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The mitogen-activated protein kinase p38 is involved in insect defense against Cry toxins from *Bacillus thuringiensis*

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Abstract

The insecticidal Cry toxins are pore-forming toxins produced by the bacteria *Bacillus thuringiensis* that disrupt insect-midgut cells. In this work we analyzed the response of two different insect orders, the Lepidopteran *Manduca sexta* and Dipteran *Aedes aegypti* to highly specific Cry toxins, Cry1Ab and Cry11Aa, respectively. One pathway activated in different organisms in response to a variety of pore forming toxins is the mitogen activated protein kinase p38 pathway (MAPK p38) that activates a complex defense response. We analyzed the MAPK p38 activation by immunodetection of its phosphorylated isoform, and the induction of p38 by RT-PCR, real time-PCR quantitative assays and immunodetection. We show that MAPK p38 is activated at postraductional level after Cry toxin intoxication in both insect orders. We detected the p38 induction at the transcriptional and traductional level, and observed a different response. In these three levels, we found that both insects respond to Cry toxin action but *M sexta* responses more strongly than *A. aegypti*. Gene silencing of MAPK p38 *in vivo*, resulted in both insect species becoming hypersensitive to Cry toxin action, suggesting that the MAPK p38 pathway is involved in insect defense against Bt Cry toxins. This finding may have biotechnological applications for enhancing the activity of some Bt Cry toxins against specific insect pests.

1. Introduction

Pore-forming toxins (PFT) are important virulent factors produced by several pathogenic bacteria. Attack by PFT represents a fundamental threat to the host after infection and consequently general host defense-responses have evolved against PFT. Among them, the mitogen activated protein kinase (MAPK) p38 pathway has been recognized as an important player, triggering survival responses in several cell types after treatment with different PFT. MAPK p38 activation after treatment with Cry (a PFT) was first described in *Caenorhabditis elegans* treated with the *Bacillus thuringiensis* (Bt) Cry5B toxin (Huffman *et al.* 2004). Several works in other studied mammalian models showed that other PFT such as aerolysin (Huffman

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et al. 2004), pneumolysin (PLY), streptolysin O (SLO), α -hemolysin (Hla), and anthrolysin O produced by different bacteria, when assayed at low doses in cultured-epithelial cell lines induced the activation of MAPK p38 pathway (Ratner, *et al.* 2006). The pore formation activity of PFT seems to play an important role since toxin deficient mutants or single-point mutations in toxin regions essential for pore formation activity were unable to induce the MAPK p38 response, suggesting that the observed phosphorylation of MAPK p38 protein correlated with formation of—at least—few pores in the membrane (Ratner, *et al.* 2006). Recently, it was shown that loss of K⁺ ions is likely involved in inducing activation of MAPK p38 as a response to α -toxin, *Vibrio cholera* cytotoxin (VCC), SLO or *Escherichia coli* hemolysin (HlyA) (Kloft *et al.* 2009).

Regarding downstream responses induced after activation of MAPK p38, it was described that one of the targets of MAPK p38 in the nematode *C. elegans* was *ttm-1* gene, an orthologue of a human divalent cation transporter, suggesting that up regulation of an efflux transporter may be important in removing cytotoxic cations from the cytosol (Huffman *et al.* 2004). Later, it was shown that the endoplasmic reticulum stress response to unfolded proteins (UPR) was also induced in *C. elegans* and in HeLa cells, as a downstream response induced after activation of MAPK p38 by two different PFT. This pathway protects cells from accumulation of unfolded proteins and increases phospholipid biogenesis to defend cells against these toxins (Bischof *et al.*, 2008). In mast-cells, low doses of SLO or listeriolysin (LLO) activates the MAPK p38 pathway resulting in up regulation of cytokines mRNA expression such as tumor necrosis factor alpha (TNF- α). This cytokine plays an important role in host defense in the murine model, recruiting inflammatory cells critical for innate and adaptive immunity (Gekara *et al.*, 2007; Stassen *et al.*, 2003).

Most of these studies were done in cultured cells not in complete animals, the only exception being studies performed with *C. elegans*. In insects, the responses to PFT Cry toxins produced by *B. thuringiensis* and specifically the participation of MAPK p38 pathway during Cry-toxin intoxication have not been described. The mechanism of action of Cry1A toxins in insect larvae involves sequential interaction with several receptors, toxin oligomerization and pore formation in the apical membrane of larval midgut cells causing osmotic shock, cell lysis and insect death (Bravo *et al.* 2004, 2007). Since it was shown that Cry5B toxin induced a defense response in the nematode *C. elegans* (Huffman *et al.* 2004), we hypothesized that other Cry toxins may induce a similar response in insects. Therefore, we analyzed the response of two different insect orders, *Manduca sexta* as a model of Lepidopteran insects and *Aedes aegypti* as a model of Dipteran insects, after intoxication with specific Cry toxins. We set up conditions for an effective RNA interference analysis by feeding dsRNA to larvae and demonstrated that the MAPK p38 pathway plays a protective role *in vivo* against Cry toxins action in both insect orders.

2. Materials and methods

2.1. Cry toxin purification

Bt strains harboring pHT315-Cry1Ab [8] or pCG6-Cry11Aa (Chang *et al.* 1993; Wu *et al.* 1994) plasmids were grown at 30°C in nutrient broth sporulation medium with 10 μ g/ml erythromycin until complete sporulation (Meza *et al.* 1996). Crystal inclusions were observed under phase contrast microscopy and purified by sucrose gradients (Thomas and Ellar, 1983). As control we used Cry1Ab-R99E (Jiménez-Juárez *et al.* 2007) and Cry11Aa-R90E (Muñoz-Garay *et al.* 2009), two different helix α -3 point mutants that were non-toxic to their corresponding insect-targets and were reported to be affected in oligomerization and pore-formation activity.

2.2. Bioassays

For bioassays using *M. sexta* larvae spore-crystals suspensions of wild type and mutant Cry1Ab (from 0 to 2,000 ng/cm²) were applied onto the diet surface in 24-well plates as described (Gómez *et al.* 2002). For clarity each well in the plate has a surface of 2 cm², we applied a volume of 35 µl per well containing the different toxin concentrations and wait until the surface is complete dry, then one larva was added per well and 24 larvae per dose. Mortality was recorded after seven days and lethal concentration (LC₅₀) estimated by Probit analysis (Polo-PC LeOra Software). Protein concentration of spore-crystal preparation was determined by the Bradford assay. For bioassays using *A. aegypti* larvae wild type and mutant Cry11Aa spore-crystal suspensions (from 0 to 10,000 ng/ml) were directly added to 100 ml H₂O. Ten fourth instar larvae were added. Mortality was recorded after 24 h and LC₅₀ values and protein concentration were determined as above.

For insect intoxication assays we used second instar *M. sexta* larvae fed with different concentrations (from 0 to 20 ng/cm²) of Cry1Ab crystal-spore suspension for different times (from 30 min to 7 days). For *A. aegypti* experiments, 4th instar larvae were feed with 0 to 2,500 ng/ml Cry11Aa crystal-spore suspension during different times (from 30 min to 24 h). Larval midguts were dissected from survivors to obtain total RNA and protein samples for RT-PCR or Western blot assays, respectively.

2.3. Western blot assays

Total protein samples were prepared from isolated *M. sexta* midguts. Midguts were homogenized in CellLytic M Cell Lysis reagent (Sigma) supplemented with protease inhibitors (Complete; Roche) and 1 mM NaVo, 20 mM NaF phosphatase inhibitors (Sigma) at 4° C. Total protein extracts from *A. aegypti* midguts were suspended and homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT) supplemented with protease complete inhibitors and phosphatase inhibitors as described above at 4° C. 20 µg of protein sample from *M. sexta* or 25 µg of protein sample from *A. aegypti* larvae were boiled 5 min in Laemmli sample loading buffer, separated in SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The proteins were detected by Western blot using commercial antibodies: monoclonal antibody M8432 to non activated MAPK p38 (dilution 1:15,000) (Sigma); monoclonal antibody M8177 to activated p38 MAPK (phosphorylated) (dilution 1:5,000) (Sigma); anti phosphorylated p38 MAPK (Thr180/Tyr 182) antibody 9215 (Dilution 1:500) (Cell Signaling); mouse anti-tubulin 180092 antibody (dilution 1:3,000) (Zemed). HRP-coupled rabbit anti-mouse IgG (dilution 1:15,000) (Zemed) or goat anti-rabbit IgG (dilution 1:10,000) (Invitrogen) were used as secondary antibodies. Results are representative experiments of three repetitions. Blots were revealed with luminol (ECL; Amersham Pharmacia Biotech) as described by the manufacturers. Molecular weight markers used in all SDS-PAGE were precision pre-stained plus standards all blue (BioRad, Hercules CA).

2.4. RT-PCR and real time PCR

Total RNA was isolated using the RNeasy Kit (Qiagen). One µg of total RNA was used for RT-PCR amplification using a First Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche). Specific oligonucleotides (Table 1) were designed to amplify *M. sexta* MAPK p38 (Ms-p38-F and Ms-p38-R) based on larval midgut ESTs generated using 454 pyro-sequencing (Pauchet *et al.* 2009). Specific oligonucleotides for MAPK p38 from *A. aegypti* (Ae-p38-F and Ae-p38-R) were designed based on the genome sequence accession number AEEL008379. Actin and RPS3 genes from *M. sexta* (accession numbers L13764 and U12708, respectively) were amplified as controls using Ms-actin and Ms-RPS3 primers described in table 1. Four replicates were performed with independent biological samples. Real time PCR was performed by Eurofins MWG GmbH Germany, on an ABI 7000 Instrument using Amplifluor Detection System (Serologicals, Clarkston, GA). *M. sexta* were fed 2.5 ng/cm² toxin during different

times from 30 min to 7 days. Actin and RPS3 were used as real time PCR normalization controls. Quadruplicate rounds of quantitative real time PCR were performed.

2.5. RNA Interference (RNAi) assays

MAPK p38 cDNA fragments were amplified from *M. sexta* and *A. aegypti* as stated above and cloned into a TOPO cloning vector using TOPO TA cloning kit (Invitrogen) and subcloned into pLitmus28i vector (HiScribe™, New England Biolabs, Beverly, MA) containing two T7 promoters flanking the multi-cloning site. These promoters enabled amplification of the cloned fragment by using a T7 oligonucleotide. The PCR product was purified with QIAquick PCR purification kit (Qiagen Valencia, CA). *In vitro* transcription of both DNA strands of the insert was performed with T7 RNA polymerase using the HiScribe RNAi Transcription Kit (New England Biolabs) as reported by the manufacturer, yielding dsRNA.

One hundred *M. sexta* neonates were fed with a single dose of one μ l drop per larvae containing 5 μ g Ms-p38 dsRNA. Each neonate *M. sexta* was located just in front of the drop. We were careful to observe that each larva ingested the μ l drop, and then they were transferred to artificial diet until they reached third instar for midgut dissection.

For gene silencing in *A. aegypti*, 200 neonates were fed for 16 h with 200 μ g of dsRNA previously encapsulated in Effectene transfection reagent (Qiagen). For encapsulating the dsRNA in Effectene, 200 μ g of dsRNA in a final volume of 4 ml of DNA-condensation buffer (EC buffer Qiagen), were mixed with 0.8 ml of Enhancer buffer (Qiagen) by vortexing and incubated 5 min at room temperature. Then the sample was mixed with 1.3 ml of Effectene by vortexing and incubated 10 min at room temperature. This sample was diluted in dechlorinated water to a final volume of 10 ml where 200 larvae were added and incubated for 16 h hours. After dsRNA feeding, the mosquito larvae were transferred to clean water and fed with regular diet, ground brewers yeast, lactalbumin and rat food Chow (1:1:1 ratio) (Bayyareddy et al. 2009) daily, until they reached fourth instar when bioassays were performed or guts were dissected for analysis by RT-PCR and Western blot. The Effectene-liposomes may be slightly toxic since feeding *A. aegypti* larvae with Effectene-liposomes causes mortality depending on time of exposure and concentration. In the conditions described here, RNA-free Effectene-liposomes caused 5 % mortality.

3. Results

To evaluate the consequence of PFT action on insect larvae we analyzed the effect of two different Cry toxins that are specifically active against two different insect orders. Cry1Ab toxin is highly active against larvae of the lepidopteran *M. sexta*, while Cry11Aa toxin is active against dipteran *A. aegypti* larvae. Using a dose-dependent toxicity assay, a lethal concentration at which 50% of the larvae die (LC₅₀) was obtained for both insect models. Cry1Ab bioassays were performed using *M. sexta* neonates by applying various concentration of the spore-crystal preparation at the surface of artificial diet, and mortality was recorded after seven days of toxin exposure. For Cry11Aa bioassays, fourth instar *A. aegypti* larvae were exposed to various concentrations of spore-crystal sample in dechlorinated water and mortality was recorded after 24 h of toxin exposure (Mulla et al. 1985; Perez et al. 2005). As controls we included spore-crystal samples containing two non-toxic mutants, Cry1Ab-R99E for *M. sexta*, and Cry11Aa-R90E for *A. aegypti*. These two mutants are unable to oligomerize properly which affects their pore formation activity (Jiménez-Juárez et al. 2007; Muñoz-Garay et al. 2009). The LC₅₀ values obtained for spore-crystal samples of both wild type and mutant toxins on their corresponding target insect are summarized in Table 2.

3.1 Activation of MAPK p38 pathway in insects after intoxication with Cry toxins

We decided to investigate the activation of MAPK p38 pathway by analyzing the induction of MAPK p38 protein phosphorylation by Cry toxins. Phosphorylation of MAPK p38 was analyzed by Western blot experiments using an anti-p38 antibody that specifically recognizes the phosphorylated isoform of MAPK p38. In *M. sexta* larvae, phosphorylation of MAPK p38 was observed at short time incubation, we fed the larvae during 30 min with spore-crystal suspension at LC₅₀ and observed that the phosphorylation of this protein increased even more after one hour of treatment (Fig 1A). Phosphorylation of MAPK p38, in *M. sexta*, is dose-dependent since one-hour treatment with 2 ng of Cry1Ab spore-crystal per cm², corresponding to the LC₅₀, resulted in a lower activation than a treatment with 20 ng of Cry1Ab spore-crystal per cm². A treatment of one hour with the spore-crystal suspension of non-active mutant Cry1Ab-R99E did not induce any activation of MAPK p38 protein (Fig 1A), suggesting that phosphorylation of MAPK p38 is an *in vivo* response of larval midgut cells to intoxication by Cry1Ab toxin. Similar results were observed in *A. aegypti* larvae (Fig. 1B). Phosphorylation of MAPK p38 was observed after 30 min treatment with Cry11Aa and increased further after one hour of toxin ingestion (LC₅₀). In comparison, no activation of MAPK p38 was observed when mosquito larvae were fed the spore-crystal suspension of the non-toxic Cry11Aa-R90E mutant (Fig. 1B).

In order to determine the effect of Cry toxins on MAPK p38 expression in both insects, the p38 protein level was analyzed by Western blot using an anti-p38 antibody after long time of intoxication with the spore-crystal suspensions. In *M. sexta* larvae, we observed an increase in MAPK p38 protein level after seven days of spore-crystal exposure, with LC₅₀. If the larvae were fed with a higher dose of spore-crystal corresponding to LC₉₀ concentration, the amount of MAPK p38 was higher than the control without toxin but lower than the LC₅₀ dose; this is probably due to the fact that LC₉₀ is a high dose where most of the larvae are death after seven days. (Fig. 2A). Similarly, RT-PCR analysis showed a clear induction by Cry1Ab of MAPK p38 transcript level detected after 24 h and up to seven days when larvae were fed with a dose of LC₅₀ (Fig. 2B). The RNAm levels of MAPK p38 in *M. sexta* larvae were low without induction with Cry1Ab toxin (see control lane in fig 2B) only after 24 h there is a clear increase in RNA levels. These data were confirmed by quantitative real-time PCR experiments, indicating that MAPK p38 transcript level increases 0.53, 1.83, 3.08 and 7.42 fold after 30 min, 24 h, 48h and seven days respectively (Fig. 2C). In contrast, very small or no changes in protein or transcript levels were observed for MAPK p38 in *A. aegypti* larvae exposed to Cry11Aa toxin (Fig. 2A and 2B).

3.2. Silencing of MAPK p38 by RNAi in *M. sexta* and *A. aegypti*

In order to determine the role of MAPK p38 pathway in protection against Cry toxins in both insect orders, we set up conditions for silencing by RNAi their corresponding MAPK p38 transcript. Previously, we demonstrated that injecting dsRNA into *M. sexta* larvae resulted in specific silencing of the Cry1Ab cadherin receptor (Soberón *et al.* 2007). However, it was recently reported that RNAi was efficiently induced in both Lepidopteran and Coleopteran insect orders by delivering dsRNA by feeding (Baum *et al.* 2007; Mao *et al.* 2007). In this work we set up the conditions to efficiently deliver dsRNA by feeding both *M. sexta* and *A. aegypti* larvae (Materials and methods). In the case of *M. sexta*, dsRNA was offered to starved and water deprived neonates and after eating the dsRNA, these exposed larvae were returned to artificial diet until they reached third instar. The MAPK p38 transcript and protein levels were then determined in third instar *M. sexta* larvae by RT-PCR and Western blot (Fig. 3). The *M. sexta* larvae that were fed with p38-dsRNA showed undetectable levels of MAPK p38 protein and transcript in contrast with larvae fed with water with an 80 % silencing efficiency (Fig. 3). As a control we analyzed expression of tubulin protein and actin and RPS3 transcripts that remained unchanged after MAPK p38 dsRNA feeding. In *A. aegypti* larvae, the silencing

of gene expression by RNAi was not a straightforward process, since mosquito larvae are filter-feeding insects. Therefore, we fed the larvae either with high amounts of dsRNA directly added to a small volume of water, or with transformed *E. coli* bacteria that were activated for expression of the dsRNA. None of these treatments resulted in any silencing of the MAPK p38 gene (data not shown). Finally we fed first instar larvae with MAPKp38-dsRNA encapsulated in Effectene-liposomes (see Materials and methods). After feeding the mosquito larvae with Effectene-liposome encapsulated dsRNA, larvae were transferred to clean water with regular diet until they reached fourth instar where MAPK p-38 protein and RNA levels were analyzed by Western blot and RT-PCR respectively. Similarly as in lepidopteran *M. sexta*, the MAPK p38 transcript and MAPK p38 protein were down regulated in contrast to tubulin protein and to actin transcript that were not modified by MAPK p38-dsRNA treatment (Fig. 3).

3.3. Role of MAPK p38 response in defense mechanisms of *M. sexta* and *A. aegypti* against Cry toxins

Finally, to determine the effect of silencing of MAPK p38 on Cry toxin susceptibility in both insect species, we analyzed the insecticidal activity of Cry1Ab and Cry11Aa spore-crystal suspension in MAPK p38-silenced and in control larvae. Both *M. sexta* and *A. aegypti* p38-silenced larvae became hypersensitive to the insecticidal action of Cry toxins (Table 2). For the p38-silenced *M. sexta* larvae, we assayed a range of Cry1Ab spore-crystal concentrations that range from 0 to 10 ng/cm² of diet. We observed an 8 fold decrease of the LC₅₀ of the *M. sexta* silenced larvae when compared with the normal *M. sexta* larvae, indicating higher susceptibility to the toxin. In *A. aegypti* larvae, RNA-free Effectene-liposomes caused a decrease of the Cry11Aa LC₅₀ in compared to untreated larvae. However, when MAPK p38-silenced *A. aegypti* larvae were tested for their susceptibility to Cry11Aa a 10-fold increase in susceptibility to the toxin, in comparison to larvae treated with RNA-free Effectene-liposomes, was observed (Table 2).

Therefore, our results suggest that the MAPK p38 pathway has a protective function against the PFT Cry1Ab and Cry11Aa toxins, in both lepidopteran and dipteran insects.

4. Discussion

Understanding the mechanism of action of PFT as well as host responses to these toxins would provide ways to deal with different pathogens and to improve the action of toxins that have biotechnological applications. Eukaryotic cells have developed a variety of defense mechanisms against PFT. Epithelial cells have evolved cellular non-immune defense mechanisms to deal with membrane impairment in their membrane permeability, such as changes in osmotic pressure, ion composition and changes in intracellular calcium concentration induced by PFT (Aroian and van deer Goot, 2007). One of these early responses involves activation of the MAPK p38 pathway. It was demonstrated that osmotic stress produced after formation of a few pores by different PFT in target cells, induces a MAPK p38-phosphorylation-response that is a crucial switch for a survival response (Huffman *et al.* 2004; Ratner *et al.* 2006; Bischof *et al.* 2008; Husmann *et al.* 2006; Aroian and van deer Goot, 2007). However, most of these studies were done in cultured cells not in complete animals. The only exceptions were the reports done with the nematode *C. elegans*, intoxicated with Cry5B toxin. It is important to mention that the mechanism of action of Cry5B in the nematode is not completely understood and may have significant differences in relation of the mode of action of other Cry toxins that are specific against insects. The main difference is that protein receptors have not been described in nematodes and that pore-forming activity of Cry5B or oligomerization of this toxin has not been demonstrated. In this work we focused to analyze the cellular response regarding to MAPK p38 activation presented by two insect species after intoxication with specific insecticidal PFT such as the Bt Cry toxins.

Here we describe intracellular effects induced by Cry toxins in insects. We demonstrate that in two different insect orders MAPK p38 pathway was specifically activated at postraductional level by phosphorylation when insect larvae were exposed to active Cry toxins at short times and that exposure to point-mutant toxins affected in oligomerization and pore formation were unable to induce this response. However, the induction response of MAPK p38 kinase in both insects differ since in *M. sexta* the transcript and protein levels were induced when larvae were exposed to Cry1Ab toxin, but in *A. aegypti* larvae no induction of MAPK p38 transcript or protein levels were observed after exposure to Cry11Aa toxin.

To analyze the role of the MAPK p38 pathway as a response to insecticidal activity of Cry toxins the expression of MAPK p38 pathway was inhibited by dsRNA. Recently it was reported that delivering of dsRNA by feeding is an efficient way for specific gene silencing in lepidopteran (Mao *et al.* 2007) and coleopteran species (Baum *et al.* 2007). Also that transgenic plants expressing these dsRNA could be used in the control of insect pests (Baum *et al.* 2007). Here, we show that delivering dsRNA by feeding is also an efficient way for gene silencing in another insect order, the dipteran *A. aegypti* larvae. However effective silencing was only observed when dsRNA was previously encapsulated in liposomes. To our knowledge this is a novel method to silence genes in the larval stage of an important mosquito vector of human diseases. We also silenced expression of MAPK p38 in the lepidopteran *M. sexta* and demonstrated that in both insect species the MAPK p38 plays a central role in defense responses *in vivo* against Cry toxins, since elimination of MAPK p38 protein leads to hypersensitivity to the PFT Cry1Ab or Cry11Aa.

The MAPK p38 pathway is a highly conserved signaling module known to participate in stress responses. Therefore its down regulation may compromise the insects' ability to respond against environmental stress. In this work we show that insects become hypersensitive to Cry toxin action after silencing MAPK p38 expression. Pore formation induced by Cry toxins probably perturbs both ionic homeostasis and intracellular ATP pool. Therefore it is crucial that target cells possess mechanisms to heal membrane lesions in order to survive. It has been shown that aerolysin treatment of CHO cells results in the activation of the SREBP pathway (Gurcel *et al.* 2006). Also it was reported that MAPK p38 is involved in activation of the *ire-1-xbp-1* branch of the UPR pathway in HeLa cells after aerolysin treatment (Bischof *et al.* 2008). In both cases the final outcome is an increase in phospholipids biogenesis, suggesting that target cells may respond to PFT by increasing membrane biogenesis in order to repair the membrane lesions (Gurcel *et al.* 2006). Alternatively another way to repair cell surface lesions is their internalization into endosomes. Supporting this scenario, Husmann *et al.* 2008 reported that *S. aureus* α -toxin is internalized by endocytosis during cell recovery. Also, Idone *et al.* 2008 showed that Ca^{+2} dependent endocytosis is involved in membrane resealing and cellular recovery after SLO exposure. Interestingly, it was shown that MAPK p38 could modulate endocytosis by regulation of Rab5 cycle and recruitment of its effectors (Cavalli *et al.* 2001; Mace *et al.* 2005). The role of endocytosis, SREBP and UPR responses in defense of Cry toxin intoxication in insects, as well as the role of genes that were induced during Cry toxin intoxication remains to be analyzed.

It is clear that eukaryotic cells may have multiple mechanisms to respond against different stress situations. Valaitis, 2008 reported that in *Lymantria dispar* larvae the treatment with active Cry1A toxins induce shedding of two glycosyl phosphatidylinositol (GPI)-anchored receptors (aminopeptidase N (APN) and alkaline phosphatase). It was proposed that receptor shedding might be a defense mechanism against Cry toxins, since ectodomains of receptors may function as competitive inhibitors preventing toxin interaction with cell surface receptors (Valaitis 2008). The APN-shedding could be inhibited by different molecules such as cyclic AMP; inhibitors of the MEK/ERK pathway such as PD98059 and U1026; suramin, a broad-spectrum antagonist of cell surface receptors and piceatannol, a potent inhibitor of the Syk

tyrosine kinase. However, inhibition of the shedding of GPI-anchored receptors by cAMP did not affect Cry1A toxicity (Valaitis 2008), suggesting that shedding of APN by cAMP may not be involved in the defense to Cry toxin action.

Finally, Loeb *et al.* 2001 reported that treatment of *Heliothis virescens* cultured midgut cells with low doses of Cry1Ac toxin stimulated the proliferation of stem cells and induced their differentiation. These data represent a complex response; since stem cell proliferation and differentiation is orchestrated by multiple feedback controls involving hormones, growth factors, smaller regulatory molecules and transcription factors. However, the role of MAPK p38 in this effect remains to be analyzed.

Our results show that insects respond to PFT by activation of the MAPK p38 transduction pathway that results in defense mechanism to Cry toxin action. Still remains to be determined the specific defense cellular responses induced by the MAPK p38 pathway. Proteomic analysis of cellular responses in silenced and non-silenced MAPK p38 larvae could reveal the proteins involved in defense to Cry toxins. However, our results show that it is possible to enhance Cry toxin action by inhibiting a specific signal transduction pathway. This may have biotechnological applications for enhancing the activity of some Bt Cry toxins against specific insect pests. Several insect pests are poorly controlled by Cry toxins and in these cases it may be feasible to enhance Cry toxin activity by feeding at the same time the specific MAPK p38 dsRNA and the Cry toxin by co-expressing both factors in the same transgenic plant.

Abbreviations

PFT	Pore forming toxins
MAPK	mitogen activated protein kinase
PLY	pneumolysin
SLO	streptolysin O
Hla	α -hemolysin
UPR	endoplasmic reticulum stress response to unfolded proteins
LLO	listeriolysin
TNF- α	tumor necrosis factor
LC ₅₀	50% lethal concentration
RNAi	RNA interference

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Figure 1. Phosphorylation of MAPK p38 in insects after intoxication with Cry toxins

Panel A, first instar *Manduca sexta* larvae were intoxicated with 2 ng/cm² (LC₅₀) or 20 ng/cm² Cry1Ab for up to one h. *M. sexta* larvae were also treated one h with the non-toxic mutant Cry1AbR99E affected in oligomerization and pore formation. Panel B, fourth instar *Aedes aegypti* larvae were fed an LC₅₀ of Cry11Aa toxin or the non-active mutant Cry11Aa R90E. The presence of phosphorylated and total MAPK p38 proteins was analyzed by western blot using specific antibodies. The blots presented here are representative figures of three independent experiments. Numbers under the blots are percentage in relation to the control band (no toxin or time 0, which was considered as 100%), after scanning the bands.

Figure 2. Activation of MAPK p38 protein synthesis in insects after intoxication with Cry toxins
 Panel A, Analysis of the MAPK p38 protein levels in *Manduca sexta* or in *Aedes aegypti* larvae by Western blot experiments using an anti-p38 antibody at various times after intoxication. First instar *M. sexta* larvae were treated with LC₅₀ or LC₉₀ Cry1Ab toxin concentrations and analyzed after seven days of intoxication. Fourth instar *A. aegypti* larvae were also treated with corresponding LC₅₀ or LC₉₀ Cry11Aa toxin concentrations and analyzed after 24 h. Detection of tubulin protein was determined as control protein. The blots presented here are representative figures of three independent experiments. Numbers under the blots are percentage in relation to the control band, after scanning the bands. The control band corresponds to no-toxin, which was labeled with a C and was considered as 100%. Panel B, Analysis of the expression of MAPK p38 RNA by RTPCR assays in *M. sexta* or in *A. aegypti* after treatment with the corresponding Cry1Ab or Cry11Aa toxins at different times. Expression of actin gene was used as control in these assays. The results presented here are representative figures of four independent experiments. Numbers under the bands are percentage in relation to the control band, after densitometry analysis. The control band corresponds to time 0, which was labeled with a C and was considered as 100%. Panel C, Regulation of MAPK p38 gene in *M. sexta* larvae after intoxication with LC₅₀ of Cry1Ab toxin analyzed by quantitative RT-PCR assays.

Figure 3. Silencing of MAPK p38 by RNAi in *Manduca sexta* and *Aedes aegypti* larvae
 MAPK p38 expression was silenced by feeding dsRNA to *M. sexta* and *A. aegypti* larvae. Panel A, The presence of total MAPK p38 protein was analyzed by western blot assays using specific antibodies. Panel B, The expression of MAPK p38 gene was analyzed by RT-PCR assays. Numbers under the bands are percentage in relation to the control band, after densitometry analysis. The control bands corresponds to non-silenced larvae, which were labeled with a C and were considered as 100%.

Table 1

Sequences of oligonucleotides used for transcript amplification.

	Oligonucleotide sequence
Ms-p38-F	5' CCC <u>AAG CTT</u> CGG CCA TAG ACA CTT TGC ATA ATA TG
Ms-p38-R	5' CCG <u>GAA TTC</u> CCG ATT ACA TTT TCA TGG TTC
Ae-p38-F	5' GAA <u>GAT CTT</u> CGG AAA TCA TGC TCA ACT
Ae-p38-R	5' GAA <u>GAT CTT</u> CGG GAC GAA ATT AAG TAC
Ms-actin-F	5' GTC GAC AAT GGC TCC GGC ATG TGC A
Ms-actin-R	5' TCT GGG TCA TCT TTT CCC TGT TGG CCT TGG GGT
Ms-RPS3-F	5' CCG ATC GGA GAT CAT CAT CAT GGC C
Ms-RPS3-R	5' CAT CCG CGA GTT GAC TTC GGT CGTC

Table 2

Toxicity of spore-crystal suspensions of wild type and mutated Cry toxins against *Manduca sexta* and *Aedes aegypti* larvae

δ -endotoxin	Insect	LC ₅₀ (95% fiducial limits)	Slope (error value)	fold increase in susceptibility
Cry1Ab	<i>M. sexta</i>	2 (0.8–4) ^a	2.5 (0.5)	1
Cry1Ab	p38-silenced	0.25 (0.15–0.4) ^a	1.8 (0.3)	8
	<i>M. sexta</i>			
Cry1Ab-R99E	<i>M. sexta</i>	>2,000 ^a	ND	ND
Cry11Aa-R90E	<i>A. aegypti</i>	>10,000 ^b	ND	ND
Cry11Aa	<i>A. aegypti</i>	30 (25–36) ^b	5.0 (0.5)	1
+ Effectene				
Cry11Aa	p38-silenced	3 (2–4) ^b	4.2 (0.4)	10
+ Effectene	<i>A. aegypti</i>			

^aLC₅₀ ng/cm²,

^bLC₅₀ ng/ml

^cNot determined