Resistance management of Bt-maize in Europe

COST 862 Workshop
Organized by the consortium:
Protecting the Benefits of Bt-Toxins from Insect Resistance
Development by Monitoring and Management,
ProBenBt, EU-funded joint project

(Programme and Abstracts)

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“Protecting the Benefits of Bt-Toxins from Insect Resistance Development by Monitoring and Management” (ProBenBt, EU-funded joint project)
EU-COST 862-Action - Scientific Management Committee Meeting

International Workshop:
Resistance management of Bt-maize in Europe

Programme

Thursday, 06. April 2006: Arrival during day
20.00 Get together in the city

Friday, 07. April 2006
08.30-08.50 Opening: Ingolf Schuphan, Wolfgang Thomas (Dean), Neil Crickmore, Juan Ferré, Charles Kessler (EU, Brussels)
08.50-09.00 General information
09.00- 09.20 Genetically differentiated population of ECB/MCB and susceptibility monitoring: PEDRO CASTANERA (Workpackage I- leader)
09.20- 09.35 Population Genetics of ECB/MCB: CHRISTIANE SAEGLITZ
09.35- 09.50 Small scale migration: DENIS BOURGUET
09.50- 10.05 Significance of microsporidia infection of ECB: LUDOVIT CAGAN
10.05- 10.20 Susceptibility screen: BARBARA MANACHINI
10.20- 10.30 Summary of WPI: PEDRO CASTAÑERA
10.30- 10.45 Discussion
10.45- 11.20 Coffee break
Friday, 07. April 2006 (continued)

11.20- 11.40  F2-screen for rare recessive Bt-resistance alleles and laboratory selection of ECB/MCB: INGOLF SCHUPHAN (WPII-leader)

11.40- 11.55  F2-screen for ECB/MCB: TIM STODOLA

11.55- 12.10  Field sampling of surviving ECB/MCB in Bt-maize: GEMA PÉREZ FARINOS

12.10- 12.25  Laboratory selection of resistant ECB/MCB: STEFANOS ANDREADIS

12.25- 12.35  Summary of WPII: INGOLF SCHUPHAN

12.35- 13.00  Discussion

13.00- 13.30  Lunch

13.45- 15.10  Development of tools to detect resistance genes: DAVID HECKEL (WP III- leader);

15.10- 15.30  Discussion

15.30- 15.45  Mode of action of Bt-toxins in ECB/MCB: JUAN FERRÉ (WP IV- leader)

15.45- 16.00  Diversity of trypsins in the Mediterranean Corn Borer and their interaction with Cry1Ab toxin: FÉLIX ORTEGO

16.00- 16.15  Mode of action of B. thuringiensis toxins active against Sesamia nonagrioides: JOEL GONZÁLEZ-CABRERA

16.15- 16.30  Active Cry1 toxins of Bacillus thuringiensis interact with leucine cotransport in midgut BBMV from Ostrinia nubilalis and Sesamia nonagrioides: MARIA GIOVANNA LEONARDI

16.30- 16.45  Binding analyses of Cry1A toxins in resistant and susceptible Ostrinia nubilalis: JUAN FERRÉ

16.45- 17.00  Discussion

17.00- 17.30  Coffee break

17.30- 18.10  Bt-Maize management plan for Europe: DAVID ANDOW

18.10- 18.30  General Discussion

20.00  Dinner
**Saturday, 08. April 2006**

08.30-08.45 ALI H. SAYYED: Can efficacy of pesticides be conserved against resistant populations of *Plutella xylostella*?

08.45-09.10 WILLIAM MOAR: Bt resistance to transgenic crops does not necessarily confer resistance to Bt sprays

09.10-09.15 Discussion

09.15-09.40 BLAIR SIEGFRIED: Selection of Bt-resistance in ECB-populations

09.40-09.45 Discussion

09.45-10.10 RICHARD L. HELLMICH: Establishing genetic foundations for managing and monitoring European corn borer resistance to transgenic Bt maize

10.10-10.15 Discussion

10.15-10.40 DAVID ANDOW: Scientific issues in Bt-maize resistance management in the US

10.40-10.45 Discussion

10.45-11.15 **Coffee break**

11.15-11.40 DETLEF BARTSCH: Bt-maize: Resistance monitoring and management demands in the EU

11.40-11.45 Discussion

11.45-12.00 Summarizing discussion

12.00-13.30 COST 862 Scientific Management Committee Meeting

13.30 **Lunch**
ABSTRACTS

(These abstracts should not be considered to be publications and should not be cited in print without the author’s permission)
For a successful resistance management it is essential to know about the extent and significance of gene flow in the pest population. To meet this demand, different techniques (AFLP, RAPD, allozyme analysis) have been established by the project partners to investigate the population genetics of European ECB and MCB populations.

Genetic differentiation was analysed and gene flow was estimated on different spatial scales: Within countries (Germany, ECB; Spain, MCB) and within Europe (Germany, Italy, France, Slovakia, Spain, Austria, Bulgaria, Romania, and Greece).

Between local populations only small genetic differences could be found for ECB and MCB. This is confirmed even on a larger European scale, by only slight geographic differences. According to these results, gene flow will in all likelihood be high enough to protect the benefits of Bt-toxins in maize by applying the high-dose/refuge resistance management strategy.
Small scale dispersal of the European corn borer

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Over the past decade, the high-dose refuge (HDR) strategy, aimed at delaying the evolution of pest resistance to insecticidal Bacillus thuringiensis (Bt) toxins produced by transgenic crops, became mandatory in the USA and is being discussed for Europe. Dispersal of the pest targeted by these toxins may have a significant influence on the speed at which Bt resistance might evolve in natural populations of these pests. Small scale dispersal of one of the main target of the Bt maize, the European corn borer (ECB, Ostrinia nubilalis Hübner, Lepidoptera: Crambidae) were estimated over almost 50 mark-release-recapture sessions performed in Germany, in France and in the Slovak Republic. Moths were released either in herbaceous field borders where ECB moths rest during the day and mate at night or directly in maize fields. The spatial distribution of recaptured moths around the release points suggests that moths probably performed two types of movement. Some of them moved but on a very local scale, while others probably left the area by a different, long-range type of dispersal. However, in these experiments, like in those previously performed for estimating ECB dispersal, the precopulatory dispersal and the mating between resident and immigrant individuals, two features influencing the efficiency of the HDR strategy, have not been quantified. Hence, we used a combination of mark-recapture experiments and biogeochemical marking over three breeding seasons to quantify these features directly in French populations of the ECB. Our results show that, at local scale, resident females mated randomly, i.e., regardless of males having experienced a dispersal event beforehand or not, as assumed in the HDR strategy. However, our results also indicate that a fraction of moths – which although variable averaged 18% for females – mated before engaging in any long-range dispersal, with no evidence for any sex-related difference. Hence, ECB moths probably mate at a more restricted spatial scale than previously assumed in the HDR strategy, a feature that may decrease its efficiency in certain circumstances such as in crop rotated landscape.
Bt-toxin susceptibility of the *Ostrinia nubilalis* Hbn. (Lepidoptera: Crambidae) larvae under influence of microsporidia infection

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Level of microsporidian infection (*Nosema pyrausta*) in the European corn borer, *Ostrinia nubilalis* Hbn. (ECB), populations was studied at several localities of Slovakia and the Czech Republic. It was found that the level of infection changed only slightly during different years at the same locality what indicate that ECB adults do not move long distances. In field conditions there was not relation between level of maize infestation caused by the ECB and level of infection of the ECB larvae by microsporidia. In laboratory conditions it was found that healthy population developed from larvae originating from infected locality did not show significantly different resistance or susceptibility to Cry IAb toxin from *Bacillus thuringiensis* (Bt) when compared to larvae originating from non infected locality. Susceptibility to toxin was determined for neonates from laboratory populations infected and non infected by *N. pyrausta* spores. LC50 (ng/cm²) values of Cry protein for laboratory populations infected and no infected by *N. pyrausta* spores evaluated with the overlay method was 90.49 and 147.77 respectively (population from Nitra locality was used; reference laboratory strain from Aachen showed the value of 180.47). It means that larvae of the ECB infected by *N. pyrausta* were more sensitive to Bt toxin. Strong effect of *N. pyrausta* to larval development and mortality was determined in laboratory conditions. The development of infected ECB larvae was slower and the weight of the larvae or pupae was lower when compared to non infected population.
Baseline susceptibility to Cry1Ab toxin of *Ostrinia nubilalis* Hb. (Lepidoptera: Crambidae) and *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae) from different European countries

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Susceptibility to Cry1Ab toxin from *Bacillus thuringiensis* (Bt) was determined for European corn borer, *Ostrinia nubilalis* (ECB), neonates from both laboratory and field populations collected from 3 different localities of Spain, France, Italy, Germany, Slovakia, and 1 locality in Greece. The baseline of susceptibility of Mediterranean corn borer *Sesamia nonagrioides* (MCB) larvae was evaluated only for the populations of Spain and Greece. ECB and MCB larvae were exposed to artificial diet treated with increasing Cry1Ab concentrations using both, toxin incorporation and overlay techniques. Mortalities were evaluated after 7 d. The LC50 (ng/cm²) values of Cry protein for different populations of ECB evaluated with the overlay method ranged from 5.7 to 9.5 for the ECB from Spain, from 122.9 to 170.6 to the ones from Italy, from 103.95 to 127.17 for Germany, from 147.77 to 473.06 for Slovakia, 198.7 for Romania, 147.7 for Austria, 346.6 for Serbia Montenegro and 29.32 for Greece. Although variation in susceptibility observed was rather high the LC ratio with respect of the reference strain (lab strain from Germany) was always close to 1 except for the Spanish population where this value ranged from 6 to 10. The LC50 (µg per ml of diet) investigated with the incorporated method ranged from 8.5 for France, 13.87 for Germany and 20.64 for Greece, up to 43.50 for Italy. Also in this case the comparison with the reference strain did not enhance any particular differences in the susceptibility. The LC50 for MCB in Greece does not show particular differences in the susceptibility ranging from 26.99 to 28.02 ng/cm². Similar results were found also for MCB in Spain (16.1-22.6 ng/cm²). In both countries, the LC ratio with respect to the LC50 of the MCB reference strain (lab strain from Spain) was always close to 1. The range of variation in Cry1Ab susceptibility indicated by mortality was rather similar except for ECB in Spain where the LC was lower than that one calculated for the reference strain. However these results suggest that the observed susceptibility differences reflect natural variation in Bt.
susceptibility among both Lepidoptera populations and provide a baseline for estimating potential shifts in susceptibility that might result from selection and exposure to Cry1Ab-expressing corn hybrids.
The F₂ Screen of ECB/ MCB


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The F₂ screen is a powerful method for monitoring and detecting rare, recessive resistance alleles in insect populations. An F₂ screen was conducted on populations of European corn borer, Ostrinia nubilalis, and Mediterranean corn borer, Sesamia nonagrioides. Populations of O. nubilalis was collected from Germany, Slovakia and Italy, and S. nonagrioides was collected from Spain and Greece. About 1345 lines of O. nubilalis and 160 lines of S. nonagrioides were screened. No alleles for resistance to Bt maize were detected. This implies that recessive resistance alleles are rarer in these European populations.
Field sampling of surviving ECB/MCB in Bt-maize


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In order to bring Bt-maize to the European Market, companies are required to conduct post-market monitoring programmes to verify Bt-maize efficacy. The main objectives of the monitoring programmes designed are: a) to establish the baseline susceptibility to the insecticidal protein in insects derived from non-transgenic fields; b) to detect changes over time in susceptibility by annual monitoring of both species on Bt maize fields. As such, a monitoring research project has been implemented on Spain for the last seven years. However, the expected increasing cultivation of transgenic insecticidal crops calls for accompanying monitoring methods to assess field resistance. Three main strategies have been explored within the scope of the ProBenBt project: a) comparing populations with a history of relatively high exposure to the toxin with conspecific populations that have had less exposure (Spain); b) light-trap cage monitoring that combines systematic mass capturing of adult insect pests and autocidal selection (Germany); and c) screening of surviving larvae to establish resistant lab populations (Slovakia and Czech Republic). None of the field collected larvae collected by the German and Spanish teams were able to survive on Bt-maize plants expressing the toxin. A laboratory strain has been initiated by the Slovakian team with 23 surviving larvae collected from 12000 plants of Bt-maize (MON-810) in Kromeriz (Czech Republic) during 2005, but they need to be tested for resistance. The implication of the results for prospective resistant monitoring, as well as the need to obtain resistant larvae for further resistance and genetic studies, are discussed.
Laboratory selection of resistant *Ostrinia nubilalis* (Lepidoptera: Crambidae) and *Sesamia nonagrioides* (Lepidoptera: Noctuidae) to Cry1Ab-toxin from different European countries

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A long term experiment was conducted to examine the potential of European corn borer, *Ostrinia nubilalis* (ECB) and Mediterranean corn borer, *Sesamia nonagrioides* (MCB) for resistant development to Cry1Ab toxin in the laboratory. Field collected larvae of ECB from Italy, Germany, Spain, Slovakia and Greece, as well as MCB larvae from Spain and Greece were used to establish laboratory cultures that were split into two strains, one selected and one unselected. A high and a low dose strategy were employed for the laboratory selection. Laboratory selection was assessed as a change in susceptibility to Cry1Ab toxin between selected and unselected strains, using either the surface treatment or the incorporation method. Regarding the low dose strategy for ECB there was a significant reduction in the susceptibility after 23 generations of selection. Differences in susceptibility between selected and unselected strains were lower when the surface treatment was used (9-fold) than with the incorporation method (16-fold). High dose strategy for ECB resulted in a rapid change in susceptibility between selected and unselected strains, observing significant difference within 4 generations. Results showed an up to 4- and 10-fold change in susceptibility after 4 and 8 generations of selection, respectively. High dose strategy for MCB led to varying results probably due to the different sources of toxin. After 8 generations of selection an up to 21-fold change in susceptibility was obtained with the Spanish strain, while no significant reduction in susceptibility was observed after 6 generations of selection regarding the Greek strain.
Development of genetic tools for detecting Bt resistance
genes in *Ostrinia nubilalis* Hb. (Lepidoptera: Crambidae)
and *Sesamia nonagrioides* LeFebvre (Lepidoptera:
Noctuidae)

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When Bt-resistance eventually appears in a given species, genetic analysis will provide crucial evidence on the identity of the resistance gene(s) involved and furnish the information required for DNA-diagnostic screening of field populations. However, this analysis is time-consuming and difficult. If it is not started until after Bt-resistance is detected in field populations, the potential for adaptive resistance management is restricted. Therefore, in order to anticipate the development of Bt-resistance in *Ostrinia nubilalis* (European Corn Borer, ECB), a genetic linkage map was constructed and used to 1) localize Bt-resistance genes that have already been identified in other species, 2) position the most likely candidate resistance genes based on knowledge of the mode of action, and 3) to identify linkage groups homologous to those identified in other species that carry mapped, but unknown, resistance genes.

The map was based on a cross between two strains from the area of Bonn, Germany; one collected from maize and the other from mugwort. The framework map consisted of more than 200 AFLP markers, covering all 31 linkage groups with a total genetic length of 1872 cM. Ribosomal protein genes were mapped to provide anchor loci for correspondence to other Lepidopteran genomes. Preliminary comparisons with *Helicoverpa armigera*, *Plutella xylostella*, and *Heliconius melpomene* indicate a high degree of synteny. Genes for two allozyme markers, TPI and MPI, previously shown to exhibit genetic differentiation among populations of ECB, were cloned and mapped. MPI is linked to the gene for the 12-domain midgut cadherin protein involved in Bt resistance in *Heliothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera*. The intron/exon structure of the ECB cadherin gene was determined, and found to be similar to other Lepidopteran species. A great deal of genetic variation was found in certain introns, which will be useful in characterizing field populations. The 12-domain midgut cadherin mRNA was also cloned from *Sesamia nonagrioides*, and found to resemble the sequence of the heliothines more than its closer relative, *Spodoptera frugiperda*. A number of aminopeptidases, known to bind Bt-toxin in other
Lepidopteran species, were cloned and mapped. Linkage groups homologous to Linkage Group 10 of *Heliothis virescens* and Linkage Group 22 of *Plutella xylostella* were also identified. These are of potential importance because highly potent Bt resistance genes have been mapped to those linkage groups. In both cases, although the molecular nature of the resistance gene has not yet been determined, they are known to be different from the "Mode 1" type of Bt resistance mediated by mutations in the 12-domain midgut cadherin gene. The linkage map is now available as a valuable resource for rapid analysis and identification of ECB Bt-resistance genes, if and when they do arise in the field.
Diversity of trypsins in the Mediterranean corn borer and their interaction with Cry1Ab toxin

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Four trypsins have been purified from midgut lumen of the Mediterranean corn borer (MCB), Sesamia nonagrioides, larvae. In parallel, a diversity of trypsin-like genes expressed in the MCB midgut has been identified. A phylogenetic analysis of MCB cDNA sequences revealed the existence of the three types of trypsin-like enzymes conserved in other lepidopteran species, including four type-I (two subtypes), four type-II (two subtypes) and one type-III. N-terminal sequencing and mass spectrometric analyses of purified trypsins have been performed in order to identify cDNAs coding for major trypsins among the diversity of trypsin-like sequences obtained. Thus, it is revealed that the four purified trypsins in MCB belong to the three well-defined phylogenetic groups of trypsin-like sequences detected: trypsin-I (type-I), trypsin-IIA and trypsin- IIB (type-II), and trypsin-III (type-III). Trypsin-I, trypsin-IIA and trypsin-III showed preference for Arg over Lys, but responded differently to proteinaceous or synthetic inhibitors. Changes in the susceptibility of the trypsin-like activity of midgut extracts from different larval instars to these inhibitors suggest that the relative proportion of these two enzymes varied through MCB larval development, being trypsin-I predominant in early instars and trypsin-IIA in late instars. To determine their role in the processing and/or inactivation of Cry toxins, purified trypsins were incubated with Cry1Ab. The digestion with trypsin-I or trypsin–III resulted in a similar processing pattern, generating a toxic fragment of 69/67 KDa. A further cleavage was obtained with trypsin-IIA, resulting in a fragment of 46/43 KDa that, however, retains its insecticidal activity. The implication of these results on the identification of protease-mediated resistance to Bt-maize is discussed.
Mode of action of *Bacillus thuringiensis* toxins active against *Sesamia nonagrioides* (Lefebvre)

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*Sesamia nonagrioides* is one of the most damaging pests of corn in Spain and other Mediterranean countries. Bt-corn expressing the *Bacillus thuringiensis* Cry1Ab toxin that was designed to control *Ostrinia nubilalis* attack has shown good control of *S. nonagrioides* populations. Here we have tested the effect of several Bt toxins against neonate larvae of *S. nonagrioides*. Furthermore, we have studied the mode of action of those Bt toxins that were found active: Cry1Ab, Cry1Ac, Cry1Ca, and Cry1Fa. Binding assays were performed with ¹²⁵I- or biotin-labeled toxins and larval brush border membrane vesicles (BBMV). Competition experiments indicated that these toxins bound specifically to the BBMV and that Cry1Aa, Cry1Ab, and Cry1Ac shared their binding site. Cry1Ca and Cry1Fa bind to different sites. In addition, Cry1Fa binds to the Cry1A’s binding site with very low affinity and vice versa (Fig. 1). Binding of Cry1Ab and Cry1Ac was found stable over time, which indicates that the observed binding is irreversible. Pore forming activity of Cry proteins on BBMV was determined using the voltage sensitive fluorescent dye DiSC3(5). Membrane permeability increased in the presence of the active toxins Cry1Ab and Cry1Fa, but not with the non-active toxin Cry1Da. In terms of resistance management, based on our results and the fact that Cry1Ca is non-toxic to *O. nubilalis*, we recommend pyramiding of Cry1Ab with Cry1Fa in the same Bt-corn plant for better long-term control of corn borers.

![Figure 1. “Receptors” model of Bt toxins binding to BBMV from *S. nonagrioides*.](image-url)
Active Cry1 toxins of *Bacillus thuringiensis* interact with leucine cotransport in midgut BBMV from *Ostrinia nubilalis* and *Sesamia nonagrioides*.

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*Bacillus thuringiensis* (Bt) toxin mode of action is a multistage process mainly studied in Lepidoptera. It involves the solubilization of the protoxins in the alkaline midgut lumen, their activation by midgut proteases and their binding to specific receptor(s) located on the apical membrane of midgut columnar cells. The final insertion of Cry1 toxins into the membrane to form a pore will lead the midgut cell to death. A complete elucidation of this complex process in each pest species is important for resistance management strategies.

The pore forming activity of Cry1Ab, Cry1Fa and Cry1Da toxins and their interaction with leucine transport mediated by the K⁺/neutral amino acid cotransporter were studied in brush border membrane vesicles (BBMV) isolated from the midgut of *Ostrinia nubilalis* and *Sesamia nonagrioides*, two harmful insect pests of maize that can be controlled by Bt toxins.

The brush border membrane fragments obtained from the midgut of both species by Ca⁺⁺-precipitation were adequately closed to form osmotically active vesicles, which retained the ability to transport leucine.

The pore formation activity of Cry1Ab was tested in *O. nubilalis*-BBMV by recording the fluorescence variation of the voltage-sensitive dye DisC₃(5) in the presence of increasing transmembrane potentials induced by inwardly directed K⁺ gradients. The consecutive addition of KCl to the extravesicular buffer, to final concentrations varying from 20 to 40, 60 and 80 mM, generated inside-positive potentials of different magnitude that were recorded in control vesicles as a progressive increase of the fluorescence signal. The formation of new pores permeable to K⁺ would generate higher diffusion potentials and consequently larger variations of fluorescence. Indeed the presence of Cry1Ab caused a significant increase of the signal, showing that this toxin, active in vivo, enhanced the membrane permeability to potassium. Similar variations of the signal were also observed in *S. nonagrioides*-BBMV incubated with the two toxins active in vivo Cry1Ab and Cry1Fa, whereas no variations were observed with the inactive toxin Cry1Da.

The lepidopteran larval midgut is characterized by a high lumen-positive transepithelial electrical potential, a strong luminal alkaline pH and an elevated K⁺ extrusion due to the coordinated activity of a vacuolar-type proton ATPase and a K⁺/2H⁺ antiporter, expressed on the membrane lining the cavity of a specialized cell, the goblet cell. Most neutral amino acids are accumulated in the insect haemolymph by K⁺-dependent cotransporters, expressed in the brush border membrane, exploiting the transmembrane K⁺-electrochemical gradient. The cotransporters can translocate the amino acids also in the absence of K⁺.

The toxins Cry1Ab in *O. nubilalis*-BBMV, and Cry1Ab and Cry1Fa in *S. nonagrioides*-BBMV, reduced in a dose-dependent manner leucine uptake, regardless of
the presence of $K^+$, while no effect was seen with the inactive toxin Cry1Da. Therefore, the inhibition of amino acid transport was strictly connected to the toxic effect in vivo and was not related to the channel formed by the toxins. Even if the molecular basis of the inhibitory effect is not yet clear, our results indicate that the active toxins affect directly the amino acid transport protein. In the feeding larva the inhibition will impair the intestinal absorption of several essential amino acids critical for larval growth and development.
Binding analyses of Cry1A toxins in resistant and susceptible Ostrinia nubilalis

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Cry1Ab toxin binding analysis was performed to determine whether resistance in laboratory-selected Ostrinia nubilalis strains is associated with target site alteration. Brush border membrane vesicles (BBMV) were prepared using dissected midguts from late instars of susceptible and resistant strains (Europe-R and RSTT) of O. nubilalis. Immunoblot analysis indicated that three different proteins bound to Cry1Ab toxin and were recognized by with an anti-cadherin serum. In comparison of resistant and susceptible strains, reduced Cry1Ab binding was apparent for all three bands corresponding to cadherin-like proteins in the Europe-R strain, while reduced binding was apparent in only one band for the RSTT strain. Real-time analysis of Cry1Ab binding to gut receptors using surface plasmon resonance (SPR) suggested slight differences in affinity in both resistant strains. Additional binding analysis was conducted using ¹²⁵I-labeled Cry1Ab, Cry1Ac and Cry1Aa. Slight differences were again observed between the resistant and susceptible strains for Cry1Ab binding. However, when binding of ¹²⁵I-labeled Cry1Aa was tested, a 10-fold reduction in the concentration of binding sites was observed in the Europe-R strain. On the other hand, transcript abundance for the O. nubilalis cadherin gene was similar in both the resistant and susceptible strains. Previous work comparing midgut protease activity between resistant and susceptible insects revealed no consistent differences (Siqueira et al., 2004, Pest Manag. Sci. 60: 1189-1196). In combination, the results of the present work suggest that differences in susceptibility to Cry1A toxins in the Europe-R strain of O. nubilalis are associated with altered receptor binding although the precise nature of this mechanism is still unclear.

Our results differ from those found in a different laboratory-selected O. nubilalis strain for which no differences were found in binding to BBMV proteins either by SPR, ligand blots, or using ¹²⁵I-labeled Cry1A proteins (Li et al., 2004, Biochem. Biophys. Res. Commun. 323: 52-57). Further analyses showed that resistance in this strain was high against the Cry1Ab protoxin (254-fold) but only low to the activated Cry1Ab toxin (12-fold), and that a trypsin-like activity was significantly reduced in the resistant insects (Li et al., 2005, Insect Biochem. Mol. Biol. 35: 847-860).
Insect resistance management (IRM) is necessary in Europe to prolong the expected lifetime of Bt maize, including those based on Cry1Ab, Cry1Fa, Cry3Bb and others. In Europe, the target pest species at greatest risk of resistance evolution are Mediterranean corn borer, *Sesamia nonagrioides*, in parts of Spain and Greece, and European corn borer, *Ostrinia nubilalis*, in parts of western Europe, and throughout central and eastern Europe. IRM in Europe will probably rely on a refuge strategy. Compared to the USA, there will likely be several alternatives toward implementing this strategy, depending on the local conditions. Additional research will be needed to demonstrate the adequacy of these local adaptations. For example, (1) other crops may function as refuges (e.g., possibly sorghum), (2) in parts of Andalucia, Spain and Central Greece IRM may need to be modified for Bt cotton, (3) coexistence measures may be sufficient to meet refuge needs for small-scale production, and (4) community or landscape refuge standards may be possible to implement and enforce.
Can efficacy of pesticides be conserved against resistant populations of *Plutella xylostella*

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*Bacillus thuringiensis* (*Bt*) crystal (Cry) toxins are expressed in various transgenic crops and are also used as sprays in integrated pest management and organic agricultural systems. The diamondback moth, *Plutella xylostella*, is one of the most destructive insect pests of crucifers worldwide. It has shown significant resistance to almost every insecticide applied in the field including biopesticides. In certain parts of the world, economical production of crucifers has become almost impossible because of its resistance to insecticides and resulting control failure. Recently we have found cross-resistance between *Bt* toxin Cry1Ac and pyrethroids. Our results showed significant reciprocal resistance between deltamethrin and Cry1Ac and degrees of cross-resistance with the other compounds. In each case the resistance phenotype appears to multifactorial. If such a broad spectrum cross resistance mechanism became established in field populations of insect pests it might prove difficult to combat with existing resistance management strategies in agricultural systems where insecticides are used.
Bt resistance to transgenic crops does not necessarily confer resistance to Bt sprays

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Because both Bt transgenic plants and Bt formulated products contain crystal (Cry) proteins, and because there has been increased concern regarding resistance development with the introduction of Bt transgenic plants, concerns have been raised that the utility of using Bt formulated products is now more at risk due to resistance concerns. This presentation will try to present various reasons as to why this concern may be premature. Several points to consider: (i) Resistance to Bt transgenic plants has yet to occur in any part of the world after up to 10 years of extensive use. The only cases of documented Bt resistance in the field occurred with Bt formulations, but only under intense selection pressure either in the subtropics-tropics, or in enclosed areas such as grain silos or glasshouses. (ii) Bt plants such as maize and cotton, are targeting insect pests not usually treated with Bt formulated products. (iii) The Cry proteins expressed in transgenic plants are not in the same form as those found in Bt formulated products. (vi) All Bt Cry proteins are not the same, and some Bt Cry proteins can overcome resistance to other Bt Cry proteins such as in the case of controlling diamondback moth, Plutella xylostella in Brassica crops. (v) Essentially all Bt formulated materials may contain various other insecticidal components associated with the Bt strain such as spore, zwittermycin, VIP proteins, chitinases, phospholipases, etc. Some of these compounds have even been shown to synergize the insecticidal activity of some Cry proteins. An example can be made using the beet armyworm, Spodoptera exigua, that was selected for resistance to a single Cry toxin, similar to what would be found in a Bt transgenic plant. This highly resistant population (>100 fold) was susceptible to the Bt Cry protein + spore, and was only 5-fold more tolerant to a Bt formulated product (compared to a susceptible population) that contained the protein that S. exigua was selected against. However, it is important to keep in mind that every insect species may react differently to these various Bt proteins and compounds, and therefore, resistance concerns should be treated on a species by species basis.
Laboratory Selection for Resistance to Bt Toxins in the European Corn Borer: Potential and Limitations

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Transgenic maize hybrids expressing toxins from Bacillus thuringiensis have been available for managing European corn borer (Ostrinia nubilalis Hübner) populations since 1996. However, there is concern that this technology will result in widespread resistance to Bt toxins. The “high dose-refuge strategy” is the currently accepted method for managing resistance in O. nubilalis and has become a requirement of registration for all transgenic events. However, this strategy is based on a number of assumptions that are difficult to validate until resistance is identified and characterized. Resistant strains resulting from laboratory selections provide a potential tool to assess these assumptions in the absence of field derived resistant populations. However, such laboratory experiments are limited by their inability to reflect the selection intensity imposed on a field population exposed to transgenic plants. Our laboratory has been selecting O. nubilalis by exposure to Bt toxins incorporated into an artificial diet since 1998. A number of different colonies that vary with regard to the nature of the resistance, degree of dominance, number of genes and ability to survive on transgenic plants have been developed. Laboratory selection with Cry1Ab produced >1000-fold resistance in two laboratory strains of European corn borer. The inheritance of resistance was autosomal and approximately additive in both strains and involved multiple genes. Laboratory selection with Cry1F resulted in greater than 3000-fold resistance levels, and the dose-response of reciprocal parental crosses indicated that the resistance was autosomal and recessive. In contrast to the Cry1Ab selected strains, backcross of the F1 generation with the selected strain revealed that a single locus or a set of tightly linked loci is responsible for the resistance. Biochemical and molecular characterization of the resistance mechanisms for both Cry1Ab and Cry1F selected strains are currently in progress. Additionally, the ability of these selected colonies to survive on expressing plants is being evaluated. The relevance of these strains in terms of their ability to improve and refine resistance management recommendations will be discussed.
Establishing genetic foundations for managing and monitoring European corn borer resistance to transgenic Bt maize


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The European corn borer (ECB) and corn rootworm are the two most serious corn pests in the U.S. Corn Belt. The long-term goal of our research is to develop sustainable ways to manage these insect pests. We take an integrated approach toward developing management strategies and tools for these pests with emphases on insect resistance management, insect ecology and genetics, and biological control. This talk will focus on one aspect, managing European corn borer resistance to *Bacillus thuringiensis* (Bt) maize. Toward this end, ECB colonies resistant to Bt are being used to better understand resistance mechanisms and to improve monitoring methods. Amplified fragment length polymorphisms (AFLP) are being used to establish an ECB linkage map for genetic mapping of Bt resistance traits. Plus, single nucleotide polymorphisms (SNP) have been detected within genes that are potentially related to insect Bt resistance, including cadherins, aminopeptidases, chymotrypsins and brainiac. These SNPs also are available for use as genetic markers. Population genetics studies using microsatellite markers have been initiated in the central Corn Belt with the goal to characterize gene flow and determine the most appropriate spatial scale for resistance monitoring. Our lab has established collaborations with many scientists in the U.S. and Canada and recently, with the development of a *Diabrotica* genetics consortium, has extended these collaborations to European scientists.
Scientific issues in Bt-maize resistance management in the US

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Bt maize has been grown in the USA for nearly 10 years and insect resistance management (IRM) has been implemented by seed companies, according to requirements of the US Environmental Protection Agency. However, several scientific issues remain unresolved: (1) Methods for monitoring resistance evolution remain unresolved. (2) Acceptable pest management of the refuge remains a scientific controversy. (3) Methods for assuring compliance to the refuge requirements need to be verified. This presentation will describe the scientific dimensions of these controversies and review the scientific evidence bearing on them.
Bt-maize: Resistance monitoring and management demands in the EU

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A plan for Post Market Environmental Monitoring (PMEM) of genetically modified (GM) plants is mandatory in all applications for deliberate release submitted under EU Directive 2001/18/EC and EU Regulation 1829/2003. PMEM aims at identifying possible unanticipated adverse effects on human health or the environment which could arise directly or indirectly from GM plants. PMEM is composed of case-specific monitoring and general surveillance of GM plants. The European Food Safety Authority (EFSA) is responsible for assessing the scientific quality of PMEM plans submitted with each application of GM crops in the EU. The EFSA GMO Panel considers that the evolution of resistance in target pests is an environmental and agronomic concern. The EFSA GMO Panel concludes that large scale cultivation of Bt maize (events Bt11 expressing CRY1Ab and event 1507 expressing CRY1F) over several years will increase the selection pressure on corn borers, which might result in the development of resistance (EFSA 2005a,b). Therefore, the EFSA GMO Panel advises that potential target pest resistance development should be monitored under so called case-specific monitoring. EFSA accepted a PMEM plan based on insect resistance management with the following key elements:

- a comprehensive grower education that will aid the grower employing the required resistance management tool of implementing a 20 % refuge for Bt maize planting areas larger than 5 hectares;
- a monitoring of the baseline susceptibility of European corn borer (*Ostrinia nubilalis*) and Mediterranean corn stalk borer (*Sesamia nonagrioides*) to CRY1F or CRY1Ab in representative EU maize cultivation areas based on the measures and monitoring techniques as described by Farinos et al. (2004);
- a plan for confirmation of pest resistance and remedial action.

However, the final adoption of Bt11 and 1507 maize varieties for cultivation in Europe is still pending, as the decision will be taken in a comitology procedure between Member States and the Commission.


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