Insect Biochemistry and Molecular Biology 43 (2013) 635–643

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Resistance to Bt maize in *Mythimna unipuncta* (Lepidoptera: Noctuidae) is mediated by alteration in Cry1Ab protein activation☆

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ARTICLE INFO

Article history:
Received 1 March 2013
Received in revised form
3 April 2013
Accepted 6 April 2013

Keywords:
Bacillus thuringiensis
Cry proteins
Toxicity
Bioassays
Resistance
Proteases
Binding assays
Resistance management

ABSTRACT

Bt maize cultivars based on the event MON810 (expressing Cry1Ab) have shown high efficacy for controlling corn borers. However, their efficiency for controlling some secondary lepidopteran pests such as *Mythimna unipuncta* has been questioned, raising concerns about potential outbreaks and its economic consequences. We have selected a resistant strain (MR) of *M. unipuncta*, which is capable of completing its life cycle on Bt maize and displays a similar performance when feeding on both Bt and non-Bt maize. The proteolytic activation of the protoxin and the binding of active toxin to brush border membrane vesicles were investigated in the resistant and a control strain. A reduction in the activity of proteolytic enzymes, which correlates with impaired capacity of midgut extracts to activate the Cry1Ab protoxin has been observed in the resistant strain. Moreover, resistance in larvae of the MR strain was reverted when treated with Cry1Ab toxin activated with midgut juice from the control strain. All these data indicate that resistance in the MR strain is mediated by alteration of toxin activation rather than to an increase in the proteolytic degradation of the protein. By contrast, binding assays performed with biotin labelled Cry1Ab suggest that binding to midgut receptors does not play a major role in the resistance to Bt maize. Our results emphasize the risk of development of resistance in field populations of *M. unipuncta* and the need to consider this secondary pest in ongoing resistance management programs to avoid the likely negative agronomic and environmental consequences.

1. Introduction

Transgenic maize engineered to express insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt maize) has become widely adopted worldwide with 51 million ha planted in 2011 (James, 2011). Bt maize hybrids were designed for controlling some of the most important pests of maize such as corn borers, rootworms and other pests (Shelton et al., 2002). However, their long-term efficacy is threatened by the possible evolution of pest resistance. Bt maize field-evolved resistance has been documented in just two lepidopteran species after 16 years of continuous cultivation: the African stem borer, *Busseola fusca* to Bt maize expressing Cry1Ab (Kruger et al., 2011; Van Rensburg, 2007) and the fall armyworm, *Spodoptera frugiperda* to the Cry1F insecticidal protein (Storer et al., 2010). Nevertheless, other target species have shown their potential to develop resistance to Bt toxins by laboratory selection (Meihls et al., 2008; Pereira et al., 2008).

At present, the only Bt maize hybrids allowed for cultivation in the EU are those based on the event MON810 (Monsanto) expressing the Cry1Ab toxin. Spanish farmers have rapidly adopted Bt maize cultivars to control two key lepidopteran pests: the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae), and the Mediterranean corn borer (MCB), *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae), by increasing the growing area from about 5% of the total in 1998 to 26.5% in 2011 (http://www.magrama.gob.es/es/calidad-y-evaluacion-ambiental/temas/biotecnologia/organismos-modificados-geneticamente-omg-/consejo-interministerial-de-ogms/superficie.aspx). However, the available Bt maize cultivars have shown a much lower efficacy for controlling some secondary Spanish maize lepidopteran pests, such as the true armyworm, *Mythimna unipuncta* Haworth (Lepidoptera: Noctuidae). Concerns that secondary pests may become a problem because of deployment of Bt crops have been recently

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http://dx.doi.org/10.1016/j.ibmb.2013.04.001

raised (Wang et al., 2009; Zhao et al., 2011). Significant damage caused by *M. unipuncta* has been reported in different Bt maize transgenic lines tested (Eizaguirre et al., 2010; Pilcher et al., 1997; Schaafsma et al., 2007). Moreover, Eizaguirre et al. (2010) showed that a small percentage of larvae of this pest can survive and complete their development on Bt maize expressing the Cry1Ab toxin. Recently, Pérez-Hedo et al. (2012) reported that multiple mechanisms (such as toxin elimination and overcompensation mechanisms) could be involved in the low susceptibility to the toxin. This finding indicates that the concentration of Cry1Ab expressed by transgenic maize hybrids represents a "low dose" scenario for *M. unipuncta*, favouring the potential development of resistance in field populations.

There are two well-studied mechanisms of resistance to Cry proteins that involve the alteration of two steps of the mechanism of action: proteolytic activation of protoxin and binding of active toxin to receptors (Ferré and Van Rie, 2002). Alterations in the activity of serine proteases (i.e. trypsin, chymotrypsin and elastase) have correlated with resistance in several lepidopteran species such as *Plodia interpunctella* (Oppert et al., 1994), *Plutella xylostella* (Sayyed et al., 2001), *Heliothis virescens* (Forcada et al., 1999), *Helicoverpa armigera* (Rajagopal et al., 2009), *Spodoptera littoralis* (Keller et al., 1996), and *O. nubilalis* (Li et al., 2004). In addition, the alteration of binding of Cry toxins to midgut receptors has been reported as the main cause of resistance in a broad number of insect species and it is the mechanism found in populations showing resistance to Bt sprays in the field (Ferré and Van Rie, 2002).

In this study we have selected a resistant strain of *M. unipuncta* against Bt maize (MON810) expressing the Cry1Ab toxin. Thereafter, we have determined the survival and performance of the *M. unipuncta* selected strain on a Bt maize variety and its nearest isogenic line. Finally, we have investigated the mechanism of resistance to Cry1Ab in the selected resistant strain.

2. Material and methods

2.1. Insect and plant material

A control colony of *M. unipuncta* was established from egg masses collected in conventional maize from a commercial field in Monzón (Huesca, Spain) in 2009 (MC strain). Due to their cannibalistic behaviour, a minimum of 600 larvae per generation was reared individually in plastic boxes (3.7 cm diameter × 2 cm height) until pupation. Larvae were fed *ad libitum* on conventional maize leaf pieces that were replaced every 3–5 days, depending on the larval size. Egg masses of *M. unipuncta* were obtained by confining batches of 10 pairs of adults in ventilated plastic boxes of 30 × 24 × 15 cm containing cotton soaked with a solution of 10% honey in water. As oviposition substrate, 3 plastic cylinders (1.6 cm diameter × 7.5 cm height), covered with overlapping layers of Parafilm® (Pechiney, Menasha, WI, USA), where females could introduce the ovipositor, were fixed at the bottom. Adult pairs were kept for 10 days and the oviposition cylinders replaced every 2 days. Oviposition cylinders with the egg masses were maintained in plastic boxes (12 cm diameter × 5 cm height) provided with moistened filter paper until hatching. Environmental conditions for rearing all stages and for egg incubation were 23 ± 0.3 °C, 70 ± 5% RH, and a photoperiod of 16:8 h (L:D), using a growth chamber (Sanyo MLR-350 H, Sanyo, Japan).

Two maize varieties were used: Bt-maize DKC6041YG (event MON810, Cry1Ab toxin); and its near isogenic conventional line DKC6040 (non-Bt maize). Plants were grown in plastic pots (25 cm diameter) using Compo Sana® Universal as a substrate and maintained in a controlled greenhouse at 25 ± 5 °C, >60% RH and 16:8 h (L:D) photoperiod.

2.2. Cry proteins

The Cry1Ab protoxin used in this study was obtained from *Escherichia coli* cultures, strain XL1-blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]), transformed with the plasmid pBD140, kindly donated by Dr. R.A. de Maagd, Plant Research International B.V., Wageningen, The Netherlands.

The cultures were grown for 96 h at 37 °C, 180 rpm, in liquid Terrific Broth medium (Laboratorios Conda S.A., Madrid, Spain) supplemented with 50 μg/ml ampicillin. The production of inclusion bodies, containing the protoxin, was monitored by phase-contrast microscopy. The cultures were pelleted by centrifugation at 12,000 × g for 10 min at 4 °C. Each gram of pellet was suspended in 3 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 5 mM EDTA, 100 mM NaCl). The cell lysis was performed by the addition of 0.8 mg of lysozyme from hen egg white (Fluka BioChemika, Buchs, Switzerland) per gram of pellet followed by 20 min incubation at room temperature with gentle agitation. The lysis process was completed by the addition of sodium deoxycholate (4 mg/g of pellet) followed by strong vortexing and incubation at 37 °C for 10 min. Contamination with DNA was removed incubating with DNase I (Sigma–Aldrich, Co. MO. USA) from bovine pancreas (0.2 mg/g of pellet) for 45 min at 37 °C. The inclusion bodies were collected by centrifugation at 24,000 × g for 20 min at 4 °C. These inclusion bodies were washed twice with ice-cold wash buffer (20 mM Tris–HCl pH 7.5, 1% Triton X-100, 1 M NaCl) and twice again with ice-cold phosphate buffered saline pH 7.4 (PBS, Roche Diagnostics GmbH, Mannheim, Germany). They were further suspended in solubilisation buffer (50 mM sodium carbonate pH 10.5, 100 mM NaCl, 10 mM dithiothreitol) and incubated overnight at room temperature with gentle agitation. Soluble protoxin was obtained in the supernatant after centrifugation at 24,000 × g for 30 min at 4 °C. The quality of preparations was tested by SDS-PAGE as described elsewhere (Sambrook et al., 1989).

To perform binding assays, Cry1Ab protoxin was incubated with bovine trypsin (Sigma–Aldrich, Co. MO. USA) at 37 °C for 2 h (1 mg trypsin per 10 mg of protoxin) to obtain activated toxin. Insoluble material was removed by centrifugation at 17,500 × g for 15 min at 4 °C. Activated Cry1Ab was purified by anion exchange chromatography in a Fast Liquid Protein Chromatography system (Pharmacia LKB, Uppsala, Sweden) using two HiTrap™Q FF 1 ml columns in tandem (GE Healthcare Bio-sciences AB, Uppsala, Sweden). The toxin preparations were fractionated by a 0–600 mM linear gradient of NaCl. The peaks were analysed by SDS-PAGE to detect those fractions containing the activated toxin. These fractions were pooled and stored at 4 °C until needed.

2.3. Verification of the insecticidal activity of purified Cry1Ab and Bt maize

A laboratory colony of *O. nubilalis* was used as a positive control to verify the biological activity of the Cry1Ab protoxin and of the Bt maize plants used in this study. Neonates <24 h ($n = 64$) were individually treated with 30 ng/cm^2 of Cry1Ab protoxin applied on the diet surface. Control larvae ($n = 32$) were treated with 50 mM sodium carbonate buffer (pH 10.5) (Farinós et al., 2004). To test the efficacy of each Bt maize plant, batches of neonates ($n = 10$) were fed on leaf pieces during 5 days. Similarly, ten larvae were fed on non-Bt maize to be used as control.

2.4. Selection process

The control MC strain was maintained in the laboratory without exposure to Bt maize or Cry toxins. After six generations, a subset of

the control (F0) was selected by feeding larvae on Bt maize leaves during the whole larval stage, to obtain the resistant MR strain (Table 1). A minimum of 500 neonate larvae was used for selection in each generation. When the number of available neonates was below 500 (generations F1 and F10), they were fed with non-Bt maize to assure that the MR strain was continued. At the end of each generation larval and adult survival was recorded. Larvae of generations F6 to F8 were used for biochemical experiments, and larvae of generations F11 and F12 for leaf disc bioassays and nutritional indices.

2.5. Nutritional indices

The nutritional indices were performed with late instars because the likelihood of L_1 instars attacking maize is less than that of larger instars migrating into maize from grassy areas (Pilcher et al., 1997). The performance of fifth instar larvae (L_5) was compared in larvae from MC and MR strains, fed on Bt or non-Bt maize. Upon moulting to the fifth instar, 20 larvae were weighed (initial fresh weight) using an analytical balance (Mettler Toledo AX205, Mettler-Toledo International Inc., Columbus, OH, USA) and individually placed in a Petri disc coated on their bottom with a 2.5% agar solution and containing weighted sets of 8 maize leaf discs of 20 mm diameter, from 12 to 13 leaf stage plants. Larvae were allowed to feed for 12 h and then, the insects, the remains of leaf discs and the faeces produced were dried at 60 °C for 48 h to determine dry weight (DW). Larval initial dry weight was estimated by calculating a fresh-dry weight conversion factor from 30 L_5 larvae from each strain (MC and MR) fed on non-Bt maize or Bt maize, dried at 60 °C for 48 h and then weighed to determine DWs. To estimate initial DW of non-Bt maize and Bt maize leaf discs, 30 sets (containing 8 leaf discs each) from each maize type were weighed separately, dried at 60 °C for 48 h and then weighed to determine DWs. The nutritional indices as described by Farrar et al. (1989) were calculated using DWs as follows: relative consumption rate [RCR = mg ingested/(insect initial DW × days)]; relative growth rate [RGR = DW gained in feeding period/(insect initial DW × days)]; efficiency of conversion of ingested matter to body mass [ECI = (RCR/RGR) × 100]; approximate digestibility [AD = ((mg ingested − DW of faeces)/(mg ingested)) × 100]; efficiency of conversion of digested matter to body mass [ECD = (DW gained in feeding period/(mg ingested − DW of faeces)) × 100].

Table 1
Selection process to obtain the resistant *Mythimna unipuncta* strain.

Generation[a]	Maize used for feeding[b]	Initial number of neonates	Larval survival (%)[c]	Adult survival (%)[d]
F0	Bt	600	1.8	1.2
F1	Non-Bt	265		
F2	Bt	560	8.9	5.9
F3	Bt	514	16.1	7.0
F4	Bt	1080	20.6	11.8
F5	Bt	1160	48.4	38.8
F6	Bt	820	53.5	42.2
F7	Bt	650	56.5	42.3
F8	Bt	590	54.4	49.5
F9	Bt	708	21.6	12.7
F10	Non-Bt	39		
F11	Bt	700	37.9	28.1
F12	Bt	600	27.2	23.2

[a] Number of generations of selection. The generation F0 was formed by neonate larvae from the sixth generation of the MC strain, which was originated from a population collected in 2009 in conventional maize from a commercial field in Monzón (Huesca, Spain).
[b] Larvae were fed with fresh Bt maize leaves from DKC6041YG (event MON810) during the whole life-cycle, except when the number of available neonates was below 500 (generations F1 and F10), that were fed with non-Bt maize (line DKC6040).
[c] Percentage of neonates that reached the pupal stage.
[d] Percentage of neonates that reached the adult stage.

2.6. Susceptibility bioassays

Susceptibility to Cry1Ab of neonate larvae (<24 h) of the control MC strain was assessed using a leaf disc dipping bioassay. Fresh maize leaf discs, 17 mm diameter, were cut (avoiding mid-rib) from 12 to 13 leaf stage of non-Bt plants. All discs were first dipped in a 0.1% Triton X-100 solution as surfactant. After drying in a laminar flow hood, discs were dipped for 10 s in 8 different concentrations of Cry1Ab, ranging from 2.5 to 1526 μg/ml in 50 mM sodium carbonate buffer (pH 10.5). Control discs were dipped in the carbonate buffer. Discs were air-dried again in a laminar flow hood and deposited in a plastic container (2 cm diameter × 2 cm height) coated with a 5 mm layer of 2.5% agar. One larva per disc was used. The bioassays were conducted at 23 ± 0.2 °C, 80 ± 5% RH and 16:8 h (L:D) photoperiod in a growth chamber (Sanyo MLR-350 H, Sanyo, Japan). For each concentration of Cry1Ab, 12 larvae replicated six times were used. After 7 days larval mortality and moult inhibition were recorded.

The susceptibility of neonates from control MC and resistant MR strains was compared performing leaf disc bioassays as described above. Five treatments were carried out: Bt maize, non-Bt maize, non-Bt maize + Cry1Ab protoxin (1526 μg Cry1Ab/ml 50 mM sodium carbonate buffer, pH 10.5), non-Bt maize + Cry1Ab toxin processed with midgut juice from the MC strain and non-Bt maize + Cry1Ab processed with bovine trypsin. In the last two treatments, the Cry1Ab protoxin was incubated for 1 h with midgut juice from the MC strain or with bovine trypsin, respectively, and the amount of protoxin was adjusted to obtain a concentration of 1526 μg Cry1Ab/ml, while keeping a ratio 1:100 (w/w) enzyme: protein (see section 2.8).

2.7. Preparation of midgut extracts and BBMV from M. unipuncta larvae

Last instar larvae either from control MC or resistant MR strains were placed on ice for 10 min to reduce their mobility and then they were dissected to obtain their midguts as previously described (Ortego et al., 1996). The midguts containing the food bolus were excised with forceps and their contents were pooled for future assays (each pool contained the midgut contents of five larvae). The midgut walls were washed with either ice-cold 0.15 M NaCl or MET buffer (0.3 M Manitol, 5 mM EGTA, 17 mM Tris–HCl pH 7.5) depending on the future use: determination of enzymatic activities or binding assays, respectively. They were pooled in 1-g fractions. All fractions, midgut contents and midgut walls were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Fractions containing pooled midgut contents or midgut walls washed with 0.15 M NaCl were thawed on ice. Then, 250 mg were mixed with 250 μl of 0.15 M NaCl. The mixture was homogenised manually with a plastic homogenizer and then centrifuged at 22,000 × g for 10 min at 4 °C. The supernatant was kept on ice to perform enzymatic assays or immediately frozen in liquid nitrogen and stored at −80 °C until used.

Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method (Wolfersberger et al., 1987) using the midgut walls washed with MET buffer obtained as described above. Once prepared, the BBMV were frozen in liquid nitrogen and stored at −80 °C until used.

2.8. Hydrolytic processing of Cry1Ab protoxin with midgut extracts

One milligram of Cry1Ab protoxin was incubated at 37 °C with 10 μg (total protein) of midguts-content extracts or BBMV from MC

and MR strains (ratio 1:100 (w/w), enzyme: protein) in 50 mM sodium carbonate pH 10.5, 150 mM NaCl. Similarly, 1 mg of Cry1Ab protoxin alone or with 10 μg of bovine trypsin was also incubated in the same conditions. Aliquots were taken at 1 and 2 h, mixed with electrophoresis sample buffer, heated at 95 °C for 10 min and subjected to SDS-PAGE (Sambrook et al., 1989) to monitor the activation process.

2.9. Binding experiments

Chromatographically purified Cry1Ab activated toxin was biotin-labelled using the Amersham™ ECL™ Protein Biotinylation module (RPN 2202, Ge Healthcare UK Limited, Little Chalfont, UK) according to the manufacturer's recommendations. Binding assays were performed essentially as published before (González-Cabrera et al., 2006). Briefly, biotinylated Cry1Ab was incubated for 1 h with BBMV from MC or MR strains in 100 μl of binding buffer (PBS supplemented with 0.1% Bovine Serum Albumin (BSA)). In competition experiments, excess unlabelled Cry1Ab activated toxin (competitor) was added before the addition of BBMV to the reaction mixture. After incubation, BBMV were pelleted at 22,000 × g for 10 min, and washed once with 500 μl of ice-cold binding buffer. The pellets were suspended in electrophoresis sample buffer, heated at 95 °C for 10 min and subjected to SDS-PAGE (Sambrook et al., 1989). The proteins contained in the gel were electro-transferred onto nitrocellulose membranes (GE Healthcare Amersham™ Hybond™-ECL; Little Chalfont, United Kingdom). The membranes were incubated 1 h in 5% Membrane Blocking Agent (GE Healthcare UK Limited, Little Chalfont, United Kingdom) and then 1 h with streptavidin-alkaline phosphatase conjugate (1:10,000; Roche Diagnostics GmbH, Mannheim, Germany). Biotinylated activated toxins were detected in the membrane by colour development, following manufacturer's recommendations, using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Diagnostics GmbH, Mannheim, Germany) as the alkaline phosphatase substrate.

2.10. Enzyme activity assays

Enzymatic activities were measured in extracts from control and resistant strains. All experiments were performed in 96-well microtiter plates (Falcon®, Becton Dickinson, NJ, USA), at least per triplicate, and blanks were used to account for spontaneous breakdown of substrates. Optical density (OD) was always measured in a microplate reader (Benchmark, Bio-Rad Laboratories, CA, USA).

Trypsin, chymotrypsin and elastase-like activities were assayed using BApNa (Nα-benzoyl-DL-arginine p-nitroanilide), SA_2PPpNa (N-succinyl-alanine-alanine-proline-phenylalanine p-nitroanilide) and SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide) as substrates, respectively, as described elsewhere but with several modifications (Ortego et al., 1996). The assays were performed in 200 μl reaction buffer (50 mM sodium carbonate pH 10.5, 150 mM NaCl) containing 0.25 mM of each substrate (added in 10 μl of DMSO) and 4 μg of total protein from midgut content extracts, for trypsin and elastase-like activities, or 0.4 μg for chymotrypsin-like activity. The reaction started after the addition of the substrate. It was incubated at 30 °C for 1 h and stopped by the addition of 100 μl of ice-cold 30% acetic acid. The OD at 415 nm was measured and the specific activity estimated as nmol substrate hydrolysed per minute per mg protein, using a molar extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961). Leucine aminopeptidase-like activity was measured following a similar protocol but with a different reaction buffer (0.1 M Tris–HCl pH 7.5, 0.15 M NaCl, 5 mM $MgCl_2$) containing 4 μg of enzyme midgut wall extracts and 1 mM LpNa (L-leucine p-nitroanilide) (added in 10 μl methanol) as substrate.

Alpha-amylase activity was measured essentially as reported by Valencia et al. (2000). Briefly, 150 μl reaction buffer (0.1 M Tris–HCl pH 7.5, 10 mM NaCl, 20 mM $CaCl_2$) containing 2 μg of total protein from midgut content extracts and 100 μg of starch were incubated at 30 °C for 30 min and stopped by the addition of 150 μl of Lugol's solution (0.02% I_2/0.2% KI). The OD at 595 nm was measured and the specific activity estimated as μg starch hydrolysed per minute per mg protein.

General esterase activity was assayed using the method reported by Dary et al. (1990). Accordingly, 170 μl of reaction buffer (0.1 M Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) containing 0.2 μg of total protein from midgut wall extracts and 0.25 mM 1-NA (1-Naphthyl acetate) as substrate (added in 5 μl ethanol) were incubated at 30 °C for 1 h and stopped by the addition of 80 μl of Fast-blue salt BN solution (0.4 mg/ml Fast-blue salt BN and 15 mg/ml of sodium dodecyl sulphate (SDS) in distilled water). The reaction mixture was then incubated again for 1 h at room temperature and the OD at 595 nm was recorded. The specific activity was estimated as nmol substrate hydrolysed per minute per mg protein.

In all cases, total protein concentration was determined by the method of Bradford (Bradford, 1976) using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) with BSA as standard.

2.11. Zymograms

Electrophoretic detection of proteolytic forms was performed by 0.1% (w/v) SDS, 12% (w/v) polyacrylamide gel electrophoresis under non-denaturing conditions using a Bio-Rad Mini-Protean II Electrophoresis Cell system. Samples consisted of pooled MC or MR larval midgut extracts (containing 1.90 and 0.75 μg of protein). The ratio of acrylamide to bis-acrylamide was 37.5:1. The gel was run at 150 V and 4 °C. After electrophoresis, gel was soaked in a 2% (w/v) gelatin (type B: from bovine skin, Sigma) solution in 0.1 M glycine-NaOH, pH 10.5 and incubated for 1 h at 35 °C. Proteolysis was stopped by transferring the gels into a staining solution [0.3% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid]. The gel was destained in 25% (v/v) methanol 10% (v/v) acetic acid. Bands of proteolytic activity were visualized against the blue background of the gel.

2.12. Data analysis

Mortality and moult inhibition data from the MC strain were used to estimate the concentrations needed to cause 50 and 90% mortality (LC_{50}, LC_{90}) and moult inhibition (MIC_{50}, MIC_{90}) by probit analysis using POLO–PC (LeOra Software, Berkeley, CA, USA), which automatically corrected for control mortality. Differences in susceptibility between MC and MR strains were compared with one way-ANOVA, followed by Tukey's multiple comparisons tests. Comparisons among RCR, RGR, ECI, AD and ECD were made with ANCOVA followed by Tukey's multiple comparisons test, using as covariate initial dry weight for RCR and RGR, assimilated mass for ECI, dry matter ingested for AD, and digested mass for ECD (Raubenheimer and Simpson, 1992). Enzymatic activities were compared by unpaired *t*-test. A significance level of $P < 0.05$ was considered for all tests.

3. Results

3.1. Selection of the resistant MR strain

Larval survival of the control MC strain (F0 generation) on Bt maize was 1.8%, and the number of individuals that reached the adult stage was 1.2% (Table 1), indicating that this population

presented some degree of tolerance to Bt maize. By laboratory selection we found that resistance to Bt maize increased rapidly when larvae were reared on Bt maize, obtaining a resistant MR strain. Remarkably, about 50% of the larvae could complete their development on Bt maize after only eight generations of selection. The low number of available neonates in generations F1 and F10 forced to feed larvae on non-Bt maize. However, a high level of resistance was rapidly reached in the following generations (Table 1).

3.2. Nutritional indices

Fifth instar larvae (L_5) from the MC strain fed on non-Bt maize presented the highest consumption (RCR) and growth (RGR) rates (Table 2). Growth of L_5 instar of *M. unipuncta* MC was negatively affected by Bt maize, and this growth suppression was mediated by a reduction in consumption, digestibility and in greater metabolic expenditures (as reflected by negatives ECI and ECD). Larvae from the MR strain presented similar nutritional indices when fed on non-Bt or Bt maize (RCR, RGR, ECI and ECD), except for the approximate digestibility (AD) that was significantly higher when the MR population fed on Bt maize. Consumption and growth rate in larvae from the MR strain (fed either on non-Bt or Bt maize) were significantly lower than in larvae from the MC strain fed on non-Bt maize, but significantly higher than in larvae from the MC strain fed on Bt maize. Nevertheless, these differences were not reflected as differential metabolic expenditures (ECI and ECD) when MR strain (fed either on non-Bt or Bt maize) and MC strain fed on non-Bt maize were compared.

3.3. Susceptibility bioassays

Mythimna unipuncta neonates readily accepted detached maize leaf discs for feeding. Lethal and moult inhibition concentrations (LCs and MICs) of Cry1Ab protoxin were estimated for neonate *M. unipuncta* larvae from the control MC strain. Values of LC_{50} [140 μg Cry1Ab/ml ($n = 576$; slope ± SE = 0.53 ± 0.08; $\chi2 = 15.2$, d.f. = 46)] and MIC_{50} [101 μg Cry1Ab/ml ($n = 576$; slope ± SE = 0.51 ± 0.07; $\chi2 = 19.3$, d.f. = 46)] were very similar, and the same occurred with LC_{90} (38281 μg Cry1Ab/ml) and MIC_{90} (33367 μg Cry1Ab/ml) values. Similar dose–response studies could not be performed with larvae from the MR strain due to their low susceptibility to the Cry1Ab protoxin

Survival of neonate larvae from MC and MR strains greatly differed among treatments (Bt maize, non-Bt maize, non-Bt maize + Cry1Ab protoxin, non-Bt maize + Cry1Ab toxin processed with midgut juice from the MC strain and non-Bt maize + Cry1Ab processed with bovine trypsin) after 7 days (Fig. 1). The highest mortality levels were found in MC larvae fed on Bt maize, non-Bt maize + Cry1Ab protoxin and non-Bt maize + Cry1Ab protoxin processed with midgut juice from the MC strain (89%, 76% and 75%, respectively). Interestingly, a mortality of 58% was observed in larvae of the resistant MR strain fed on maize leaf discs dipped in Cry1Ab toxin processed with midgut juice from the control MC strain. This value is significantly higher than the mortality displayed by MR in the rest of the treatments with the toxin Cry1Ab or Bt maize, which did not exceed 21% (Fig. 1). The mortality of larvae of both strains fed on non-Bt maize was below 6 %.

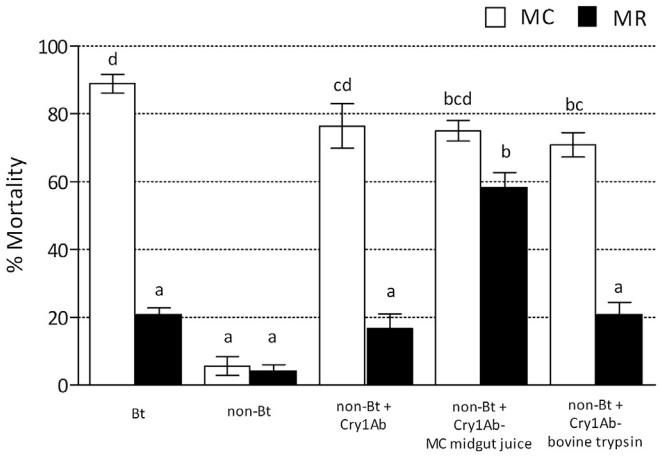

Fig. 1. Mortality (mean ± SE) of *M. unipuncta* neonates from a control (MC) and a resistant (MR) strain fed on maize leaf discs of Bt maize *var.* DKC6041YG, expressing Cry1Ab (Bt), its near isogenic line DKC6040 (non-Bt), and the isogenic line dipped in 1526 μg Cry1Ab protoxin/ml (non-Bt + Cry1Ab) or in 1526 μg Cry1Ab protoxin/ml incubated with midgut juice from the MC strain (non-Bt + Cry1Ab-MC midgut juice) or with bovine trypsin (non-Bt + Cry1Ab-bovine tryspsin). Mortality was assessed after 7 days of exposure. Means were compared by one way-ANOVA, followed by Tukey's multiple comparisons test ($P < 0.05$). Different letters above bars indicate significant differences.

3.4. Activation of Cry1Ab protoxin

The incubation of Cry1Ab protoxin with midgut content extracts from control MC and resistant MR larvae from *M. unipuncta* yielded a clearly different pattern in both cases (Fig. 2A). After 1 h incubation, MC produced a strong single band of ~67–69 kDa, corresponding to a processed Cry1Ab protein, having a molecular weight lower than that produced by bovine trypsin (~74 kDa). In contrast, MR processed the toxin less efficiently into two bands, having similar weight to those produced by bovine trypsin and MC gut extracts. Increasing the incubation time did not alter the pattern observed in the activation of Cry1Ab by MC extracts or bovine trypsin. However, in case of activation with MR extracts, such increase resulted in a reduction in the intensity of the higher molecular weight band in favour to the lower one.

Differences in processing were also observed when using BBMV isolated from MC and MR to activate the Cry1Ab toxin (Fig. 2B).

Table 2
Nutritional indices of *Mythimna unipuncta* L_5 larvae fed for 12 h on Bt and non-Bt maize.

Strain[a]	Maize	Nutritional indices[b]				
		RCR (mg/mg/day)	RGR (mg/mg/day)	ECI (%)	AD (%)	ECD (%)
MC	Non-Bt	4.84 ± 0.4 c	1.67 ± 0.1 c	34.41 ± 1.7 b	51.56 ± 2.4 bc	71.04 ± 5.8 b
	Bt	0.21 ± 0.0 a	−0.05 ± 0.0 a	−33.56 ± 14.3 a	9.27 ± 1.3 a	−485.25 ± 154.4 a
MR	Non-Bt	2.79 ± 0.3 b	0.73 ± 0.1 b	23.21 ± 3.9 b	41.32 ± 6.1 b	58.49 ± 4.6 b
	Bt	3.34 ± 0.3 b	0.83 ± 0.1 b	27.04 ± 3.0 b	50.19 ± 3.6 c	62.67 ± 10.4 b

Means were compared by ANCOVA followed by Tukey's multiple comparisons tests (as covariate was used initial dry weight for RCR and RGR; assimilated mass for ECI; dry matter ingested for AD; digested mass for ECD). Values followed by the same letter within a column are not significantly different from each other ($P < 0.05$).

[a] MC: Control strain; MR: Resistant strain.

[b] RCR: relative consumption rate; RGR: relative growth rate; ECI: efficiency of conversion of ingested matter to body mass; AD: approximate digestibility; ECD: efficiency with which digested food is converted to body substance. Values are the mean ± standard error of 20 replicates.

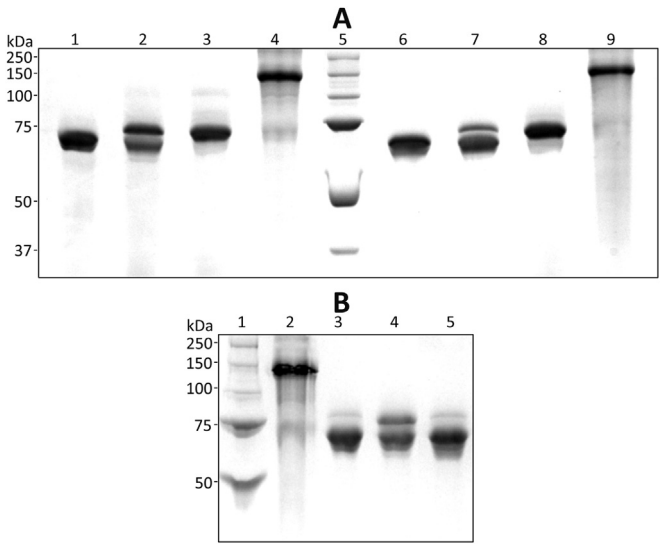

Fig. 2. Activation of Cry1Ab protoxin with extracts from larvae of control (MC) and resistant (MR) strains of *M. unipuncta*. Ratio: 1 mg protoxin/10 μg total protein from enzyme extracts, BBMV or trypsin. (**A**) **Lanes 1 and 6**: Cry1Ab incubated with midgut content extracts from MC, **Lanes 2 and 7**: Cry1Ab incubated with midgut content extracts from MR, **Lanes 3 and 8**: Cry1Ab incubated with bovine trypsin (Sigma–Aldrich Co. LLC. MO, USA), **Lanes 4 and 9**: Cry1Ab protoxin (Control), **Lane 5**: Precision Plus Protein Standard (Bio-Rad Laboratories GmbH, Munich, Germany). **Lanes 1-4** incubation 1 h at 37 °C, **Lanes 6-9** incubation 2 h at 37 °C. (**B**) **Lane 1**: Precision Plus Protein Standard (Bio-Rad Laboratories GmbH, Munich, Germany), **Lane 2**: Cry1Ab protoxin (Control), **Lane 3**: Cry1Ab incubated with bovine trypsin (Sigma–Aldrich Co. LLC. MO, USA), **Lane 4**: Cry1Ab incubated with BBMV from MR, **Lane 5**: Cry1Ab incubated with BBMV from MC. Incubation 2 h at 37 °C.

Interestingly, BBMV isolated from MC processed the Cry1Ab protoxin to a main band of higher molecular weight to that obtained using gut extracts, producing a similar pattern to that observed in the digestion by bovine trypsin (a main band of ~74 kDa). However, two main bands were observed in the activation produced by BBMV isolated from the MR strains, one similar to that produced by bovine trypsin and the other of higher molecular weight.

3.5. Enzymatic activities

To test differences in enzymatic activity between MC and MR, several synthetic substrates were incubated with the midgut content or midgut wall extracts from both strains (Table 3). Trypsin-like activity was tested using BApNa as substrate and it was found that MR had 29% lower activity than MC. Similar reductions in activity were obtained when testing chymotrypsin- and elastase-like activities, using SA_2PPpNa and SA_3pNa as substrates, respectively. In these cases the differences were even higher with MR showing 48% lower activity for chymotrypsin and up to 84% lower activity for elastase. On the other hand, no significant differences were found between MC and MR when the activity of α-amylase and esterases was tested using starch and 1-NA as substrates, respectively. Finally, LpNa was used to test leucine aminopeptidase activity and, in this case, MR showed 56% higher activity than MC.

Similar proteolytic patterns were obtained in the zymograms when midgut larval extracts of control MC and resistant MR strains of *M. unipuncta* were compared (Supplementary Fig. 1).

3.6. Binding assays

Biotin labelled Cry1Ab activated toxin bound specifically to BBMV prepared from MC and MR (Fig. 3). A single band, corresponding to the labelled activated toxin, was observed in the assays

Table 3
Enzymatic activity of midgut extracts from control MC and resistant MR strains of *Mythimna unipuncta*.

Enzymatic activity[a]	Substrate	Specific activity[b]	
		MC	MR
Trypsin	BApNa	106 ± 1	75 ± 5**
Chymotrypsin	SA_2PPpNa	1480 ± 40	770 ± 90**
Elastase	SA_3pNa	43 ± 5	7 ± 2**
α-amylase	Starch	1502 ± 93	1528 ± 94
Esterase	1-NA	354 ± 12	399 ± 9
Leucine aminopeptidase	LpNa	27 ± 7	42 ± 4*

** Significantly different from MC (Unpaired *t-test*, two tail, $P < 0.01$); *$P < 0.05$.
[a] Enzymatic activities were determined using midgut contents (trypsin, chymotrypsin, elastase and α-amylase) or midgut walls (esterase and leucine aminopeptidase) extracts.
[b] Specific activities are expressed as nmol substrate hydrolysed/min/mg total protein, except for that of α-amylase which is expressed as μg starch hydrolysed/min/mg total protein. Values are the mean ± standard error of at least triplicated experiments.

performed with BBMV from MC (Fig. 3A). The same band was observed using BBMV from MR but in this case, there was also another band of higher molecular weight (>75 kDa) than that of the labelled toxin (Fig. 3A). The assay performed incubating BBMV from both strains, with or without the labelled activated toxin, evidenced that the signal >75 kDa corresponds to a protein other than the labelled toxin since this band was clearly observed in the lane $BBMV_{MR}$ containing BBMV from MR without labelled Cry1Ab (Fig. 3B).

Homologous competition assays were performed, first, to test if the binding to BBMV was specific or not and second, to determine if there are strong qualitative/quantitative differences in the specificity of binding between MC and MR strains (Fig. 3C). The assays showed that the binding was specific in both cases since an excess of unlabelled Cry1Ab led to a decrease in the intensity of bands corresponding to the labelled activated toxin. In addition, the analysis of bands intensity showed that the reduction in the binding of labelled activated toxin due to an increase in the amount of unlabelled toxin was almost the same in both strains with only a slight difference in the intensity of bands at 100 × excess (Fig. 3C).

4. Discussion

We have obtained a resistant strain of *M. unipuncta* by laboratory selection. The control MC strain, established from egg masses collected in commercial fields of non-Bt maize, showed some basal tolerance to Bt maize. We were able to increase the percentage of larvae capable of survive and complete their development on Bt maize from 1.2% (MC) to about 50% (MR) in only eight generations of selection. Interestingly, when the susceptibility to the Cry1Ab toxin was examined in the MC strain, similar values of LC_{50} and MIC_{50} were found, and the same occurred with LC_{90} and MIC_{90} values. These results would suggest that sub-lethal doses of toxin have none or little effects on larval development of this species. Our data reveal that the MR strain has lower RCR and RGR values than the MC strain when both were fed on non-Bt maize. However, MR L_5 larvae exhibited similar metabolic expenditures when fed either on Bt or non-Bt maize than the MC strain fed on non-Bt maize, as reflected on ECI and ECD values. Moreover, the approximate digestibility of the MR resistant strain fed with Bt maize was significantly higher than when fed with non-Bt maize and similar to that of the MC strain fed on non-Bt maize, indicating that nutrient utilization was not impaired. These findings highlight the risk of resistance development in *M. unipuncta* for Bt maize sustainability, since L_5 larvae can migrate from other crops and grassy areas to maize during the growing season (Pilcher et al., 1997).

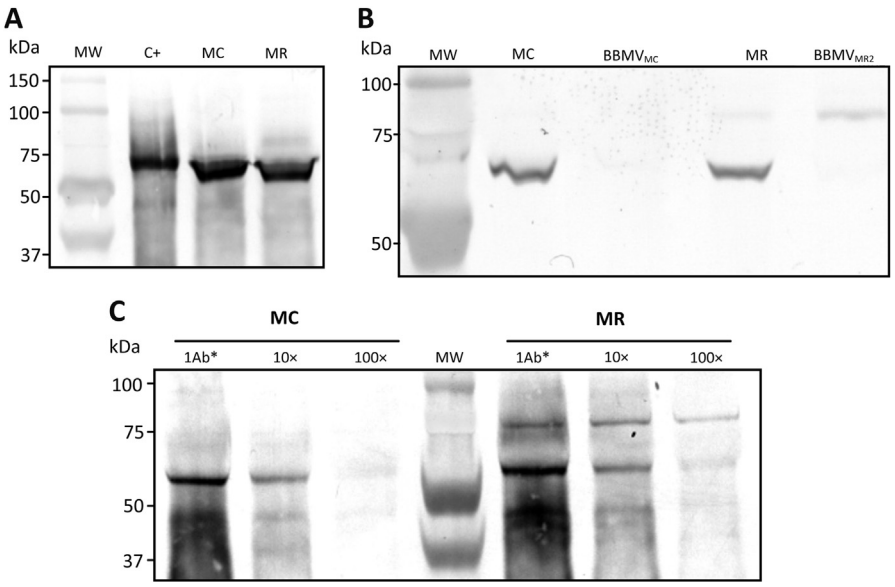

Fig. 3. Binding of biotin-labelled Cry activated toxins to BBMV from control (MC) and resistant (MR) strains of *M. unipuncta*. (**A**) **Lane MW**: Precision Plus Protein Standard Dual colour (BioRad Laboratories GmbH, Munich, Germany), **Lane C+**: biotin-labelled Cry1Ab (110 ng), **Lane MC**: biotin-labelled Cry1Ab (280 ng) with 15 μg total protein from BBMV from MC, **Lane MR**: biotin-labelled Cry1Ab (280 ng) with 15 μg total protein from BBMV from MR., (**B**) **Lane MW**: Precision Plus Protein Standard Dual colour, **Lane MC**: biotin-labelled Cry1Ab (100 ng) with 10 μg total protein from BBMV from MC, **Lane B-MC**: 10 μg total protein from BBMV from MC, **Lane MR**: biotin-labelled Cry1Ab (100 ng) with 10 μg total protein from BBMV from MR, **Lane B-MR**: 10 μg total protein from BBMV from MR. (**C**) **MC** [**Lane 1Ab***: biotin-labelled Cry1Ab (170 ng) with 10 μg total protein from BBMV from MC, **Lane 10** × : biotin-labelled Cry1Ab (170 ng)/non-labelled Cry1Ab (1.7 μg-**10** ×) with 10 μg total protein from BBMV from MC, **Lane 100** × : biotin-labelled Cry1Ab (170 ng)/non-labelled Cry1Ab (17 μg-**100** ×) with 10 μg total protein from BBMV from MC] **Lane MW**: Precision Plus Protein Standard Dual colour, **MR** [**Lane 1Ab***: biotin-labelled Cry1Ab (170 ng) with 10 μg total protein from BBMV from MR, **Lane 10** × : biotin-labelled Cry1Ab (170 ng)/non-labelled Cry1Ab (1.7 μg-**10** ×) with 10 μg total protein from BBMV from MR, **Lane 100** × : biotin-labelled Cry1Ab (170 ng)/non-labelled Cry1Ab (17 μg-**100** ×) with 10 μg total protein from BBMV from MR].

The activation of Cry1Ab from a non-toxic ~130 kDa protoxin to a biologically active or toxic fragment of ~65 kDa is a crucial step in the mechanism of action of Bt (Schnepf et al., 1998). It is well known that differences in the activation process may induce changes in the specificity of a given toxin (Haider et al., 1986) and more importantly, if this process is impaired, the insects may develop resistance (Li et al., 2004; Oppert et al., 1994; Sayyed et al., 2001; Rajagopal et al., 2009). In this work we evidenced a reduction in the activity of trypsin-, chymotrypsin- and elastase-like proteolytic enzymes in larvae of the resistant MR strain, commonly involved in Cry protoxin activation (Díaz-Mendoza et al., 2007), which correlates with impaired capacity of their midgut extracts to activate the Cry1Ab protoxin. Interestingly, the reduction on the activity of proteolytic enzymes observed in larvae of the MR strain is not accompanied by a reduction on the activity of other luminal enzymes involved in the digestion process, such as α-amylase, or on the activity of intracellular gut esterases that could participate in stress-response (Oakeshott et al., 2005). Moreover, resistance in larvae of the MR strain was reverted when they were fed on non-Bt maize treated with Cry1Ab toxin activated with midgut juice from the control MC strain. All these data indicate that resistance in the MR strain is mediated by alteration of toxin activation rather than to an increase in the proteolytic degradation of the protein.

It is noteworthy that the leucine aminopeptidase (LAP) activity was significantly higher in the MR insects. To the best of our knowledge, there is no report associating this activity to the activation of Cry proteins. Thus, no changes in LAP activity were observed in an *O. nubilalis* resistant strain showing a reduction of Bt protoxin activation (Li et al., 2004). Increased LAP activity has been reported as an adaptive response to toxin stress in a resistant strain from the Colorado potato beetle, *Leptinotarsa decemlineata*, and appears not to be associated with the activation of Cry proteins or with the role of aminopeptidases as receptors for the Cry toxins (Loseva et al., 2002). Moreover, it is possible that the increase in LAP activity can be due to an adaptation of the larvae to the reduction of other activities involved in proteolysis. Similar phenomenon was observed when larvae from *L. decemlineata* and beet armyworm (*Spodoptera exigua*) were fed with leaves from transgenic plants expressing protease inhibitors specific for endopeptidases (Lara et al., 2000; Ortego et al., 2001).

Our results showed that the reduction in proteolytic activity correlates with an alteration in the activation of Cry1Ab in resistant insects. The assays performed showed that the activation process was slower in the resistant individuals, with a significant amount of protein not fully activated after 2 h (two bands of ~74 kDa to ~67–69 kDa), while the same amount of protein was completely activated after 1 h (a unique band of ~67–69 kDa) with enzyme extracts from the control MC strain. These data are in agreement to that observed in resistant colonies of *O. nubilalis* and *H. armigera,* in which improper processing of the protoxin could also be associated with resistance (Li et al., 2004; Rajagopal et al., 2009). More importantly, our data demonstrated that neonate larvae of the MR strain were resistant to the Cry1Ab protoxin, but when the toxin was activated with midgut juice from the MC strain (band of ~67–69 kDa) resulted equally toxic to control MC and resistant MR larvae. In fact, resistance in larvae of the MR strain could not be reverted when treated with Cry1Ab toxin activated with bovine trypsin (a unique band of ~74 kDa), indicating that trypsin activated Cry1Ab need to be further activated in larval midguts. Taken together, our data indicate that resistance in the MR strain is due to the lack of processing to the active form of ~67–69 kDa. No differences in the proteolytic forms were visualised in zymograms of gut extracts from the MC and MR strains, suggesting that the differences in proteolytic activity were quantitative rather than qualitative.

As activation and binding progress dynamically, it is likely that after the ingestion of the truncated toxin from transgenic leaves, resistant insects remove most of this toxin from the midgut before it can be fully activated to exert its toxic action. This is supported by

the high rate of toxin elimination reported for this species (Pérez-Hedo et al., 2012), which may partially account for its low susceptibility to the MON810 expressed toxin, and contributes significantly to minimize the toxic effect on the resistant MR strain. Digestive proteases have also been involved in Bt resistance by further processing or degradation of the activated toxin (core of about 65 kDa) that may inactivate the toxin, and it was suggested as the mechanism of resistance in some cases (Forcada et al., 1996; Keller et al., 1996). As we did not observe such an over-processing or degradation pattern in our assays, we can say that this is certainly not contributing to the resistance in MR strain.

The implication of membrane associated proteases in the activation of Cry proteins has been assessed in several species and it seems that they can play an important role in those steps of the mechanism of action taking place after the toxin binding to the membrane, but before triggering the cascade of events leading to insect death. It has been demonstrated that Cry1 proteins undergo an "extra" activation step after the binding to the membrane in some lepidopteran species and that this activation enhances the formation of pores (Gómez et al., 2002; Bravo et al., 2007). Moreover, activation of other Cry proteins performed with BBMV from *L. decemlineata* (Loseva et al., 2002; Rausell et al., 2004) and *Culex quinquefasciatus* (Dai and Gill, 1993) showed digestion patterns different to that obtained with midgut contents, also indicating the presence of different proteases in the membrane or at least that these proteases are further processing the toxin after its interaction with the membrane. However, our data show no further processing of the toxin after incubation of the Cry1Ab protoxin with BBMV from control and resistant larvae. Indeed, a different pattern to that obtained with enzyme extracts was observed, suggesting that the set of proteases associated to the membrane is not able to fully activate the protoxin. Likewise, it has been reported in *Manduca sexta* that trypsin activated Cry1Aa does not need further activation from proteases associated to BBMV to form pores (Kirouac et al., 2006).

The alteration in binding is the most studied and well characterised mechanism of resistance to Bt toxins and has been described before in several insect resistant strains (Ballester et al., 1999; Van Rie et al., 1990). In our assays we found that trypsin activated Cry1Ab is able to bind specifically to membrane receptors from both, control and resistant larvae. Besides, based in our competition assays, it is also possible to say that binding affinity was similar for both strains. Thus, the alteration in the affinity of Cry proteins binding to midgut receptors appears to play a minor role in the resistance to Bt maize in the MR strain. Interestingly, when BBMV were incubated alone, a biotinylated protein of >75 kDa was detected in the MR strain, but not in the MC strain. It is known that biotin is commonly used as cofactor by several enzymes (Samols et al., 1988; Tu and Hagedorn, 1992), and that bands other than the labelled toxins have been observed before in similar blots from other species (Du and Nickerson, 1996; González-Cabrera et al., 2006). We hypothesized that the midgut membrane of resistant larvae have changed its composition to include at least one protein with biotin bound that it is recognised by streptavidin. It is possible that these proteins are not specifically involved in the mechanism of resistance but framed in a general context of changes in membrane composition to overcome the toxic effect of Cry proteins.

We have shown that *M. unipuncta* has the potential to develop high levels of resistance to Bt maize, mediated by alteration in Cry1Ab activation. Although there are no yet reports of *M. unipuncta* outbreaks in Bt maize fields, our results suggest that the susceptibility of this secondary pest to Bt maize is far from a "high dose" scenario, favouring the potential development of resistance in field populations. Accordingly, this secondary pest should be considered on ongoing resistance management program to limit the development of resistance in field populations and avoid the likely negative agronomic and environmental consequences.

Acknowledgements

We are thankful to Dr. Matilde Eizaguirre (UdL) for providing the initial insect colony. This work was supported by grants of the " Ministerio de Economia y Competitividad" (AGL2012-34289) and CSIC (CSIC-201040E049). J. G. C. was recipient of a post-doctoral grant from the CSIC (JAE doc).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.04.001.

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