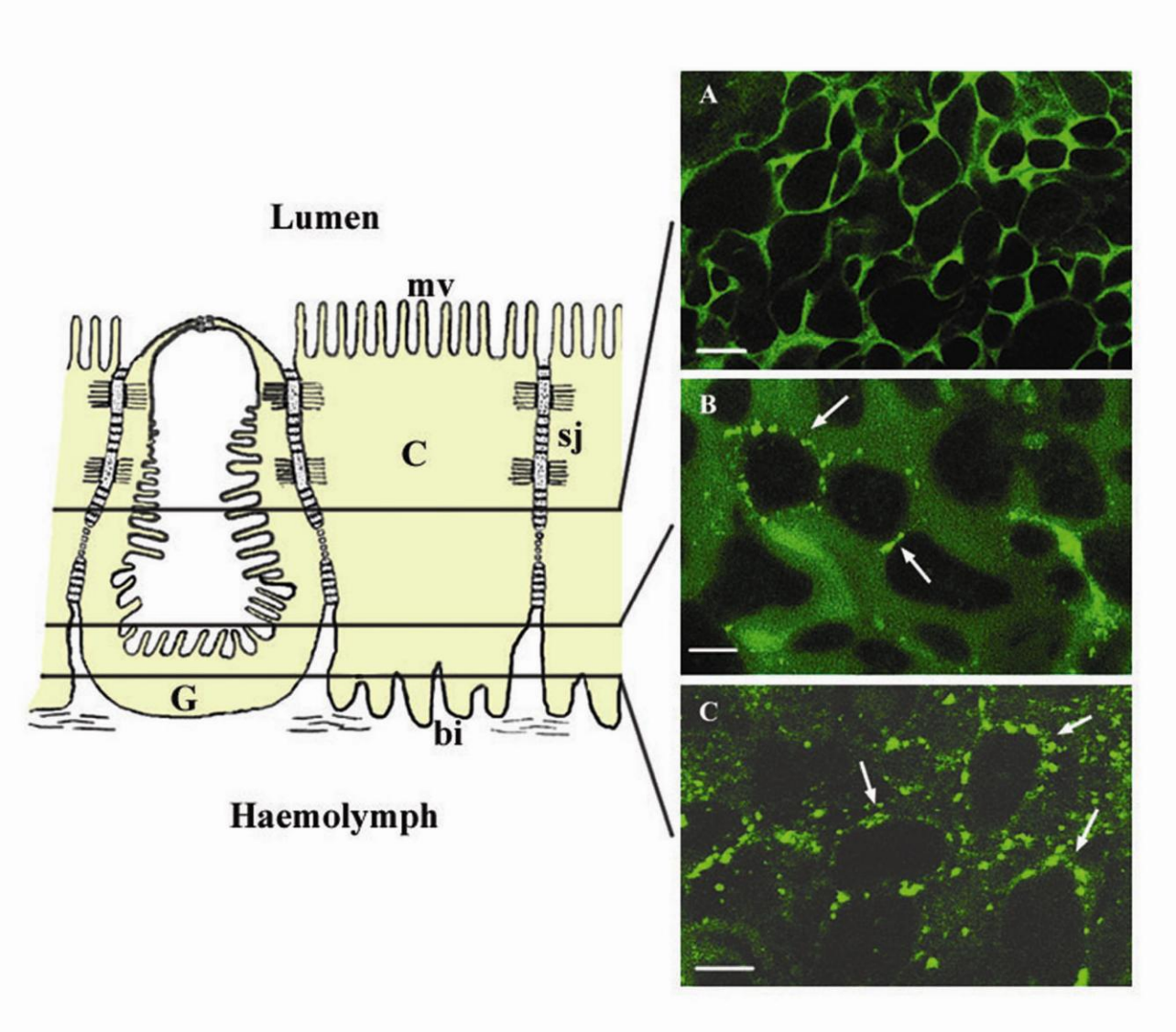


Figure 5