Isaac Ishaaya · Subba Reddy Palli
A. Rami Horowitz *Editors*

Advanced Technologies for Managing Insect Pests

Springer

Editors
Isaac Ishaaya
Department of Entomology
The Volcani Center
Agricultural Research Organization
Bet Dagan, Israel

A. Rami Horowitz
Department of Entomology
Gilat Research Center
Agricultural Research Organization
Gilat, Israel

Subba Reddy Palli
Department of Entomology
College of Agriculture
University of Kentucky
Lexington, KY, USA

ISBN 978-94-007-4496-7 ISBN 978-94-007-4497-4 (eBook)
DOI 10.1007/978-94-007-4497-4
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2012943161

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Chapter 10
Comparative Aspects of Cry Toxin Usage in Insect Control

András Székács and Béla Darvas

1 Introduction

Bacillus thuringiensis Berliner microorganisms are aerobe, Gram positive, endospore-forming insect pathogenic bacteria, discovered by Ishiwata in 1901 from silkworm (*Bombyx mori* L.) and described by Berliner in 1915 (Hilbeck and Schmidt 2006; Roh et al. 2007). *B. thuringiensis* strains are ubiquitously present in our environment, as soil-borne bacteria and as insect larval pathogens. The identification of the protein composition in the parasporal bodies of numerous *B. thuringiensis* strains and the discovery of the unique physico-chemical features and biological specificity of the protein toxins (termed Cry toxins) has led to several landmark events in pest control practices.

Initially *B. thuringiensis* products (spores and δ-endotoxins) were applied (Table 10.1), but later, with the discovery of several *Bt* strains and their genetic investigations, two or more strains of *Bt* subspecies were mixed together in a way that facilitates the formation of combination of *cry* genes settled on plasmids. *Bt* strains EG2348, EG2349 and EG2371 (Ecogen, Inc.) were created through a process called transconjugation, a phenomenon known to occur in nature and considered analogous to hybridization in higher organisms. In the next step two or more subspecies (serological or pathological variants) of *Bt* were mixed together. Thus, *Bt* strains EG7826, EG7841 (Ecogen, Inc.) and GC-91 (AGC Ltd.) have also been produced through this process. These novel strains may be considered modified *Bt* strains, although the term hybrid *Bt* strain (gene exchange within a species) would be more accurate.

A. Székács (✉) • B. Darvas
Department of Ecotoxicology and Environmental Analysis, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary
e-mail: a.szekacs@cfri.hu; drdarvas.bela@chello.hu

I. Ishaaya et al. (eds.), *Advanced Technologies for Managing Insect Pests*,
DOI 10.1007/978-94-007-4497-4_10,

Table 10.1 Application forms of *Bacillus thuringiensis*

Subspecies	Strain	Cry toxin types	Selected trade names/codes	Type
aizawai	SA-2	Cry1	XenTari	NAT
aizawai	MYX833	Cry1C	M/C	CC
israelensis	SA-3	Cry4, Cry1	Acrobe, Aquabac, Bacticide, Bactilarvae, Bactoculis, Bakthane, Gnatrol, Larvatrol, Prehatch, Sentry, Teknar, VectoBac, Vectobar, Vectocid	NAT
japonensis	buibui	Cry8Ga1	M-Press	NAT
kurstaki		Cry1, Cry2	Agrobac, Bactec, Bactuside, Baritone, Baturad, Biobit, Biolep, BioPas, Collapse, Cordalene, Costar, Delfin, Dipel, Foray, Forwarbit, Halt, Insectobiol, Javelin, Lipel	NAT
kurstaki	EG2348	Cry1Aa, Cry1Ac, Cry2A	Condor, Ecotech Pro, Rapax, Wormox	NAT
kurstaki	EG2349	Cry1	Bollgard[a]	NAT
kurstaki	EG2371	Cry1	Cutlass[a], Ecotech Bio	NAT
kurstaki/*P. fluorescens*	MYX7275	Cry1A	MVP	CC
kurstaki/*P. fluorescens*	MYX104	Cry1Ac	Guardjet, M-Peril, MVP II,	CC
kurstaki x *aizawai*	GC-91	Cry1Ac, Cry1C	Agree, Design, Turex	HYB
kurstaki x *aizawai*	EG7826	Cry1Ac, Cry1F	Lepinox	HYB
kurstaki x *aizawai*	EG7841	Cry1Ac	Crymax	HYB
kurstaki x *aizawai*/*P. fluorescens*	MYX300	Cry1Ac, Cry1C	Mattch	CC
kurstaki x *tenebrionis* x *kumamotoensis*	EG2424	Cry1, Cry3	Foil[a], Jackpot	HYB
kurstaki x *tenebrionis* x *kumamotoensis*	EG7673	Cry3Aa, Cry3Bb	Raven	HYB
tenebrionis (= *morrisoni, san diego*)	SA-10	Cry3, Cry1	M-One[a], Novodor	NAT
tenebrionis/*P. florescens*		Cry3A	M-Trak[a]	CC

Comments: *NAT* – native *B. thuringiensis*, *HYB* – hybrid *B. thuringiensis*, *CC* – δ-endotoxin (CellCap)
[a]withdrawn from the market

The bioencapsulation and delivery system CellCap is a proprietary technology of Mycogen Co. for enhancing field persistence. The *cry* gene(s) coding for the desired endotoxin(s) is (are) isolated from *Bt* strains and transferred into a *Pseudomonas fluorescens* Migula host isolated from the phylloplane. In the production of CellCap preparations, genetically modified (GM) *P. fluorescens* cells are cultured in large-scale fermentors. Unlike *Bt* cells, which undergo lysis at the end of the fermentation cycle, the *P. fluorescens* cell walls remain intact. *P. fluorescens* cells are then killed in the fermentor before harvest using a proprietary physico-chemical procedure. This process also fixes the cell wall by cross-linking its components, creating a stable, dead cell biocapsule that encapsulates and protects the Cry toxins. Thus, the active component of any CellCap product contains no living cells: it rather consists of the selected Cry toxin(s) encapsulated within a dead cell biocapsule. The best-known GM *P. fluorescens* strains, containing different *cry* genes, are MYX104, MXY300, MYX833 and MYX7275 (Mycogen Co.) (Table 10.1).

Beside the introduction and broadening applications of *Bt*-bioinsecticides as means of environmentally friendly insect control, another *Bt*-based molecular biological application of increasing significance has been the development of insect resistant GM plants by the insertion of *cry* transgenes into the plant genome, the expression of which being responsible for the biosynthesis of Cry toxin proteins. This enables the resultant transgenic *Bt* plants to produce these microbial proteins and gain protection against sensitive insect pests through a mechanism similar to the pathogenicity of *B. thuringiensis* strains. As the molecular basis of the insect pathogenicity of the two approaches are identical, these agricultural practices are often considered equivalent. The low environmental and ecological impact of *Bt*-based bioinsecticides (Darvas and Polgár 1998) is observably so low that these formulated preparations gained acceptance even in ecological (organic) agriculture, the practice of which completely rejects the use of synthetic pesticides. In turn, *Bt* plants have also been proposed to be as environmentally safe as *Bt* insecticides. Nonetheless, although very closely related to each other in their biochemical mode of action, there are fundamental differences between these two insect control practices.

2 *Bt* Bioinsecticides

Bacillus thuringiensis strains applied in agricultural or hygienic (mosquito larva) treatment practices characteristically form parasporal bodies consisting of δ-endotoxins during sporulation. Certain varieties also contain parasporin, a recently described toxin causing cellular toxicity on tumor cells (Crickmore et al. 2009). In addition, several exotoxins (α-, β-, M-, etc.) and Vip (vegetative insecticidal protein) toxins may also be formed at the end of the vegetative stage of the bacteria, if food sources are limited for further vegetative periods (Bravo et al. 2007; Crickmore et al. 2009). Strains producing α-exotoxin (lecitinase C) and β-exotoxin (thermostable adenine nucleotide inhibiting RNA-polymerase) have been banned due to severe side-effects (mutagenicity and teratogenicity) of the latter.

The first insecticide containing *B. thuringiensis* was introduced in France in 1938 under the trade name of Sporeine and contained the *B. thuringiensis* subsp. *thuringiensis* pathotype. The HD-1 strain of *B. thuringiensis* subsp. *kurstaki*, isolated by Dulmage in 1970, has been found to be two orders of magnitude more active against agricultural pests (van Frankenhuyzen 1993), allowing broad application of *Bt* bioinsecticides. One of the major *Bt*-bioinsecticides is Dipel, developed from the HD-1 strain by Abbott Laboratories (1992).

2.1 Bt-Based Toxins: Structure and Classification

The δ-endotoxin proteins are further divided into two main groups, the pore-forming Cry (crystalline) and Cyt (cytolytic) toxins. Cry toxins are structurally related three-domain proteins consisting of an α-helix (domain 1), participating in the insertion into membranes, and two β-sheets (domains 2 and 3), taking part in the binding to the lectin receptors (Schnepf et al. 1998; Bravo et al. 2007). In Cyt toxins two α-helices surround a β-sheet, forming a simple α-β domain (Li et al. 1996). Cry toxins bind to special midgut receptors (Schnepf et al. 1998), while Cyt toxins form pores on the cell membrane through direct interaction with membrane lipids (Promdonkoy and Ellar 2003).

Bacillus thuringiensis strains used to be classified into 69 serotypes and 13 subgroups based on the H antigens of their flagellae and certain biochemical characteristics as there also exist strains without flagellae (van Frankenhuyzen 1993; Lecadet et al. 1999). These large toxin groups can be further divided by toxin structure (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, etc.). Most of the *Bt* strains produce several toxin types, for example *B. thuringiensis* subsp. *kurstaki* HD-1 produces Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry2B toxins (Arvidson et al. 1989; Lisansky et al. 1997). As the classification of several new toxins was problematic in this setup, a new system has been introduced on the basis of primary protein structure (amino acid sequence) similarities. Thus, the so far described 179 Cry and 9 Cyt toxins have been reclassified into 55 (Cry1 – Cry55) and 2 (Cyt1 – Cyt2) main toxin types, with several subtypes in each (e.g., Cry1Aa, Cry1Ba) (Crickmore et al. 1998, 2009). In addition, several toxins of structures different from the above are also known, including Bin (binary), Mtx (mosquitocidal) and Vip type toxins (Bravo and Soberón 2008). Cyt toxins not utilized in plant protection have cytolytic and hemolytic activity, and exert effects mainly in larvae of Diptera, or synergize the effect of other Cry toxins (Bravo et al. 2007; Gómez et al. 2007).

2.2 Mode of Action of Cry Toxins

Cry toxins exert *per os* type activity, and are divided in groups that each affect individuals within the same insect order. On this basis, Cry toxins can be sorted

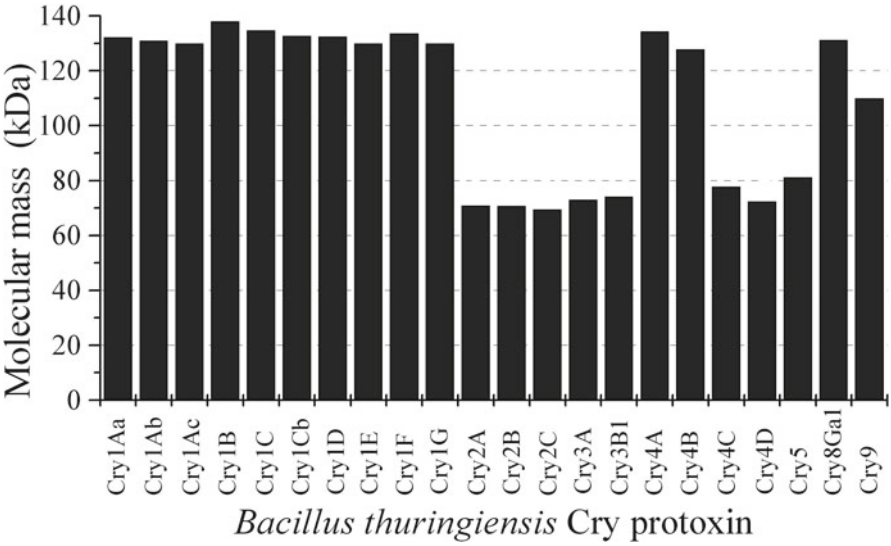

Fig. 10.1 Molecular mass of Cry protoxins

into five groups: exerting effects on Cry1 – mostly lepidopteran (*aizawai, kurstaki*, etc.), Cry2 – lepidopteran and dipteran (*kurstaki*), Cry3 – coleopteran (*tenebrionis*, *kumamotoensis*, etc.*)*, Cry4 – Diptera (*israelensis)* larvae, Cry8 – coleopteran (*japonensis*), Cry9 – lepidopteran (*tolworthi*) specific toxins (Crickmore et al. 2009; van Frankenhuyzen 2009).

As for the mode of action of the most important Cry toxins, their effects lead, in several steps, to the lysis of the cells in the midgut epithelium. The Cry toxins, as formed in the *B. thuringiensis* bacteria, are proteins of 70–140 kDa molecular mass (Fig. 10.1), stabilized by disulfide bonds, and therefore, hard to decompose. This form is termed protoxin. The C-terminal domain in the characteristic three-domain structure of Cry1 protoxins is essential for exerting toxicity, and is believed to play a role in the formation of the bacterial parasporal bodies and to maintain the unique solubility properties of the protoxin crystals (de Maagd et al. 2003). Moreover, these C-terminal domains occur highly conserved, showing high (>90%) homology among Cry1 toxins. The N-terminal region of the toxic part of the crystalline toxins is more variable, showing 40–90% homology. The N-terminal region of the protoxin is markedly hydrophobic, while the C-terminal domain is dominantly hydrophilic. Proteases (trypsin, chymotrypsin, etc.) in the insect midgut cleave these protoxins to 55–65 kDa size activated toxins. The process occurs at high pH (10–11). Cry1A protoxins contain 16 cystein moieties (12 of which are conserved), and upon proteolytic removal of the first 28 amino acids and the C-terminal part of the protoxin, there remain no cystein in the remaining trypsin-resistant activated toxin. Of the 34 lysine moieties, only 3 remain in the trypsin-cleaved activated toxin, and even these and the arginine moieties present must be buried in the protein structure (causing resistance to further hydrolysis by trypsin). Chemical modification

of approximately 12 of the tyrosine moieties in the toxic segment resulted in decrease in the cytolytic activity, while modification of the lysine and cystein moieties did not affect toxicity, indicating that these tyrosine moieties are located on the molecular surface of the activated toxin (Visser et al. 1993).

Cry toxins are lectin type proteins that undergo oligomerization upon binding to the lectin-specific receptors of the cell membranes in the midgut epithelium. The oligomer forms irreversible insertion into the lipid membrane, and thus opens pores in the cell membrane, induce colloid-osmotic swelling, disturbing the ion balance of the cell and causing its lysis (Knowles and Ellar 1987; Gill et al. 1992; Knowles 1994). Peristalsis of the gut stops, and the insect ceases feeding. The vegetative body of *B. thuringiensis* enters the larval coleoma through the microinjury (Schnepf et al. 1998), but any other microrganisms living in the gut tract may cause sepsis at that stage. Recent studies (Mason et al. 2011; Graf 2011) indicate that mortality upon creating pores in the epithelium may take place by septicemia caused by a midgut microorganism, *Enterococcus faecalis* entering the hemolymph of the larvae. Whatever mechanism causes sepsis, it makes the successful use of Cry toxin preparations and even the later development of *Bt* plants possible, as the lethal effect is triggered alone by the Cry toxin protein (Broderick et al. 2006), produced either by a microorganism or a plant expressing a *cry* transgene.

Collapse of the ion balance is sufficient for mortal paralysis in certain insect species (van Frankenhuyzen 1993). Sensitivity of insect species to given Cry toxins and consequently efficacy of different toxins on insect species varies.

2.3 Analysis of Cry Toxins

Quantitative detectability of the active ingredient content of biocides is essential both for technological control and environmental risk assessment. As the biological activity of *Bt* bioinsecticides is not necessarily directly proportional to the detectable content of given Cry toxins, this remains an unsolved problem for *Bt* preparations. Cry toxins are proteins, therefore, methods for their biochemical analysis possibly include high performance liquid chromatography (HPLC), gel or capillary electrophoresis, and immunoanalytical techniques. HPLC methods are mostly not suitable due to the instability and highly adsorptive character of the protein during separation. Therefore, electrophoretic, immunological and bioassay test methods are being used to identify and quantify Cry proteins (Hickle and Fitch 1990). The most commonly used immunoanalytical formats are lateral flow devices and 96-well microplate-based enzyme-linked immunosorbent assay (ELISA) (Grothaus et al. 2006). ELISAs are rapid and cost-effective methods in Cry toxin analysis, and numerous ELISAs have been developed and made commercially available for Cry endotoxins. Other immunoanalytical and specific receptor binding assay techniques exist: the rocket immunoelectrophoretic precipitation assay (Winkler et al. 1971), microsphere-based immunoassays (Ermolli et al. 2006a, b; Fantozzi et al. 2007), sensors (e.g., surface plasmon resonance biosensor, Okumura et al. 2001) and

immunomagnetic electrochemical sensor (Volpe et al. 2006) have been developed for Cry toxins. Among these numerous analytical methods, ELISAs remain the methods of choice for their versatile applicability (Hickle and Fitch 1990; Hammock et al. 1991), and the use of ELISAs for Cry1Ab monitoring in *Bt* bioinsecticides and *Bt* plants has been reported extensively (Grothaus et al. 2006; Ermolli et al. 2006a; Palm et al. 1994; Adamczyk et al. 2001; Xie and Shu 2001; Zwahlen et al. 2003; Douville et al. 2001; Harwood et al. 2005; Székács et al. 2005, 2010a, b, 2012; Baumgarte and Tebbe 2005; Margarit et al. 2006; Nguyen and Jehle 2007; Crespo et al. 2008; Chen et al. 2009). It is important to emphasize that all commercially available ELISA methods have been developed using bacterial protoxins (see later), and therefore, they are directly applicable to bacterial preparations only.

Bt formulations are complex mixtures containing large amounts of damaged spores, intact δ-endotoxin crystals, residual amounts of fermentation medium and bacterial cell wall debris. The insecticidal efficacy of *Bt* formulations was characterized by specifying their bacterial spore content, yet it did not necessarily correlate with toxicity on insects. Attempts have also been made to standardize the endotoxin content of these preparations (Crespo et al. 2008), but did not succeed for different Cry toxin compositions due to varying actual fermentation conditions. Instead, solely biological activity requirements have been accepted, expressing the toxic efficacy on insects in International Units (IU) (van Frankenhuyzen 1993). A great boost occurred in the 1980s with the broadening use of *B. thuringiensis* subsp. *israelensis* preparations active on Diptera (mosquitoes and black flies) (Goldberg and Margalit 1977; de Barjac 1978; Federici et al. 1990) and Cry3-based preparations active on coleopteran (Gelernter 2004; Oppert et al. 2011). Yet *Bt* bioinsecticides of the highest importance remained the lepidopteran-specific preparations containing Cry1 and Cry2 type *Bt*-based endotoxins (e.g., Dipel).

The Cry endotoxin content of Dipel has been described inconsistently. Approximately 20–30% of the dry cell mass is constituted by Cry endotoxin crystals (Baum and Malvar 1995; Schnepf et al. 1998), nearly 80% of which is Cry1A (a, b, c) and approximately 20% is Cry2 (A, B) (Abbott Laboratories 1992). Nonetheless, actual δ-endotoxin concentrations in commercial and experimental formulations, determined by ion exchange chromatography, were found to be much lower, 0.3–1.7% δ-endotoxin (Bernhard and Utz 1993). Consequently, endotoxin content of distributed preparations varies extensively (U.S. EPA/OPP Pesticide-Related Database Queries; NPIRS National Pesticides Information Retrieval System; http://ppis.ceris.purdue.edu/).

It is of outstanding importance to consider the actual form of the Cry endotoxins within the *Bt* bioinsecticides, such as Dipel. The crystalline structure of these toxins is stabilized by disulfide bonds, and the crystal structure varies across toxin proteins. Due to the numerous disulfide bonds per protein molecule, the crystals are quite stable to solubilization at neutral pH. As a result, only a small minority of the toxin protein is immediately bioavailable (solubilizable), the vast majority of the crystal mass being only bioaccessible (temporarily non-bioavailable). Thus, just the solubilizable portion of the toxin content is analyzed promptly by ELISA; the bioaccessible part can be detected by ELISA only upon decomposition of the disulfide bonds

stabilizing the crystal structure. Lastly, a minor part of the entire toxin content in the parasporal bodies is non-bioavailable: this amount of endotoxin is decomposed during the process of breaking the crystal structure, and therefore, cannot exert its potential biological activity by direct bioavailability or bioaccessibility.

The endotoxin composition in *Bt* bioinsecticides is illustrated on Fig. 10.2 using as an example the Cry1Ab endotoxin in Dipel. Dipel contains protoxins that require enzymatic activation by a hydrolytic process. Cry1Ab protoxins of molecular mass of 131 kDa form bipyramidal crystals stabilized by a maximum of 16 disulfide bonds per molecule: 14 of the cysteins are found at the C-terminal of the protein (Huber et al. 1981; Bietlot et al. 1990; Vazquez-Padron et al. 2004). These crystals are soluble only at high pH (Hickle and Fitch 1990) or in the presence of reducing agents of the disulfide bonds (e.g., mercaptoethanol or dithiothreitol). Solubilized Cry1Ab protoxin molecules undergo enzymatic cleavage in the alkaline medium of the insect midgut, and their cleavage by peptidases (such as trypsin) produces an activated toxin of approximately 63–65 kDa molecular mass, which is resistant to further hydrolysis (Chestukhina et al. 1982; Choma et al. 1990; Schnepf et al. 1998; Oppert 1999; Hilbeck 2001; Douville et al. 2001).

The nominal concentration of a common formulation of Dipel is 3.2%, meaning that the preparation contains 32 mg/g bacterial protein. While the biological efficacy was consistent, actual protein and Cry1Ab/Cry1Ac toxin concentrations of Dipel were found to be highly variable depending on the product batches (possibly differing from each other in actual fermentation conditions). Thus, total protein concentrations in various batches (obtained in separate product packages and in different years) ranged between 22.4 ± 2.2 and 51.4 ± 5.8 mg/g according to the bicinchoninic acid (BCA) method (Smith et al. 1985). Cry1Ab/Cry1Ac toxin content detectable by ELISA, however, was much more variable and depended on product batch and sample preparation conditions. The bacterial preparation extracted with neutral buffer (pH 7) resulted in detected Cry1Ab/Cry1Ac concentrations between 4.8 ± 0.6 and 60.2 ± 3.7 μg/g, with an average of 20.6 μg/g. This concentration is considered as the bioavailable Cry1Ab/Cry1Ac toxin content of the preparation. Dissolving the crystal structure under alkaline conditions results in increasing immobilization of the bioaccessible toxin stock and in higher detectable toxin concentration. Optimal solubilization was achieved at pH 10 (better than at pH 12), which could be further improved by the use of 50 mM mercaptoethanol or dithiothreitol in the extraction buffer reaching Cry1Ab/Cry1Ac concentrations of 84.5 ± 6.9 μg/g and 8.16 ± 0.87 mg/g without and with solubilizing agent, respectively. From these determinations, per hectare dosages of bioavailable and bioaccessible Cry1Ab/Cry1Ac toxin (20.6 mg/ha and 0.085–8.16 g/ha, respectively) can be easily calculated from the registered dose of 1 kg/ha for Dipel.

Fig. 10.2 (continued).*Bt* plants (e.g., *MON 810* maize) contain a single, truncated form of the Cry1Ab protein (approximately 91 kDa molecular mass), termed preactivated toxin, expressed at various concentrations in the plant tissues. Entering the insect digestive tract, this preactivated Cry1Ab toxin undergoes enzymatic cleavage resulting in the same hydrolysis-resistant core, activated Cry1Ab toxin. (The graphical representation of Cry1Ab toxin forms is adopted from Séralini 2010)

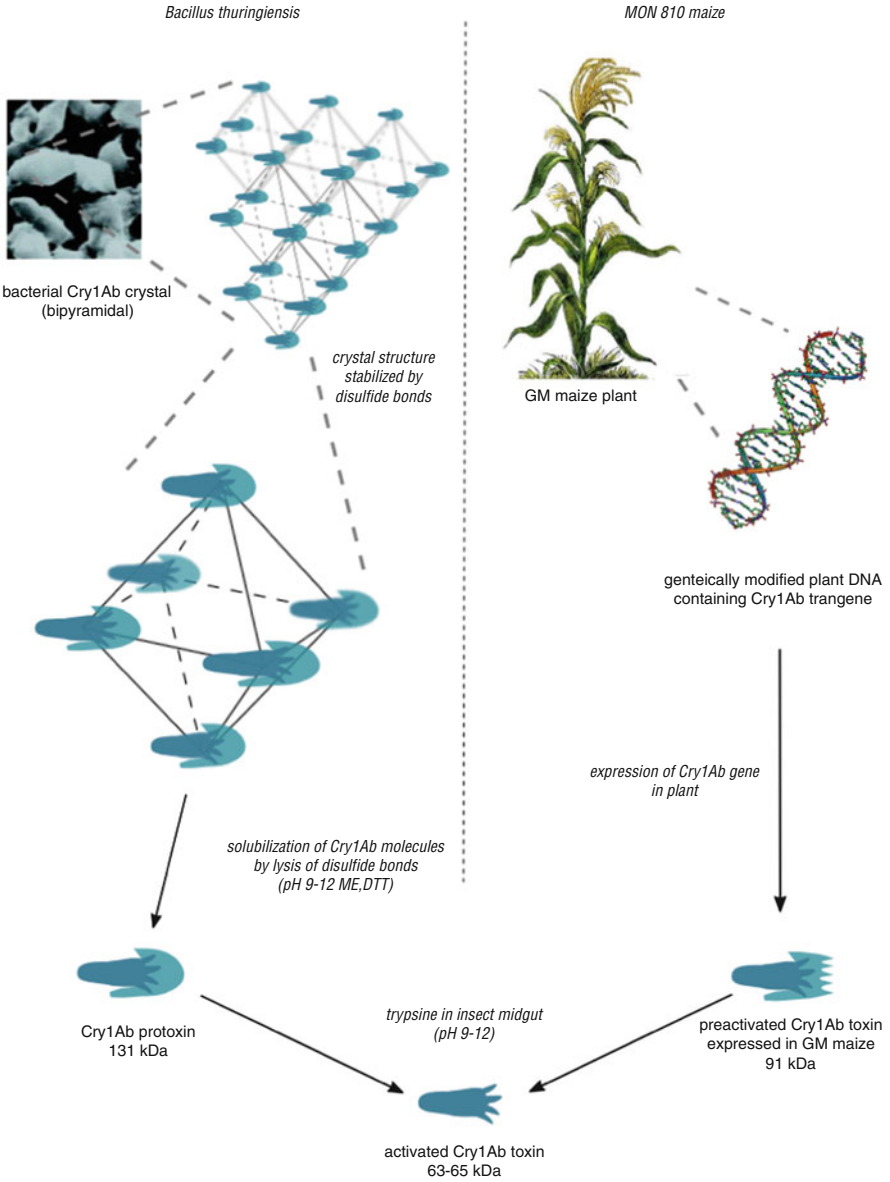

Fig. 10.2 Schematic representation of the various forms and activation of Cry1Ab protein in bacterial *Bt* preparations (*left*) and *Bt* crops (*right*). *Bt* bioinsecticides (e.g., Dipel) contain, among several other Cry protoxins, Cry1Ab protoxin molecules (131 kDa molecular mass) in bipyramidal crystals stabilized by disulfide bonds. Upon cleavage of the crystal structure by high pH or disulfide-reducing agents (e.g., mercaptoethanol, dithiothreitol), Cry1Ab protoxin molecules are solubilized. The solubilized Cry1Ab protoxin content undergoes enzymatic activation in the insect midgut to form the activated Cry1Ab toxin (63–65 kDa molecular mass) responsible for insecticidal action.

3 *Bt* Plants

At present there are 130 single or stacked event (i.e., producing one or several insecticide substances and/or tolerating one or more herbicide active ingredients) GM plant varieties under registration in the European Union, and a great part of these have been modified for pest resistance. It has been mentioned as an advantage of *Bt* plants that they provide continuous protection against the target pest and related species with similar modes of action. The active substance is not subject to certain environmental effects (direct UV radiation and rain) that could possibly lower its efficacy. It has been considered as a disadvantage, however, that the pollen of *Bt* maize containing the *cry* gene originated from *B. thuringiensis* may fertilize the flowers of traditional varieties of the same species (intraspecific hybridization) or their relatives (interspecific hybridization).

Bt plants registered or considered for registration within the European Union are cotton, maize and soybean (12 genetic events, and one additional withdrawn earlier). These GM plants, produced by three multinational firms (Monsanto Corporation, Pioneer Hi-Bred/Dow Agrosciences LLC/DuPont and Syngenta), target lepidopteran and coleopteran pests, and may be resistant to certain herbicides (Table 10.2). Although the genetic event *MON 810* has been registered for cultivation in the European Union, nine Member States (Austria, Hungary, Greece, Poland, Italy, France, Germany, Luxemburg and Bulgaria) announced national moratoria against the cultivation of this maize variety group.

Bt potato and additional *Bt* maize and cotton varieties have been registered outside the European Union. For example, the GM potato variety Russet Burbank/NewLeaf of Monsanto, producing Cry3A toxin and resistant against potato beetle was planted in the United States and Canada between 1995 and 2001. However, GM potato never captured a large share of the market because an efficient and cheaper insecticide against the potato beetle was commercialized at the same time, and public pressure on food processors led them to stop using this type of crop (US National Research Council 2010). StarLink maize varieties producing Cry9C toxin, registered only for animal feed between 1999 and 2000 (Castle et al. 2006), were rapidly withdrawn upon their widespread occurrence in the human food chain. These varieties have never been introduced in the European registration system, which is substantially more precautious than North America, partly due to agro-technological and legistative differences between these continents.

Bt plants produce large amounts of Cry toxin protein (Cry toxin/ha), and this toxin encapsulated in the plant cells remain for long periods in the environment. The effects of the toxin on the arthropods involved in the decomposition of the stubble- and on soil-microbial populations deserve further attention. Numerous current studies reveal low environmental impacts (US National Research Council 2010), yet the exact combination of all processes involved is not yet known. Pollen containing Cry toxin drifting off the fields may modify the habitat quality of the area and its borders, and therefore, can cause risks to rare and nationally protected butterflies. Butterflies living on nettle (*Urtica dioica*) and *Rubus* spp. at the perimeters or on

Table 10.2 Main single and stacked genetic events for lepidopteran and coleopteran resistance authorized in the European Union

Crop	Owner of the variety	Cry toxin types	Genetic event (Trade names)	Authorization		Type of genetic modification
				Type	Stage	
Cotton	Monsanto	Cry1Ac	*MON 531* (Bollgard)	FF	R	Lepidopteran-resistant (*nptII*)
		Cry1Ac, Cry2Ab	*MON 15985* (Bollgard II)	FF	R	Lepidopteran-resistant (*nptII*)
Maize	Monsanto	Cry1Ab	*MON 810* (MaizeGard, YieldGard)	FF, IP, C	R	Lepidopteran-resistant
		Cry3Bb1	*MON 863* (MaxGard)	FF, IP	R	Coleopteran-resistant
		Cry3Bb1	*MON 88017* (YieldGard VT RW)	FF, IP	D	Coleopteran-resistant + herbicide-tolerant (*glyphosate*)
		Cry1A.105, Cry2Ab2	*MON 89034* (YieldGard VT Pro)	FF, IP	D	Lepidopteran-resistant
	Pioneer Hi-Bred/Dow/DuPont	Cry1F	*DAS-1507* (Herculex I)	FF	D	Lepidopteran-resistant
		Cry34Ab1, Cry35Ab1	*DAS-59122-7* (Herculex RW)	FF	D	Coleopteran-resistant + herbicide-tolerant (*glufosinate*)
	Syngenta	Cry1Ab	*SYN-BT011-1*	FF	P	Lepidopteran-resistant + herbicide-tolerant (*glufosinate*)
		Cry3A	*SYN-IR604* (Agrisure RW)	FF, IP	D	Coleopteran-resistant
		Vip3Aa19e	*SYN-IR162-4*	FF, IP	D	Lepidopteran-resistant
Soybean	Monsanto	Cry3A	*MON 87701*	FF, IP	D	Lepidopteran-resistant

Comments: *FF* – food and feed, *IP* – import and processing, *C* – cultivation, *D* – documentation submitted, *P* – product, *R* – authorization under renewal, *nptII* – containing antibiotics (kanamycin) resistance gene

thorn apple (*Datura stramonium*) at the first 50 m of corn fields are at high risk of exposure, in particular, larvae of the peacock butterfly (*Nymphalis io*) in Central Europe (Darvas et al. 2004; Lauber et al. 2010; Lauber 2011).

Rapid insect resistance development has been observed with products producing a single Cry toxin (Tabashnik et al. 2008; Bagla 2010; Storer et al. 2010; Gassmann et al. 2011). To delay the development of such pest resistance, so-called "pyramid" *Bt* crops, producing two or more Cry toxins active against the same pest are being developed. Such pyramid strategy is best applicable, when selection for resistance to one of the toxins applied does not cause cross-resistance to the other(s) (Zhao et al. 2005; Gassmann et al. 2009; Tabashnik et al. 2009b). Nonetheless, although the evolution of pest resistance to pyramid *Bt* crops is slower, resistance development is driven by the same evolutionary processes as for single *Bt* Cry toxin varieties (Ives et al. 2011). *Bt* maize varieties producing toxins other than Cry, such as Vip3Aa19e (*SYN-IR162-4*) without cross-resistance with Cry1 toxins may also provide a solution to the problem of Cry1 toxin resistant strains of lepidopteran pests.

3.1 Coexistence of Bt Plants with Conventional Varieties

Probably one of the practically most important risks of GM crops is the possibility of gene flow. The reason is that the transgene is contained in and transmitted by the reproductive organs of the GM plants. Therefore, in the course of the cultivation of GM varieties, especially in the wind- (e.g., maize) and insect-pollinated (e.g., canola) plant species, the escape of the transgene with pollen (biological gene flow) or with seed/reproductive organs mixed with conventional varieties (physical gene flow) cannot be prevented, jeopardizing natural biodiversity, as well as traditional and organic farming. At present, gene flow is more problematic for glyphosate tolerant GM crops (for a review, see Székács and Darvas 2012), but also applies to *Bt* crops. Due to such possibilities of biological and physical gene flow, even the most severe coexistence law can only provide short-term solutions. In the interest of the elimination of gene flow it is necessary for the pollen not to contain the transgene or at least not in an operational state (Heszky 2011a).

Data on maize indicate that a distance larger than 25 and 250 m is needed to keep admixture below the European Union labeling threshold of 0.9% (that used to be the limit of detection of the early PCR techniques) and for 0.1% threshold (as favored by organic farming organizations), respectively. Oilseed rape represents a more complex issue in Europe, because apart from pollen flow, persistence of volunteers in arable fields and their perimeters, as well as interspecific hybridization with wild relatives also play a role (van de Wiel and Lotz 2006).

Different levels of GM traces in seeds (ranging from 0.01% to 0.5%) are considered in the European Union for quantifying the final adventitious GM presence in crops, especially in seed production. Three solutions are frequently mentioned in order to reduce gene flow: (*i*) the isolation distances between GM and non-GM fields; (*ii*) sowing a non-GM maize buffer strip around GM fields; and finally (*iii*) using GM varieties with different flowering duration compared with non-GM

Varieties. The latter is highly effective but is dependent on meteorological conditions and is hampered by associated yield losses (Messean et al. 2006).

The viewpoint in organic production is that the product cannot be declared organic if it contains any detectable GMO content (in other words tolerance for GMO content is 0%). In case of 0.5% GMO content in traditional maize seed, the calculated amount of individual GM plants that will emerge with a crop density of 70,000 plants per hectare is 350 individual GM plants. Projected to 1.2 million hectares (corresponding to of maize production of Hungary, the second biggest maize seed producer in European Union), the amount of emerged GM maize represents 420 million individual GM plants, equivalent to 6,000 ha of pure GM maize cultivation size. Considering 0.1% GMO content in the seed, the corresponding result is 60 GM plants per hectare and 70 million GM maize plants, representing 1,000 ha of pure GM maize cultivation size (Heszky 2011b).

The direction of biological gene flow can be diverse, which is unfortunately rarely discussed: (*i*) Gene flow *via* pollen can occur from GM to traditional Varieties. This is regulated by the current coexistence law. Gene flow, however, can occur in the reverse direction as well: conventional variety may also contaminate GM varieties, which may cause biological risk e.g., in the case of glyphosate resistance; (*ii*) Gene flow *via* pollen can also occur from GM varieties to other GM varieties, which is not regulated by the current coexistence law; (*iii*) From grass, trees, open pollinated fruit species, etc. gene flow *via* pollen may occur from GM varieties to wild ecotypes of the same plant species and also in the reverse direction (Heszky 2011b).

Intraspecific hybridization among cultivars is a major issue among foreign pollinated plants, especially in seed production, where pollen competition does not occur (detasseled or male sterile plants). The necessary isolation distance may reach 1,000 m in maize seed production if the tolerance limit is approximately 0.1% of foreign seed. Maize cultivars may be subdivided into three means groups by flowering time: early, normal and late pollination cultivars (see FAO numbering). The occurrence of silk or maturing of female flowers of maize follows the tasseling pattern. Pollen emission lasts 10–14 days in general for hybrid species, although it may last even twice as long in the external five rows, where individual plants may be at different developmental stages, well-known as border effect. Cross-pollination may occur only among cultivars of similar flowering time. In case of cross-pollination, however, the *cry1* gene transferred *via* pollen produces Cry1 toxin in the seed of originally non-modified plants already in the same year, as seen by RT-PCR and ELISA techniques. Therefore, although measures ensuring co-existence of GM and non-GM crops have been devised for many countries in Europe, Asia, and America, long term co-existence of a conventional a GM variety with same flowering time in case of cross-pollination is an ecological nonsense.

3.2 Compatibility of Bt Plants with Integrated Pest Management

A clear advantage of *Bt* crops is their specific mode of action. The broad range of *cry* genes provides the possibility of their applicability against various insect orders.

The utility of traditional formulated *Bt* products is well documented (Ravensberg 2011). Although sprayable *Bt* preparations with oral activity (e.g., *kurstaki* – Dipel; *israelensis* – VectoBac) are well applicable, certain unfavorable features have been revealed: the distribution of Cry toxin is uneven on the plant surface to be protected, the spray does not provide protection against pests that feed inside the plant; the toxin is decomposed upon UV irradiation; and the preparation is washed off from plants by rain (Roh et al. 2007).

In contrast, *Bt* plants provide relatively continuous protection against target pests and related species. This, however, means that the truncated Cry toxin is synthesized by the *Bt* plant continuously, regulated by the gene construct introduced into the plant and by the own genetic program of the plant, independently from the actual occurrence and population dynamics of the insects, causing an extensive presence of the Cry toxins in plants. In this context, *Bt* plants do not comply with the principles of integrated pest management (IPM), as the occurrence of the toxin is not limited to the duration of the possible damage by the pest, and does not implement any threshold value to the acceptable damage level. Although *Bt* crops can bring significant advantages under given climatic conditions, including reduction in use of broad-spectrum insecticides that is one of the primary goal of IPM (Cannon 2000; Romeis et al. 2008; US National Research Council 2010), there is opposition regarding the acceptance of *Bt* crops in IPM. Regardless of how mild there transgenic protein may be considered, the use of these crops currently cannot fulfill the main ecological principle of IPM that any protection step against any given pest is justified only if pest damage exceeds a critical threshold level.

Moreover, the Cry toxin varieties expressed in *Bt* plants are not necessarily the same as those in the corresponding *Bt* bioinsecticides. For example, maize varieties in the *MON 810* variety group produce a single preactivated Cry1Ab toxin of approximately 91 kDa molecular mass (Fig. 10.2), a truncated form of the bacterial Cry1Ab protoxin that undergoes enzymatic cleavage in the insect midgut, resulting in the same hydrolyzed, 63–65 kDa active toxin as Dipel (Hilbeck 2001; Székács et al. 2010a). Besides the obvious biochemical consequences, this fact of not identical Cry1Ab active ingredient in *Bt* bioinsecticides and *Bt* plants has connotations of utmost importance in pesticide/crop registration and in analysis of the active ingredient content.

As for registration issues, on the basis of the above, *MON 810* maize produces an active substance that is not a registered bioinsecticide ingredient. The Cry1Ab active ingredient of *MON 810* maize varieties is preactivated Cry1Ab toxin (91 kDa), yet toxicology studies in the registration documentation have been carried out with either bacterial protoxin (one of the active ingredients of Dipel, 131 kDa) or with the active toxin (63–65 kDa). This may be considered by some as a formal issue, yet a rather important one, as no pesticide active ingredient, regardless how similar it is to a registered one, can be exempt from individual registration and (eco)toxicological evaluation. This is well known in the registration of pesticides or pharmaceuticals, where complete toxicological evaluation is required for authorization of an active ingredient even if it differs structurally only slightly (e.g. in a single substituent) from a registered active ingredient. The same should also apply to insecticidally active proteins, such as Cry toxins.

It is often claimed that the registration of *Bt* crops has gone through an extensive review process that has been deemed thorough in more than 23 countries, where these crops are used. Such evaluations, however, are mostly administrative, based on the documentation of the genetic event supplied by the owner and corresponding data from the scientific literature. Moreover, in the scope of registration of new *Bt* crops in the European Union, event based documentation are submitted to a selected Member State called the rapporteur country of the given event. In case of maize, rapporteur countries include France (*MON 810*, *SYN-BT011*), Germany (*MON 863*, *SYN-IR162*), Spain (*DAS-1507*), the Czech Republic (*MON 88017*), the Netherlands (*MON 89034*, *DAS-59122*) and the United Kingdom (*SYN-IR604*). It is hardly justifiable, why decisions about cultivated *Bt* maize varieties are made in countries of slight importance in European maize production, and why not in leading maize producers such as Hungary or Italy, beside France.

Toxicological assessment of the variety documentations is not unambiguous, either, as seen for example in the case of maize variety *MON 863*. In 2002, Monsanto Company submitted an application to the German authorities to import *MON 863* maize into the European Union. The submission contained a 13-week rat feeding study, performed by a third company (Covance Labs), but statistically analyzed by Monsanto (Lemen et al. 2002; Hammond et al. 2006). Based on the results EFSA's experts (2004) stated, "The results of 90-day sub-chronic rodent studies do not indicate adverse effects from consumption of *MON 863* and *MON 810* and the Panel concluded that there are no concerns over their safety." A Court of Appeal action in Germany in June 2005 allowed public access to all the raw data from this 13-week rat-feeding study, on the basis of which Séralini et al. (2007) performed an independent analysis, and arrived to a conclusion that *MON 863* consumption affected the two main organs of detoxification: liver (in case of females) and kidney (in case of males). It appears that the statistical methods used by Monsanto were not sufficiently detailed to see disruptions in biochemical parameters, in order to evidence possible pathological signs. The EFSA GMO Panel re-evaluated the statistical methods (EFSA 2007), and stated that the observed differences in test parameters were not indicatives of adverse effects, and the new statistical analysis had not raised toxicologically relevant issues. In parallel, Doull et al. (2007) also came to the same conclusion regarding *MON 863*, and studying a Cry1Ab toxin producing maize variety in a three-generation feeding study, Kiliç and Akay (2008) found no statistically significant differences in relative organ weights of rats, except for minimal histopathological changes in liver and kidney. Changes in creatinine, total protein and globulin levels were also determined in biochemical analysis.

Further statistical analysis done by de Vendômois et al. (2009), however, clearly revealed for three events (*MON 810 – cry1Ab* gene, *MON 863 – cry3Bb* gene, *MON 603 – cp4-epsps* gene) new, sex- and often dose-dependent side-effects upon consumption. Effects were mostly associated with the kidney and liver, although varying among the three events. Further effects were also noticed in the heart, adrenal glands, spleen and the haematopoietic system. Upon criticism received from Monsanto (2010); de Vendômois et al. (2010) summarized the debated alimentary chronic risks, and suggested they may come from unpredictable insertional mutagenesis or

metabolic effects, or from new pesticide residues. Therefore, as chronic health effects including cancerous, hormonal, reproductive, nervous or immune diseases are increasing worldwide, gender differences and the non-linear dose- or time-related effects should be particularly considered in toxicology, mainly in attempts to reveal hormone-dependent diseases and first signs of toxicities (Séralini et al. 2009). Yet, although certain 90-day feeding tests were performed, longer studies are very rare. Upon worldwide commercialization of GM crops, especially stacked events, the standard toxicological evaluation is even more seriously inadequate as the so-called "cocktail effects" are not taken into consideration.

An additional example of the controversies in the toxicological evaluations is the mammalian toxicological assessment in the application for renewal of authorization of *MON 810* maize (European Food Safety Authority 2009b). The assessment refers to a 90-day rat feeding study with grain of *MON 810* maize (Hammond et al. 2006), in which the overall health, body weight, food consumption, clinical pathology parameters (hematology, blood chemistry, urinalysis), organ weights, and gross and microscopic appearance of tissues were found to be comparable between groups fed with diets containing *MON 810* maize and conventional maize varieties. In contrast to this conclusion, principal component analysis clearly revealed sex- and often dose-dependent new side effects linked with consumption of GM maize of genetic events *MON 810* (containing Cry1Ab toxin), *MON 863* (containing Cry3Bb1 toxin) and *NK603* (glyphosate tolerant). Effects were mostly associated with the dietary detoxifying organs (de Vendômois et al. 2009). Moreover, Séralini et al. (2011) found the results of Hammond et al. (2006) highly controversial, claiming that 90-day tests are insufficient in length to evaluate chronic toxicity, and the hepatorenal toxicity signs observed may indicate the onset of chronic diseases. Similar toxicological considerations apply for stacked trait GM plants with both Roundup (glyphosate) tolerance and transgenic insecticidal Cry toxin-based insect resistance. Current findings indicate that Cry1 toxins cause cell death at high concentrations (above 100 ppm), while reduce caspase 3/7 activation induced by Roundup on a human embryonic kidney cell line (Mesnage et al. 2011), indicating that Cry toxins are not inert on non-target human cells and can interact with the side-effects of glyphosate.

As for the analytical consequences of the differing Cry1Ab toxin proteins in *Bt* bioinsecticides and *Bt* crops, the ELISA systems devised against the bacterial protoxins are improper to be used in the analytical sense to directly apply to the quantitative determination of plant-expressed toxins. It is obvious to concede that antibodies generated about the protoxin will show different (lower) affinity to the truncated forms of the toxin. Thus, ELISA systems against Cry1Ab/Cry1Ac bacterial protoxins cannot be applied without correction to the measurement of plant toxin levels by using analytical standards of the protoxin protein. A simple possibility to overcome this problem is the use of plant-produced toxins as analytical standards, however, the preactivated Cry1Ab protein produced by *MON 810* maize is commercially not available. A more elaborate approach has been the determination of the cross-reactivity (CR) of the ELISA systems with the activated toxin. On the basis of established enzymatic activation protocols (Lilley et al. 1980; Lambert et al. 1996;

Shao et al. 1998; Miranda et al. 2001; Mohan and Gujar 2003), this has been achieved for Cry1Ab (Székács et al. 2010a), and two commercial ELISA systems have been shown to have CR to the active toxin of 0.41–0.56. It has to be emphasized, that all values reported in the scientific literature, obtained by protoxin-based immunoassays, are subject to correction with such CR values. In other words, reported toxin concentration values underestimate the actual levels if they do not take the lower CR of the plant expressed Cry1Ab toxin with the antibodies into consideration. To overcome this problem, ELISA systems against the plant-expressed toxin(s) should be developed and made available, or at least the plant-expressed toxin(s) should be made readily available as a protein standard.

3.3 Biomass and Expressed Cry1Ab Toxin Levels in Bt Maize

Maize biomass production is affected by a number of parameters, the most important of which being the plant variety, agrotechnologies and weather conditions. Cultivating various *Bt*, near-isogenic and commercial maize varieties in the period between 2001 and 2011 at the Ecological Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences (Julianna-major, Nagykovácsi, Hungary) (Székács et al. 2005, 2010a, b; Takács et al. 2011), the overall maize (FAO number near 400) biomass produced by various varieties ranged between 70.9 and 96.2 t/ha. The mass proportion of each plant organ was determined in each year for each variety, and was found consistent among different years and varieties with the highest mass being the cobs (measured with all husk leaves and pistils), 31.9–42.6% of the entire biomass, followed by the foliage 21.1–32.0%, and stalk 21.7–23.0% (Fig. 10.3).

Cry1Ab toxin content was determined for all *Bt* maize varieties, with the CR between activated toxin and protoxin (Székács et al. 2010a) and the uneven distribution within organs (Székács et al. 2010b) considered. Thus, in 2001 Cry1Ab toxin concentration was demonstrated to be 9.6–17.2, 2.3–5.3 and 1.4 μg/g in the leaves, roots and stalk of *MON 810* maize DK-440 BTY, showing seasonal fluctuation (Székács et al. 2010a) with maximal toxin content in the leaves in the vegetative five-leaf (V5) phenological stage. The per hectare production of plant-expressed Cry1Ab toxin was calculated, and was found to range between 147 and 456 g of Cry1Ab toxin/ha during the 2001–2011 period. Calculating the Cry1Ab toxin distribution among plant tissues (Fig. 10.3) revealed important observations: (*i*) The vast majority of the Cry1Ab toxin is found in the foliage (69.0–72.1%) (Székács et al. 2010a). (*ii*) Toxin content in the seeds represents a minor portion (1.9–6.8%). (*iii*) *MON 810* maize expresses the Cry1Ab toxin at moderate concentrations in the root as well, and as a result, a significant proportion (7.7–9.7%) of the overall produced toxin quantity is found in the roots and inevitably remain, along with other plant parts, in the stubble.

These toxin levels may further be elevated by soil fertilization, the use of long maturation maize varieties and the use of stacked genetic events. Soil quality, especially

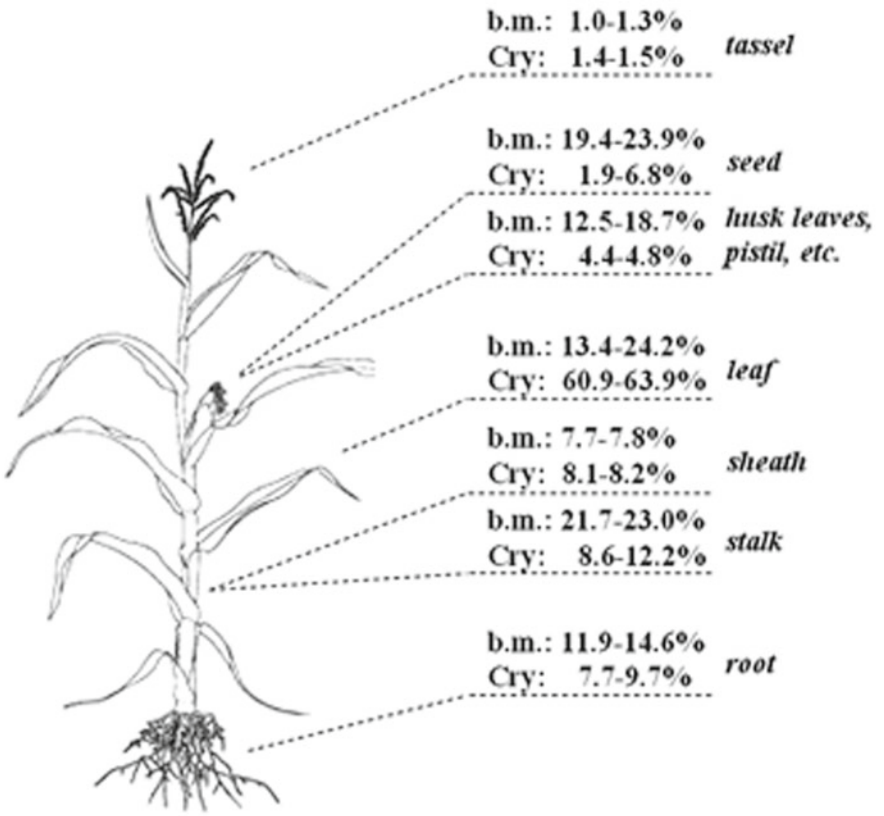

Fig. 10.3 Average biomass (b.m.) and Cry1Ab toxin production (Cry) by *MON 810* maize varieties. Meanwhile the largest biomass is *represented by the seed, leaf and stem* (approximately equal to each other), the largest proportion of the transgenic Cry1Ab toxin content is found in the leaves

the use of N-fertilizers was shown to exert a strong influence on Cry1Ab toxin expression by increasing biomass production by up to 80–85% (Bruns and Abel 2003; Ma and Subedi 2005) and consequently Cry1Ab toxin levels in *MON 810* by up to 3.7-fold (Bruns and Abel 2004). Thus, in the study of Bruns and Abel (2003, 2004) examining the effect of N-fertilization on Cry1Ab toxin production in the leaves of two *MON 810* maize varieties (Pioneer 33V08 Bt és DK-626 Bt) at V2 phenological stage and in the husk leaves of three varieties (AgriGold 6729 Bt, Pioneer 33V08 Bt, DeKalb DBT 418 Bt) at R3 phenological stage, an 1.4–1.5-fold increase was seen in Cry1Ab concentration in the leaves at 224–336 kg/ha N-fertilizer (NH_4NO_3) application, and over threefold increase in the husk leaves at nearly 300 kg/ha N-fertilization in two varieties (AgriGold 6729 Bt, Pioneer 33V08 Bt) and none in the third one (DeKalb DBT-418 Bt). The absolute amount of Cry1Ab toxin produced is further increased by the higher biomass of the maize varieties due to N-fertilization. In our experience fertilization increased the biomass produced by

DK-440 BTY in pot experiments up to 1.5–2.0-fold. Fertilization, therefore, may multiply Cry1Ab toxin production both through boosting Cry1Ab biosynthesis (along with other physiological biosynthesis processes in photosynthesizing tissues) and through increased biomass production. Cry1Ab toxin production varies among *Bt* maize varieties of different genetic events (Fearing et al. 1997; Baumgarte and Tebbe 2005; Nguyen and Jehle 2007; Székács et al. 2010a). Therefore, the use of long maturation (high biomass) maize variety of high toxin production (e.g., DK-818) may also result in such double enhancement effect: a *Bt* variety of DK-818 produced Cry1Ab toxin in the leaves at concentrations about 80% higher than in certain short maturation varieties (e.g., DK-440 BTY), while resulting in increased biomass production by 1.9–3.2-fold, to 180 t/ha. In that case, the overall quantity of produced Cry1Ab could reach as high as 1,930 g/ha. Apparently, the combination of individual genetic events in stacked GM crops may have an effect on the expression of the given transgenes, as a twofold increase has been observed in Cry1Ab toxin levels in stacked event maize varieties than in single event *MON 810* cultivars (European Food Safety Authority 2005). Moreover, the active ingredient is not subject to such strong environmental effects (UV radiation, rain) than in spray application that would lower their efficacy.

Yield increases are often mentioned as major advantages of *Bt* crops (Betz et al. 2000), particularly when pest pressure is high, but such yield advantages are related to avoided loss by pest damage, not the growth or production capacity of the maize variety. In the case of Cry1-expressing maize varieties, achievable yields depend on damage by the European corn borer, and therefore, as indicated in the literature (Ma and Subedi 2005) and in national distinctness, uniformity and stability (DUS) tests (e.g., in Hungary), maize yields remain unchanged in areas, where this pest is insignificant (Füsti Molnár 2011; Darvas et al. 2011).

3.4 Utility and Duration of Cry1Ab Toxin Production in Plant Tissues

In contrast to cause-directed crop protection involving spraying pesticide applications, GM plants express Cry toxin also during periods, when it is not necessary for pest control and also in plant tissues, where it is not needed. It has also been mentioned as a benefit that *Bt* crops reduce the need for broad spectrum insecticides, however, this does not apply to all climatic conditions. For example significant occurrence of the European corn borer in Hungary is rare (once in every 10 years), therefore, farmers do not even protect their crops against this pest. In such regions, cost efficacy cannot be justified for the very same reason. In addition, Cry1Ab toxin does not exert any effect on other maize pests such as aphids and mites or soil inhabiting coleopteran species. Moreover, *MON 810* maize resistant to European corn borer produces significant amounts of Cry1Ab toxin in its roots (approximately one quarter of the level produced in the leaves, where European corn borer feeds only in its L1 stage), while this pest does not damage the root at all. The roots release Cry1Ab into

the soil *via* their exudate (Icoz and Stotzky 2008b) during the entire vegetation period, and the toxin may be taken up by plants cultivated subsequently (Icoz et al. 2009). Although more studies are needed about such exudation of Cry toxins into the soil, numerous studies indicated little or no effect on soil organisms (Blackwood and Buyer 2004; Griffiths et al. 2006; Cortet et al. 2007; Icoz et al. 2008; US National Research Council 2010; Zeilinger et al. 2010; Tan et al. 2010), while others found low but significant effects of *Bt* maize on microbial community structure in soil (Turrini et al. 2004; Castaldini et al. 2005; Oliveira et al. 2008). In their extensive evaluation, Icoz and Stotzky (2008b) concluded that the effects of *Bt* maize on the soil biota are transient, but possible long-term impacts cannot be excluded.

The decrease in the incidence of corn ear in infestation by *Fusarium* species co-occurring with damage by larvae is considered a benefit (Munkvold 2003; Clements et al. 2003; Folcher et al. 2010). Yet, visual signs of fungal infection do not necessarily correlate with mycotoxin content, the composition of which reflects to proportions of various *Fusarium* species. Seed infection may occur not only by surface injuries (chewing by insects), but also by infestation through the pistil, characteristic to certain *Fusarium* species (Darvas et al. 2011). Thus, the level of given mycotoxins (e.g., zearalenone) does not correlate with the rate of larval damage (Folcher et al. 2009). In the case of *Bt* maize (*MON 810* and *SYN-EV176* variety groups), only a limited decrease of certain mycotoxins has been verified (Papst et al. 2005).

Seeds of *MON 810* maize produce significantly lower amounts of Cry1Ab toxin than the leaves (approximately one-tenth) (Székács et al. 2010a). Therefore, this maize variety cannot offer an optimal method to control corn ear damage by the larvae of the cotton bollworm (*Helicoverpa armigera* Hübner) and the European corn borer (Darvas et al. 2011). The high Cry1Ab toxin content in the foliage also raises the question of safe use of *MON 810* maize as silage. At least, 60% of the initial Cry1Ab protein concentration remained in the fresh silage (Kamota et al. 2011). Cry1Ab content in silage exhibited no clear-cut pattern of decrease over the time of 4 months. Thus, average Cry1Ab toxin content was detected to be 1.88 ± 0.71 μg/g *MON 810* silage (Rauschen and Schuphan 2006). In case of lactating dairy cows, there was no difference in ruminal degradability, determined separately for maize silage and grain of *MON 810*. Nutritional value and production efficacy for *MON 810* maize silage was similar to its near isogenic line (Donkin et al. 2003). No transgenic DNA from maize containing stacked (2GM) *cry1Ab* and *mepsps* genes, or Cry1Ab protein produced in the plant were detected in milk from cows fed with GM corn silage (Calsamiglia et al. 2007). A long-term study over 25 months was conducted to evaluate the effects of *MON 810* maize (silage, kernels and whole-crop cobs) on performance of lactating dairy cows. Cows fed with *MON 810* maize were exposed daily to Cry1Ab protein intake of 6.0 and 6.1 mg in the first and second lactation of the trial, respectively. There were no consistent effects of feeding with *MON 810* or its isogenic line on milk composition and body condition (Steinke et al. 2010). Health studies on mammalian model as rats (Hammond et al. 2006) met strong criticism (de Vendômois et al. 2009; Séralini et al. 2011) as mentioned earlier.

As seen above, *Bt* plants produce high amounts of Cry1Ab toxin per hectare – depending on the proportion of the vegetative plant parts in the biomass. The alarmingly high toxin amount in the foliage raises severe concerns regarding its utility for feed/silage or even leaving foliage in the stubble. In contrast, toxin content is of lesser concern in the seeds, maybe that is the reason, besides high insect specificity of the toxin, why seed consumption issues of *MON 810* maize have not emerged. Toxin expression in the roots and pollen, where it is not needed for plant protection purposes and causes unwanted exposure and ecotoxicological consequences prompts further development of *Bt* crops towards tissue-specific gene expression systems. The time of degradation of Cry toxins in the soil have been shown to depend on several factors, including climatic conditions (temperature, soil water content), soil characteristics (pH, composition) and soil microbial life. Cry toxins have been shown to be rapidly degraded microbially in soil (Clark et al. 2005). The plant-expressed toxin, however, being protected against decomposition in the plant cells, may persist in the soil (Baumgarte and Tebbe 2005; Icoz et al. 2008). There are numerous studies indicating no persistence or environmental effects of Cry toxins (Hopkins and Gregorich 2003; Pagel-Wieder et al. 2007; Icoz and Stotzky 2008a), others however, are less comforting. Results indicate 1–8% of the toxin content in stubble can be detected 1 year later, upon harvest. This can still be a substantial amount in the case of large vegetative mass varieties, compared to toxin amounts released with the bioinsecticide Dipel. Toxin accumulation and biological effects observed on insects show a pattern dependent on soil type (Tapp and Stotzky 1998). Moreover, Cry1 toxin persistence is a function of the entire amount of Cry toxin produced, its distribution among plant tissues, production and decomposition dynamics, as well as additional abiotic factors (Zwahlen et al. 2003). Certain collembolan species show a tendency to avoid maize debris containing Cry1Ab toxin in stubble as compared to the isogenic variety (Bakonyi et al. 2006, 2011), and their fecundity is decreased on this food type, that may partially explain low decomposition rate of a *MON 810* maize variety (DK-440 BTY) in stubble.

3.5 Secondary Effects Through Cry1 Toxin Containing Pollen and Stubble

Non-target organisms may get exposed through feeding to Cry toxins produced by *Bt* crops. The main routes of exposure include drifted pollen settled on food plants for phytophagous species, Cry toxin consuming pray and host for predators and parasitoids, plant debris and residues for decomposing organisms, flower and pollen for pollinating insects, and community connections for symbiontic organisms. Although Cry toxins (including Cry1Ab) have been claimed to pose no risk to various non-target organisms in numerous studies due to low toxicity or lack of exposure (Romeis et al. 2006), possible affectedness of non-target organisms related to the target pest is outstandingly problematic. Pollination by Cry1 producing crops present a possible risk in the pollination period to the habitats of larvae of protected

and rare lepidopteran insects living in the weedy perimeters of cultivation sites, if pollen containing Cry1 toxin is settled to these habitats and endures there for longer periods.

The analysis of pollen shedding revealed that a 300–600 pollen/cm^2 density is not rare on the top leaves of maize, but exceptional outside the maize fields (Pleasants et al. 2001; Darvas et al. 2004; Li et al. 2005). Ample intervals appear at the edges of maize fields for deposited pollen, depending on the pollen yield of the hybrid, the prevailing wind direction and pressure, and leaf surface characteristics of the plants on which maize pollen is deposited (Pleasants et al. 2001; Darvas et al. 2004). *Bt* maize pollen is ingested by non-target organisms that consume weeds emerging on maize fields. In a laboratory assay, larvae of *Danaus plexippus* (L.), reared on milkweed leaves dusted with pollen from *Bt* maize (N4640-*Bt*), consumed less plant material, grew slower and suffered higher mortality than larvae reared on leaves dusted with maize pollen without Cry1 toxin (Losey et al. 1999). These results were later questioned, and the impact of *Bt* maize pollen from commercial hybrids was suggested negligible on monarch butterfly populations (Sears et al. 2001) due to low levels of exposure. In subsequent experiments, increased mortality, delayed development of lepidopteran larvae and decreased larval, pupal and adult weight were revealed in experiments performed with pollen containing Cry1 toxin (*MON 810* or *SYN-Bt11*: ~90 ng Cry1Ab/g pollen) using densities typical at maize field adjacencies (Jesse and Obrycki 2000; Dively et al. 2004; Anderson et al. 2005).

The majority of lepidopteran adults visit agricultural areas for feeding and oviposition, so the diversity of flowering plants in the neighboring environments severely affects the butterfly community. Only extended monitoring can exactly reflect the effects of the environmental changes (Lang 2004).

There are several protected butterfly species in Europe, especially in the Pannonian Biogeographic Region, where the number of protected species is more than 200 (European Environment Agency 2002; Darvas et al. 2004). In this region larvae of *Nymphalis urticae* (L.), *Nymphalis io* (L.), *Nymphalis c-album* (L.), *Vanessa atalanta* (L.) feeding on *Urtica dioica* L.; *Argynnis niobe* (L.), *Argynnis pandora* (Dennis et Schiffermüller), *Brenthis ino* (Rottemburg), *Spialia sertorius* (Hoffmannsegg) feeding on *Rubus* spp.; and *Acherontia atropos* (L.) feeding on *Datura stramonium* L. may be affected principally (Darvas et al. 2004; Lauber et al. 2010; Lauber 2011). *Urtica dioica* and *Rubus* spp. are common at the perimeters and *D. stramonium* is frequent weed at the 50 m in the maize field.

In Germany, toxicity of *Bt* maize pollen to *Papilio machaon* L. was also studied. First instar larvae were exposed to different pollen densities applied to leaf disks of *Pastinaca sativa* L. for 48 h. The LD_{50} with regard to larvae surviving to adulthood was ~14 pollen grains of *SYN-EV176* consumed by first instar larvae (Lang and Vojtech 2006).

A mathematical model analyzed exposure of larvae of some non-target species: for example *N. io* and *V. atalanta* in four European countries. A dose-mortality relationship was integrated with a dose-distance relationship to estimate mortality both within maize field and at varying distances from the field edge. Perry et al. (2010) concluded the estimated environmental impact was low. Lang et al. (2011) found

that the incomplete and uncertain input data cause a higher uncertainty than indicated by Perry et al. (2010), and the possibility that the effects might be worse than predicted. Moreover, Perry et al. (2010) assumed larvae of *V. atalanta*, and *N. io* equally susceptible to Cry1Ab. They cited Darvas et al. (2004) as a reference for such equitoxicity, even though the cited paper contains no data about species sensitivity. In contrary, we reported LC_{50} of Dipel being 15.14 ppm to *V. atalanta* and 4.39 ppm to *N. io* first instar larvae (for example for the EFSA GMO Panel in Parma at June 11, 2008 – see Rodics et al. 2011; Lauber 2011). In another version of the mathematical model extended to non-target effects of Cry1F toxin in Bt maize pollen (Perry et al. 2012), therefore, the sensitivity of non-target insects is considered purely on a theoretical basis. The value of a mathematical model rests on the basis of the certainty of its input data (the biological effect in this case), which is highly questionable for the given model. Moreover, no acceptable mortality threshold applies in environmental risk assessment regarding protected species. In other world only the lack of exposure can limit the risk of a toxic substance to protected species, but any rate of mortality is unacceptable if the species is exposed to the substance. Pollen drifted from maize fields modifies habitat characteristics of protected species, which is not allowed by the Habitat Directive of the European Union (European Council 1992).

A frequently mentioned justification of pest control on the basis of plant-expressed toxin is the argument that broad-spectrum insecticides have more severe toxic effects on non-target organisms (Romeis et al. 2006). This may be true for various crops, climatic conditions and pests, but not for *MON 810* maize, the Pannonian Biogeographic Region (e.g., Hungary) and the European corn borer, which is not a regular pest in Hungary, and therefore, there is no reasons to use chemical or agro-biotechnological protection against it.

The large, globular particles of maize pollen settle in a relatively rapid course. Nearly 80% of it is settled within 6 m, but a small proportion may reach as far as several hundred meters. Pollen containing Cry toxin may reach natural aquatic habitats as well, where it may travel long distances without decomposition of the toxin. Rosi-Marshall et al. (2007) found that pollen containing Cry1 toxin along with other plant debris may enter aquatic ecosystems, where may impede the development of given trichopteran species and increase their mortality. Chambers et al. (2010) detected developmental delay of the development, but not the abundance or biomass of a trichopteran species (*Lepidostoma liba* Ross). Bøhn et al. (2008, 2010) demonstrated inhibitory effects of plant debris containing Cry1 toxin on the development and reproduction of the great water flea (*Daphnia magna* Straus), an indicator organism for water quality. None of these species belong to the group of known sensitivity to Cry1 toxins. Cry1Ab toxin protected from decomposition in the plant tissue could be detected from pollen transported by surface waters as late as 6 months after harvest (Tank et al. 2010).

The amount of Cry toxin containing pollen drifting from the maize field can be substantially reduced, yet not eliminated, by a refugee zone (border lines) with the isogenic line. Moreover, such refugee zone cannot solve the problem around canals across and temporarily water-covered spots on cultivation sites. The required size of

the critical zone is proportional with the expressed Cry toxin content in the pollen, characteristic to the crop variety. A satisfactory solution to this problem would be if the *cry* gene would not be present in the pollen.

The Pannonian Biogeographic Region i.e., the Carpathian basin is of outstanding importance in Europe in respect of conservation of biodiversity. Hungary represent 81% of this region. This justifies the definition of strict specifications for co-existence of GM organisms in the biogeographic zone. Legal regulations fully complying with requirements on the conservation of natural resources and the precautionary principle are required.

Environmental risk assessment of agrochemical or agrobiotechnological substances is based on the identification and estimation of negative effects and a subsequent evaluation of real exposures in agricultural practice. Secondary effects on non-target species are often tested experimentally on model organisms to describe the potential effects. Decision-makers at different authorities and boards (e.g., EPA, FDA, USDA, EFSA) attempt to balance experimental results and offer a general solution on the basis of special findings. In turn, several debates emerge. Regulatory frameworks should advocate the tiered approach to assess possible non-target effects. According to Romeis et al. (2006), in risk assessment of *Bt* plants on non-target organisms, early tier (i.e., laboratory) tests are conducted to determine whether an organism is susceptible to the Cry toxin under worst case conditions. The main problem is that laboratory conditions usually do not represent worst cases, and the agent with selectivity at order level may often exert indirect tritrophic effect through sensitive parasitoids or predators of an insensitive pest. In their comments, Andow et al. (2006) argued that several of the proposed conclusions and recommendations are restrictive and premature. It is essential for a suitable environmental risk assessment to include direct and indirect effects on natural enemies, which may not be resolved in a mechanistic decision procedure. Lang et al. (2007) emphasized that laboratory settings with ample food supply and favorable climatic circumstances ensure that experimental animals are in a good condition, provide an advantageous status to cope with exposure to Cry toxins. In worst case scenarios, however, additional stressors such as low temperature, rain, food shortage, or especially parasites and diseases are likely to exacerbate the effect. For example, *N. io* larval populations are regularly reduced by an endemic pathogen (cypovirus 2) and certain parasitoids (e.g., *Sturmia bella* (Meigen), Tachinidae and *Microgaster subcompleta*, Nees, Ichneumonoidea and *Pteromalus puparum* L., Pteromalidae) in the Pannonian Biogeographic Region. These controlling agents may divide a single *N. io* population into different susceptible and tolerant subpopulations, modulating the effect of an additional pathogenic factor such as food containing Cry1Ab toxin (Lang et al. 2007; Lauber 2011).

3.6 Pest Resistance to Cry Toxins

Sublethal effects (that possibly occur with spray applications upon the wash-out effect of natural precipitation or with *Bt* crops with low gene expression levels or

insufficient exposure of the pest) may contribute to the occurrence of Cry resistance or cross-resistance. This phenomenon is attributed to two main factors, sublethal effects caused by the transgenic Cry1Ab toxin on pest sub-populations and Cry toxin composition of the *Bt* crop (namely a single toxin, preactivated Cry1Ab toxin). Sublethal effects may contribute to the occurrence of Cry resistance due to low gene expression levels and thus insufficient exposure of the pest. In laboratory experiments, the model species, Indian meal moth (*Plodia interpunctella* Hübner) was found to develop resistance already in the 10th generation (Darvas 2011), indicating rapid obsoletion of *Bt* maize varieties. What makes this problem even more troublesome is that *P. interpunctella* larvae resistant to *MON 810* show tolerance also to Dipel. Such cross-resistance means that development of resistance to Cry1 toxins, may lead to loss of applicability of both *Bt* crops and conventional *Bt* preparations.

The rapid onset of resistance development is explained by the fact that *MON 810* maize contains a single Cry toxin (preactivated Cry1Ab) only. Microbial *Bt*-bioinsecticides, in contrast, contain several Cry toxins, and therefore, resistance development is more hindered. Although the mode of action of Cry toxins is similar, their pathways do differ from each other at least in the receptor protein in the insect midgut. The severity of the resistance problem is well indicated by the fact that the occurrence of resistance has been an accentuated and critical issue in environmental risk assessment (beside non-target effects and toxin loads on the environment) within the re-registration of the *MON 810* variety group in the European Union (European Food Safety Authority 2009a).

During 2005–2006, field-evolved Cry1 toxin resistance has been documented on three noctuid species: *Spodoptera frugiperda* (J. E. Smith) to Cry1F toxin in Puerto Rico, *Busseola fusca* (Fuller) to Cry1Ab toxin in *Bt* maize in South Africa, and *Helicoverpa zea* (Boddie) to Cry1Ac and Cry2Ab toxins in *Bt* cotton in the Southeastern United States (Tabashnik et al. 2008, 2009a). In 2001, Cry1Ab-resistant individuals of *O. nubilalis* were identified from a field collection from Kandiyohi, Minnesota, based on increased survival at a diagnostic Cry1Ab concentration. The resistant strain exhibited later over 800-fold resistance to Cry1Ab. Resistance was primarily autosomal, and was controlled by more than one locus or multiple alleles at one locus (Crespo et al. 2009).

The resistance management approach, often termed as "high-dose refuge strategy" works best if the dose of the toxin ingested by insects on *Bt* plants is high enough to kill all or nearly all of the aforementioned hybrid progeny (Gould 1998). Meihls et al. (2008) reported rapid resistance development without refuges, and slower or no occurrence of resistance with refuges in the case of *Diabrotica* species. Variety owners suggest the use of isogenic maize (10–20% proportion) in the fields of *Bt* maize to sustain susceptible pest populations. This purpose can be served by the isogenic border line sown in order to avoid pollen drift from the *Bt* maize field.

The refuge theory has several flaws in the practical sense: (*i*) the pest is being "bred" on substantial areas, bringing pest damage to a constant level, which is practically nonsense; (*ii*) in refuge zones where the *MON 810* x non-GM variety hybrids are frequent, different seeds in a cob result in survivorship for Lepidopteran cob pest and help the selection of a Cry1 resistant subpopulation; (*iii*) the developmental time is substantially different for survivor insects in the *MON 810* field and in the

refuge zone (insects may develop twice as slow on *Bt* maize than on the isogenic line). This situation results in a modified time-table for the subsequent lepidopteran generations in the given year, lowering the chance for the summer generations of *O. nubilalis* or *H. armigera* to mate. Susceptible insects could survive in the overwintering population (first generation), but the presently popular stalk crusher technology destroys *O. nubilalis* larvae in the stalks.

Bt maize varieties producing Cry3 toxin were developed against *Diabrotica* species. Expression of the *cry3* gene is usually poor, and in consequence a small portion of the *Diabrotica* spp. larvae may survive. This is an ideal setup for selection of a Cry3 resistant subpopulation. Gassmann et al. (2011) reported field evolved resistance: Western corn rootworm displayed significantly higher survival on Cry3Bb1 maize in Iowa in 2009, *MON 863* maize variety having been commercialized since 2003. No significant correlation was found among populations for survival on Cry34/35Ab1 (*DAS-59122*) and Cry3Bb1 (*MON 853*, *MON 88017*) maize, suggesting a lack of cross-resistance between these Cry3 toxins.

The occurrence of cross-resistance depends on the specificity of the toxin-receptor interaction. At least four Cry receptors have been identified in larvae of diamondback moth (*Plutella xylostella* L.): there are (also) separate receptors for Cry1Aa, Cry1Ba and Cry1Ca toxins, while Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa and Cry1Ja toxins distributively bind to the fourth receptor (Ferré and Van Rie 2002). However, this explains only partially the strong cross-resistance of Cry1C-resistant *P. xylostella* larvae to CrylAb, CrylAc and Cry1F toxins (CrylAc and Cry1F found in WideStrike). Low of mediocre cross-resistance was seen with Cry1Aa and Cry9C toxins (the latter in StarLink). Cross-resistance did not occur with Cry1Bb, CrylJa and Cry2A toxins (the latter in Bollgard II or YieldGard VT Pro) (Liu et al. 2001). Due to various biochemical mechanisms behind resistance, cross-resistance against toxins with different receptors (e.g., Cry1Ac and Cry2Aa) may occur (Jurat-Fuentes et al. 2003).

As a result, stacked event crops expressing several toxins in parallel, appearing in increasing numbers nowadays in registration and cultivation, may limit the problem of Cry resistance, if prudent resistance studies were included in their development. The overall amount of Cry toxins produced by these crops is consequently higher, as the given Cry toxins (i.e., several Cry toxins used in resistance management or *Bt* crop variety groups producing Cry toxins specific to both the corn borer and corn rootworm) each must be produced above sublethal doses.

4 Conclusions

Based on the above, *Bt*-based bioinsecticides and crops cannot be considered by far as equivalent technologies. Their application differs as *Bt* bioinsecticides allow singular applications, while *Bt* crops exert a continuous production of the Cry toxin. This results in higher environmental doses of the plant-expressed toxin(s) than in the case of the *Bt* bioinsecticide. For example a single treatment of Dipel bioinsecticide at the registered dosage (1 kg/ha) contains 4.8–60.2 mg/ha (average 20.6 mg/ha) of

bioavailable Cry1Ab toxin, while the amount of bioaccessible amount of Cry1Ab toxin is 0.085–8.16 g/ha. In contrast, the production of plant-expressed Cry1Ab toxin was found to be 147–456 g Cry1Ab toxin/ha, representing 18–56 treatments with Dipel (on the basis of its maximally detected bioaccessible Cry1Ab toxin content, 8.16 g/ha). The level of plant-expressed Cry1Ab toxin can be further elevated by soil fertilization (2.3–6.8-fold) and the use of long maturation maize varieties (2.5–5.8-fold), representing, in worst case scenarios, in 625–1,930 treatments with Dipel. Moreover, it has to be mentioned that stacked genetic events may further elevate toxin production (twofold). These ratios are even higher if lower bioaccessible Cry1Ab protoxin content biopesticides or bioavailable Cry1Ab toxin contents are considered.

Beside toxin ratios, another characteristic difference is that while *Bt* bioinsecticides are composed of several crystalline toxins, single genetic event *Bt* crops express only a single toxin molecule. This has severe consequences in resistance development, which may be alleviated, yet not eliminated by the use of "pyramid" *Bt* event varieties, expressing several Cry toxins acting on the same insect order, as the evolutionary driving force remain the same. The active ingredient of *Bt* bioinsecticides are bacterial protoxins stabilized in crystalline form and requiring enzymatic activation, while *Bt* plants (e.g., *MON 810*) express a truncated form of the protoxin, so-called preactivated toxin. This has severe consequences in product registration, as the active ingredient toxin in the *Bt* crop is not the registered active substance of the corresponding *Bt* bioinsecticide, and the required toxicology studies have been carried out not with the plant-expressed preactivated toxin, but with the bacterial protoxin or the enzyme-activated active toxin. Moreover, commercial ELISA systems utilizing antibodies against the bacterial protoxin and analytical standards of that protoxin consistently underdetect actual toxin content in *Bt* plants due to their lower cross-reactivities to the plant-expressed preactivated toxin. As a result, all reported results obtained by protoxin-based ELISAs, including manufacturer documentation, are subject to correction. And finally, although *Bt* crops have been widely advocated to be included in integrated pest management (IPM) practices or even in ecological agriculture, *Bt* crops cannot fulfill the main ecological principle of IPM that any protection measures should be timed only to the period(s) when pest damage exceeds the critical level, and therefore, regardless how environmentally mild their active ingredient is, do not comply with IPM.

References

Adamczyk JJ Jr, Adam LC, Hardee DD (2001) Field efficacy and seasonal expression profiles for terminal leaves of single and double *Bacillus thuringiensis* toxin cotton genotypes. J Econ Entomol 94:1589–1593

Anderson PL, Hellmich RL, Prasifka JR, Lewis LC (2005) Effects on fitness and behavior of monarch butterfly larvae exposed to a combination of Cry1Ab-expressing corn anthers and pollen. Environ Entomol 34:944–952

Andow DA, Lövei GL, Arpaia S (2006) Ecological risk assessment for *Bt* crops. Nat Biotechnol 24:749–751

Arvidson H, Dunn PE, Strnad S, Aronson AI (1989) Specificity of *Bacillus thuringiensis* for lepidopteran larvae: factors involved *in vivo* and in the structure of a purified protoxin. Mol Microbiol 3:1533–1543

Bagla P (2010) Hardy cotton-munching pests are latest blow to GM crops. Science 327:1439

Bakonyi G, Szira F, Kiss I, Villányi I, Seres A, Székács A (2006) Preference tests with collembolas on isogenic and *Bt*-maize. Eur J Soil Biol 42:S132–S135

Bakonyi G, Dolezsai A, Mátrai N, Székács A (2011) Long-term effects of *Bt*-maize (*MON 810*) consumption on the Collembolan *Folsomia candida*, over multiple generations: a laboratory study. Insects 2:243–252

Baum JA, Malvar T (1995) Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Mol Microbiol 18:1–12

Baumgarte S, Tebbe CC (2005) Field studies on the environmental fate of the Cry1Ab *Bt*-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. Mol Ecol 14:2539–2551

Bernhard K, Utz R (1993) Production of *Bacillus thuringiensis* insecticides for experimental and commercial uses. In: Entwistle PF, Cory JS, Bailey MJ, Higgs S (eds) *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Wiley, New York, pp 255–267

Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul Toxicol Pharmacol 32:156–173

Bietlot HPL, Vishnubhatla I, Carey PR, Pozsgay M, Kaplan H (1990) Characterization of the cysteine residues and disulphide linkages in the protein crystal of *B. thuringiensis*. J Biochem 267:309–315

Blackwood CB, Buyer JS (2004) Soil microbial communities associated with *Bt* and non-*Bt* corn in three soils. J Environ Qual 33:832–836

Bøhn T, Primicerio R, Hessen DO, Traavik T (2008) Reduced fitness of *Daphnia magna* fed a *Bt*-transgenic maize variety. Arch Environ Contam Toxicol 55:584–592

Bøhn T, Traavik T, Primicerio R (2010) Demographic responses of *Daphnia magna* fed transgenic *Bt*-maize. Ecotoxicology 19:419–430

Bravo A, Soberón M (2008) How to cope with insect resistance to *Bt* toxins? Trends Biotechnol 26:573–579

Bravo A, Gill SS, Soberón M (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49:423–435

Broderick NA, Raffa KF, Handelsman J (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc Natl Acad Sci USA 103:15196–15199

Bruns HA, Abel CA (2003) Nitrogen fertility effects on *Bt* delta-endotoxin and nitrogen concentrations of maize during-early growth. Agron J 95:207–211

Bruns HA, Abel CA (2004) Effects of nitrogen fertility on *Bt* endotoxin levels in *Bt* hybrid maize. In: 4th proceedings of the international crop science congress. Poster 3.8. The Regional Institute Ltd, Gosford, Australia. (http://www.cropscience.org.au/icsc2004/poster/3/8/453_brunsha.htm)

Calsamiglia S, Hernandez B, Hartnell GF, Phipps R (2007) Effects of corn silage derived from a genetically modified variety containing two transgenes on feed intake, milk production, and composition, and the absence of detectable transgenic deoxyribonucleic acid in milk in Holstein dairy cows. J Dairy Sci 90:4718–4723

Cannon RJC (2000) *Bt* transgenic crops: risks and benefits. Integr Pest Manag Rev 5:151–173

Castaldini M, Turrini A, Sbrana C, Benedetti A, Marchionni M, Mocali S, Fabiani A, Landi S, Santomassimo F, Pietrangeli B, Nuti MP, Miclaus N, Giovannetti M (2005) Impact of *Bt* corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. Appl Environ Microbiol 71:6719–6729

Castle LA, Wu G, McElroy D (2006) Agricultural input traits: past, present and future. Curr Opin Biotechol 17:105–112

Chambers CP, Whiles MR, Rosi-Marshall EJ, Tank JL, Royer TV, Griffiths NA, Evans-White MA, Stojak AR (2010) Responses of stream macroinvertebrates to *Bt* maize leaf detritus. Ecol Appl 20:1949–1960

Chen M, Ye G, Liu Z, Fang Q, Hu C, Peng Y, Shelton AM (2009) Analysis of Cry1Ab toxin bioaccumulation in a food chain of *Bt* rice, an herbivore and a predator. Ecotoxicology 18:230–238

Chestukhina GG, Kostina LI, Mikhailova AL, Tyurin SA, Klepikova FS, Stepanov VM (1982) Crystal-forming proteins of *Bacillus thuringiensis*. Arch Microbiol 132:159–162

Choma CT, Surewicz WK, Carey PR, Pozsgay M, Kaplan H (1990) Unusual proteolysis of the protoxin and toxin from *Bacillus thuringiensis*. J Protein Chem 9:87–94

Clark BW, Phillips TA, Coats JR (2005) Environmental fate and effects of *Bacillus thuringiensis* (*Bt*) proteins from transgenic crops: a review. J Agric Food Chem 53:4643–4653

Clements MJ, Campbell KW, Maragos CM, Pilcher C, Headrick JM, Pataky JK, White DG (2003) Influence of Cry1Ab protein and hybrid genotype on fumonisin contamination and *Fusarium* ear rot of corn. Crop Sci 43:1283–1293

Cortet J, Griffiths BS, Bohanec M, Demsar D, Andersen MN, Caul S, Birch ANE, Pernin C, Tabone E, de Vaufleury A, Ke X, Krogh PH (2007) Evaluation of effects of transgenic *Bt* maize on microarthropods in a European multi-site experiment. Pedobiologia 51:207–250

Crespo ALB, Spencer TA, Nekl E, Pusztai-Carey M, Moar WJ, Siegfried BD (2008) Comparison and validation of methods to quantify Cry1Ab toxin from *Bacillus thuringiensis* for standardization of insect bioassays. Appl Environ Microbiol 74:130–135

Crespo ALB, Spencer TA, Alves AP, Hellmich RL, Blankenship EE, Magalhäesa LC, Siegfried BD (2009) On-plant survival and inheritance of resistance to Cry1Ab toxin from *Bacillus thuringiensis* in a field-derived strain of European corn borer, *Ostrinia nubilalis*. Pest Manag Sci 65:1071–1081

Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813

Crickmore N, Zeigler DR, Schnepf E, Van Rie J, Lereclus D, Baum J, Bravo A, Dean DH (2009) *Bacillus thuringiensis* toxin nomenclature. (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/)

Darvas B (2011) GM plants and resistance – resistance-management. In: Darvas B, Székács A (eds) Hungarian background on views of 1st generation genetically modified plants. Agricultural Committee of the Hungarian Parliament, Budapest, Hungary, pp 140–141. (http://www.kormany.hu/download/2/9d/20000/GenetEM.pdf)

Darvas B, Polgár LA (1998) Novel type insecticides: specificity and effects on non-target organisms. In: Ishaaya I, Degheele D (eds) Insecticides with novel modes of action, mechanism and application. Springer, Berlin, pp 188–259

Darvas B, Csóti A, Gharib A, Peregovits L, Ronkay L, Lauber É, Polgár AL (2004) Some data to the risk analysis of *Bt*-corn pollen and protected Lepidoptera species in Hungary. Növényvédelem 40:441–449 (in Hungarian)

Darvas B, Bánáti H, Takács E, Lauber É, Szécsi Á, Székács A (2011) Relationships of *Helicoverpa armigera*, *Ostrinia nubilalis* and *Fusarium verticillioides* on *MON 810* maize. Insects 2:1–11

de Barjac H (1978) Une nouvelle variété de *Bacillus thuringiensis* très toxique pour les moustiques: *Bacillus thuringiensis* subsp. *israelensis* serotype 14. C R Acad Sci Paris D 286:797–800

de Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet 37:409–433

de Vendômois JS, Roullier F, Cellier D, Séralini G-E (2009) A comparison of the effects of three GM corn varieties on mammalian health. Int J Biol Sci 5(7):706–726

de Vendômois JS, CellierD VC, Clair E, Mesnage R, Séralini G-E (2010) Debate on GMOs health risks after statistical findings in regulatory tests. Int J Biol Sci 6:590–598

Dively GP, Rose R, Sears MK, Hellmich RL, Stanley-Horn DE, Calvin DD, Russo JM, Anderson PL (2004) Effects on monarch butterfly larvae (Lepidoptera: Danaidae) after continuous exposure to Cry1Ab-expressing corn during anthesis. Environ Entomol 33:1116–1125

Donkin SS, Velez JC, Totten AK, Stanisiewski EP, Hartnell GF (2003) Effects of feeding silage and grain from glyphosate-tolerant or insect-protected corn hybrids on feed intake, ruminal digestion, and milk production in dairy cattle. J Dairy Sci 86:1780–1788

Doull J, Gaylor D, Greim HA, Lovell DP, Lynch B, Munro IC (2007) Report of an expert panel on the reanalysis by Séralini et al. (2007) of a 90-day study conducted by Monsanto in support of the safety of a genetically modified corn variety (MON 863). Food Chem Toxicol 45:2073–2085

Douville M, Gagné F, Masson L, McKay J, Blaise C (2001) Tracking the source of *Bacillus thuringiensis* Cry1Ab endotoxin in the environment. Biochem Syst Ecol 33:219–232

Ermolli M, Fantozzi A, Marini M, Scotti D, Balla B, Hoffmann S, Querci M, Paoletti C, Van den Eede G (2006a) Food safety: screening tests used to detect and quantify GMO proteins. Accredit Qual Assur 11:55–57

Ermolli M, Prospero A, Balla B, Querci M, Mazzeo A, Van Den Eede G (2006b) Development of an innovative immunoassay for CP4EPSPS and Cry1AB genetically modified protein detection and quantification. Food Addit Contam 23:876–882

European Council (1992) Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. OJ L 206, 22.7.1992, pp 1–7

European Environment Agency (2002) Europe's biodiversity – biogeographical regions and seas. In: Pinborg U, Larsson TJ (eds) European Environment Agency, Copenhagen, Denmark. (http://www.eea.europa.eu/publications/report_2002_0524_154909)

European Food Safety Authority (2004) Opinion of the scientific panel on genetically modified organisms on a request from the Commission related to the notification for the placing on the market of insect protected genetically modified maize MON 863 and MON 863 x MON 810, for import and processing, under Part C of Directive 2001/18/EC from Monsanto. EFSA J 49:1–25

European Food Safety Authority (2005) Opinion of the scientific panel on genetically modified organisms on an application (Reference EFSAGMO-DE-2004-03) for the placing on the market of insect-protected genetically modified maize MON 863 x MON 810, for food and feed use, under Regulation (EC) No 1829/2003 from Monsanto. EFSA J 252:1–23

European Food Safety Authority (2007) Statement of the scientific panel on genetically modified organisms on the analysis of data from a 90-day rat feeding study with MON 863 maize. doi:10.2903/j.efsa.2007.753. (http://www.efsa.europa.eu/en/efsajournal/pub/753.htm)

European Food Safety Authority (2009a) Technical meeting between EFSA GMO panel environmental experts and environmental experts from Member States (May 26, 2009). European Food Safety Authority, Parma, Italy. (http://www.efsa.europa.eu/en/events/event/gmo090526.htm)

European Food Safety Authority (2009b) Applications (EFSA-GMO-RX-MON810) for renewal of authorisation for the continued marketing of (1) existing food and food ingredients produced from genetically modified insect resistant maize MON810; (2) feed consisting of and/or containing maize MON810, including the use of seed for cultivation; and of (3) food and feed additives, and feed materials produced from maize MON810, all under Regulation (EC) No 1829/2003 from Monsanto. EFSA J 1149:1–85

Fantozzi A, Ermolli M, Marini M, Scotti D, Balla B, Querci M, Langrell SR, Van den Eede G (2007) First application of a microsphere-based immunoassay to the detection of genetically modified organisms (GMOs): quantification of Cry1Ab protein in genetically modified maize. J Agric Food Chem 55:1071–1076

Fearing PL, Brown D, Vlachos D, Meghji M, Privalle L (1997) Quantitative analysis of CryIA(b) expression in *Bt* maize plants, tissues, and silage and stability of expression over successive generations. Mol Breed 3(3):169–176

Federici BA, Lüthy P, Ibarra JE (1990) Parasporal body of *Bacillus thuringiensis israelensis*. Structure, protein composition, and toxicity. In: de Barjac H, Sutherland DJ (eds) Bacterial control of mosquitoes and black flies*:* biochemistry, genetics and applications of *Bacillus thuringiensis israelensis*. Rutgers University Press, New Brunswick, pp 16–44

Ferré J, Van Rie J (2002) Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 47:501–533

Folcher L, Jarry M, Weissenberger A, Gérault F, Eychenne N, Delos M, Regnault-Roger C (2009) Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium mycoflora* in maize (*Zea mays* L.) fields. Crop Prot 28:302–308

Folcher L, Delos M, Marengue E, Jarry M, Weissenberger A, Eychenne N, Regnault-Roger C (2010) Lower mycotoxin levels in *Bt* maize grain. Agron Sustain Dev 30:711–719

Füsti Molnár G (2011) Results of the Hungarian variety evaluation of genetically modified varieties awaiting government authorization. In: Darvas B, Székács A (eds) Hungarian background on views of 1st generation genetically modified plants. Agricultural Committee of the Hungarian Parliament, Budapest, Hungary, pp 125–128. (http://www.kormany.hu/download/2/9d/20000/GenetEM.pdf)

Gassmann A, Carrière Y, Tabashnik BE (2009) Fitness costs of insect resistance to *Bacillus thuringiensis*. Annu Rev Entomol 54:147–163

Gassmann AJ, Petzold-Maxwell JL, Keweshan RS, Dunbar MW (2011) Field-evolved resistance to *Bt* maize by western corn rootworm. PLoS One 6(7):e22629. doi:10.1371/journal.pone.0022629

Gelernter W (2004) The rise and fall of *Bacillus thuringiensis tenebrionis*. Phytoparasitica 32:321–324

Gill SS, Cowles EA, Pietrantonio PV (1992) The mode of action of *Bacillus thuringiensis* endotoxins. Annu Rev Entomol 37:615–634

Goldberg LJ, Margalit J (1977) A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univittatus*, *Aedes aegypti* and *Culex pipiens*. Mosq News 37:355–358

Gómez I, Pardo-López L, Munoz-Garay C, Fernandez LE, Pérez C, Sánchez J, Soberón M, Bravo A (2007) Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides 28:169–173

Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726

Graf J (2011) Shifting paradigm on *Bacillus thuringiensis* toxin and a natural model for *Enterococcus faecalis* Septicemia. mBio 2. doi:10.1128/mBio.00161-11

Griffiths BS, Caul S, Thompson J, Birch AN, Scrimgeour C, Cortet J, Foggo A, Hackett CA, Krogh PH (2006) Soil microbial and faunal community responses to *Bt* maize and insecticide in two soils. J Environ Qual 35:734–741

Grothaus GD, Bandla M, Currier T, Giroux R, Jenkins GR, Lipp M, Shan G, Stave JW, Pantella V (2006) Immunoassay as an analytical tool in agricultural biotechnology. J AOAC Int 89: 913–928

Hammock BD, Gee SJ, Harrison RO, Jung F, Goodrow M, Li Q-X, Lucas AD, Székács A, Sundaram KMS (1991) Immunochemical technology in environmental analysis: addressing critical problems. In: Van Emon J, Mumma RO (eds) Immunochemical methods for environmental analysis, vol 442, ACS Symp Ser. American Chemical Society, Washington, DC, pp 112–139

Hammond BG, Dudek R, Lemen JK, Nemeth MA (2006) Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. Food Chem Toxicol 44:1092–1099

Harwood JD, Wallin WG, Obrycki JJ (2005) Uptake of *Bt* endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. Mol Ecol 14:2815–2823

Heszky L (2011a) Scientific problems associated with the cultivation of transgenic (GM) crops. In: Darvas B, Székács A (eds) Hungarian background on views of 1st generation genetically modified plants). Agricultural Committee of the Hungarian Parliament, Budapest, Hungary, pp 130–135. (http://www.kormany.hu/download/2/9d/20000/GenetEM.pdf)

Heszky L (2011b) Coexistence is professionally unacceptable, practically unaccomplishable. In: Darvas B, Székács A (eds) Hungarian background on views of 1st generation genetically modified plants). Agricultural Committee of the Hungarian Parliament, Budapest, Hungary, pp 119–122. (http://www.kormany.hu/download/2/9d/20000/GenetEM.pdf)

Hickle LA, Fitch WL (1990) Analytical chemistry of *Bacillus thuringiensis*. An overview. In: Hickle LA, Fitch WL (eds) Analytical chemistry of *Bacillus thuringiensis*, vol 432, ACS Symp Ser. American Chemical Society, Washington, DC, pp 1–8

Hilbeck A (2001) Implications of transgenic, insecticidal plants for insect and plant biodiversity. Perspect Plant Ecol Evol Syst 4:43–61

Hilbeck A, Schmidt JEU (2006) Another view on *Bt* proteins – How specific are they and what else might they do? Biopesticides Int 2:1–50

Hopkins DW, Gregorich EG (2003) Detection and decay of the *Bt* endotoxin in soil from a field trial with genetically modified maize. Eur J Soil Sci 54:793–800

Huber HE, Luthy P, Rudolf H-R, Cordier J-L (1981) The subunits of the parasporal crystal of *Bacillus thuringiensis*: size linkage and toxicity. Arch Microbiol 129:14–18

Icoz I, Stotzky G (2008a) Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil. Transgenic Res 17:609–620

Icoz I, Stotzky G (2008b) Fate and effects of insect-resistant *Bt* crops in soil ecosystems. Soil Biol Biochem 40:559–586

Icoz I, Saxena D, Andow DA, Zwahlen C, Stotzky G (2008) Microbial populations and enzyme activities in soil *in situ* under transgenic corn expressing Cry proteins from *Bacillus thuringiensis*. J Environ Qual 37:647–662

Icoz I, Andow D, Zwahlen C, Stotzky G (2009) Is the Cry1Ab protein from *Bacillus thuringiensis* (*Bt*) taken up by plants from soils previously planted with *Bt* corn and by carrot from hydroponic culture? Bull Environ Contam Toxicol 83:48–58

Ives AR, Glaum PR, Ziebarth NL, Andow DA (2011) The evolution of resistance to two-toxin pyramid transgenic crops. Ecol Appl 21:503–515

Jesse LCH, Obrycki JJ (2000) Field deposition of *Bt* transgenic corn pollen: lethal effects on the monarch butterfly. Oecologia 125:241–248

Jurat-Fuentes JL, Gould FL, Adang MJ (2003) Dual resistance to *Bacillus thuringiensis* Cry1Ac and Cry2Aa toxins in *Heliothis virescens* suggests multiple mechanisms of resistance. Appl Environ Microbiol 69:5898–5906

Kamota A, Muchaonyerwa P, Mnkeni PNS (2011) Effects of ensiling of *Bacillus thuringiensis* (*Bt*) maize (MON810) on degradation of the crystal 1Ab (Cry1Ab) protein and compositional quality of silage. Afr J Biotechnol 10(76):17484–17489

Kiliç A, Akay MT (2008) A three generation study with genetically modified *Bt* corn in rats: biochemical and histopathological investigation. Food Chem Toxicol 46:1164–1170

Knowles BH (1994) Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. Adv Insect Physiol 24:275–308

Knowles BH, Ellar DJ (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. Biochim Biophys Acta 924:509–518

Laboratories A (1992) *Bt* products manual. Abbott, North Chicago

Lambert B, Buysse L, Decock C, Jansens S, Piens C, Saey B, Seurinck J, van Audenhove K, van Rie J, van Vliet A, Peferoen M (1996) A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. Appl Environ Microbiol 62:80–86

Lang A (2004) Monitoring the impact of *Bt* maize on butterflies in the field: estimation of required sample sizes. Environ Biosafety Res 3:55–66

Lang A, Vojtech E (2006) The effects of pollen consumption of transgenic *Bt* maize on the common swallowtail, *Papilio machaon* L. (Lepidoptera, Papilionidae). Basic Appl Ecol 7:296–306

Lang A, Lauber É, Darvas B (2007) Early tier tests are not sufficient for GMO risk assessment. Nat Biotechnol 25:35–36

Lang A, Brunzel S, Dolek M, Otto M, Theißen B (2011) Modelling in the light of uncertainty of key parameters: a call to exercise caution in field predictions of *Bt*-maize effects. Proc R Soc B 278:980–981

Lauber É (2011) The Cry1-toxin content of MON 810 and the affectivity of its pollen on Hungarian protected lepidopteran species (Supervisor: Darvas B.) PhD dissertation, Corvinus University of Budapest, Budapest, pp 1–102 (in Hungarian)

Lauber É, Peregovits L, Ronkay L, Csóti A, Székács A, Darvas B (2010) Protected lepidopteran larvae and Cry1Ab toxin exposure by *Bt* maize pollen in the Pannonian Region. In: 9th European congress of entomology, programme and book of abstracts. Hungarian Entomological Society, Budapest, Hungary, p 205

Lecadet MM, Frachon E, Dumanoir VC, Ripouteau H, Hamon S, Laurent P, Thiéry I (1999) Updating the H-antigen classification of *Bacillus thuringiensis*. J Appl Microbiol 86:660–672

Lemen JK, Hammond BG, Riordan SG, Jiang C, Nemeth M (2002) 13-Week dietary subchronic comparison study with MON 863 corn in rats preceded by a 1-week baseline food consumption determination with PMI certified rodent diet #5002. Monsanto Co. No: MSL-18175. (http://www.greenpeace.de/fileadmin/gpd/user_upload/themen/gentechnik/Monsanto_Rattenfuetterungsstudie.pdf)

Li J, Koni PA, Ellar DJ (1996) Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J Mol Biol 257:129–152

Li W, Wu K, Wang X, Wang G, Guo Y (2005) Impact of pollen grains from *Bt* transgenic corn on the growth and development of Chinese tussah silkworm, *Antheraea pernyi* (Lepidoptera: Saturniidae). Environ Entomol 34:922–928

Lilley M, Ruffell RN, Somerville HJ (1980) Purification of the insecticidal toxin in crystals of *Bacillus thuringiensis*. J Gen Microbiol 118:1–11

Lisansky SG, Coombs J, Dale T, Frederick R (1997) Biopesticides – markets, technology, registration and IPR companies. CPL Scientific Information Services Ltd., Newbury

Liu Y-B, Tabashnik B, Meyer SK, Crickmore N (2001) Cross-resistance and stability of resistance to *Bacillus thuringiensis* toxin Cry1C in diamondback moth. Appl Environ Microbiol 67: 3216–3219

Losey JE, Rayor LS, Carter ME (1999) Transgenic pollen harms monarch larvae. Nature 399:214

Ma BL, Subedi KD (2005) Development, yield, grain moisture and nitrogen uptake of *Bt* corn hybrids and their conventional near-isolines. Field Crop Res 93:199–211

Margarit E, Reggiardo MI, Vallejos RH, Permingeat HR (2006) Detection of *BT* transgenic maize in foodstuffs. Food Res Int 39:250–255

Mason KL, Stepien TA, Blum JE, Holt JF, Labbe NH, Rush JS, Raffa KF, Handelsman J (2011) From commensal to pathogen: translocation of *Enterococcus faecalis* from the midgut to the hemocoel of *Manduca sexta*. mBio 2. doi:10.1128/mBio.00065-11

Meihls LN, Higdon ML, Siegfried BD, Miller NJ, Sappington TW, Ellersieck MR, Spencer TA, Hibbard BA (2008) Increased survival of western corn rootworm on transgenic corn within three generations of on-plant greenhouse selection. Proc Natl Acad Sci USA 105:19177–19182

Mesnage R, Clair E, Gress S, Then C, Székács A, Séralini G-E (2011) Cytotoxicity on human cells of Cry1Ab and Cry1Ac *Bt* insecticidal toxins alone or with a glyphosate-based herbicide. J Appl Toxicol. doi:10.1002/jat.2712

Messean A, Angevin F, Gómez-Barbero M, Menrad K, Rodríguez-Cerezo E (2006) New case studies on the coexistence of GM and non-GM crops in European agriculture. European Commission Joint Research Centre Institute for Prospective Technological Studies. Technical Report EUR 22102 EN, pp 1–112

Miranda R, Zamudio FZ, Bravo A (2001) Processing of Cry1Ab [delta]-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem Mol Biol 31:1155–1163

Mohan M, Gujar GT (2003) Characterization and comparison of midgut proteases of *Bacillus thuringiensis* susceptible and resistant diamondback moth (Plutellidae: Lepidoptera). J Invertebr Pathol 82:1–11

Monsanto (2010) Monsanto response: de Vendômois et al. 2009 (A comparison of the effects of three GM corn varieties on mammalian health) regarding: MON 863, MON 810 and NK603. Monsanto Scientific Affairs. (http://www.monsanto.com/newsviews/Documents/SpirouxdeVendimois.pdf)

Munkvold GP (2003) Cultural and genetic approaches to managing mycotoxins in maize. Annu Rev Phytopathol 41:99–116

Nguyen TH, Jehle JA (2007) Quantitative analysis of the seasonal and tissue-specific expression of Cry1Ab in transgenic maize *MON 810*. J Plant Dis Prot 114:82–87

Okumura S, Akao T, Mizuki E, Ohba M, Inouye K (2001) Screening of the *Bacillus thuringiensis* Cry1Ac δ-endotoxin on the artificial phospholipid monolayer incorporated with brush border membrane vesicles of *Plutella xylostella* by optical biosensor technology. J Biochem Biophys Methods 47:177–188

Oliveira AP, Pampulha ME, Bennett JP (2008) A two-year field study with transgenic *Bacillus thuringiensis* maize: effects on soil microorganisms. Sci Total Environ 405:351–357

Oppert B (1999) Protease interactions with *Bacillus thuringiensis* insecticidal toxins. Arch Insect Biochem Physiol 42:1–12

Oppert BS, Morgan TD, Kramer KJ (2011) Efficacy of *Bacillus thuringiensis* Cry3Aa protoxin and protease inhibitors toward coleopteran storage pests. Pest Manag Sci 67:568–573

Pagel-Wieder S, Niemeyer J, Fischer WR, Gessler F (2007) Effects of physical and chemical properties of soils on adsorption of the insecticidal protein (Cry1Ab) from *Bacillus thuringiensis* at Cry1Ab protein concentrations relevant for experimental field sites. Soil Biol Biochem 39:3034–3042

Palm CJ, Donegan K, Harris D, Seidler RJ (1994) Quantification in soil of *Bacillus thuringiensis* var. *kurstaki* δ-endotoxin from transgenic plants. Mol Ecol 3:145–151

Papst C, Utz HF, Melchinger AE, Eder J, Magg T, Klein D, Bohn M (2005) Mycotoxins produced by *Fusarium* spp. in isogenic, *Bt vs*. non-*Bt* maize hybrids under European corn borer pressure. Agron J 97:219–224

Perry JN, Devos Y, Arpaia S, Bartsch D, Ehlert C, Gathmann A, Hails RS, Hendriksen NB, Kiss J, Messéan A, Mestdagh S, Neemann G, Nuti M, Sweet JB, Tebbe CC (2012) Estimating the effects of Cry1F *Bt*-maize on non-target Lepidoptera using a mathematical model of exposure. J Appl Ecol 49:29–37

Perry JN, Devos Y, Arpaia S, Bartsch D, Gathmann A, Hails RS, Kiss J, Lheureux K, Manachini B, Mestdagh S, Neemann G, Ortego F, Schiemann J, Sweet JB (2010) A mathematical model of exposure of nontarget Lepidoptera to *Bt*-maize pollen expressing Cry1Ab within Europe. Proc R Soc B 277:1417–1425

Pleasants JM, Hellmich RL, Dively GP, Sears MK, Stanley-Horn DE, Mattila HR, Foster JE, Clark P, Jones GD (2001) Corn pollen deposition on milkweeds in and near cornfields. Proc Natl Acad Sci USA 98:11919–11924

Promdonkoy B, Ellar DJ (2003) Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*. Biochem J 374:255–259

Rauschen S, Schuphan I (2006) Fate of the Cry1Ab protein from *Bt*-maize MON810 silage in biogas production facilities. J Agric Food Chem 54(3):879–883

Ravensberg WJ (2011) Critical factors in the successful commercialization of microbial pest control products. Prog Biol Control 10:295–356

Rodics K, Homoki H, Bakonyi G, Darvas B, Székács A (2011) The hereafter of Hungarian scientific lectures for EFSA GMO Panel (Parma, June 11, 2008). In: Darvas B, Székács A (eds) Hungarian background on views of 1st generation genetically modified plants. Agricultural Committee of the Hungarian Parliament, Budapest, Hungary, pp 155–169. (http://www.kormany.hu/download/2/9d/20000/GenetEM.pdf)

Roh JY, Choi JY, Li MS, Jin BR, Je YH (2007) *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. J Microbiol Biotechnol 17:547–559

Romeis J, Meissle M, Bigler F (2006) Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. Nat Biotechnol 24:63–71

Romeis J, Shelton A, Kennedy GG (eds) (2008) Integration of insect-resistant genetically modified crops within IPM programs. Springer, Dordrecht

Rosi-Marshall EJ, Tank JL, Royer TV, Whiles MR, Evans-White M, Chamber C, Griffiths NA, Pokelsek J, Stephen ML (2007) Toxins in transgenic crop byproducts may affect headwater stream ecosystems. Proc Natl Acad Sci USA 104:16204–16208

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, Siegfried BD, Dively GP (2001) Impact of *Bt* corn pollen on monarch butterfly populations: a risk assessment. Proc Natl Acad Sci USA 98:11937–11942

Séralini G-E (2010) Ces OGM qui changent le monde. Flammarion, Paris

Séralini G-E, Cellier D, de Vendômois JP (2007) New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contam Toxicol 45:2073–2085

Séralini G-E, de Vendômois JS, Cellier D, Sultan C, Buiatti M, GallagherL AM, Dronamraju KR (2009) How subchronic and chronic health effects can be neglected for GMOs, pesticides or chemicals. Int J Biol Sci 5:438–443

Séralini G-E, Mesnage R, Clair E, Gress S, de Vendômois JS, Cellier D (2011) Genetically modified crops safety assessments: present limits and possible improvements. Environ Sci Eur 23(10):1–10

Shao Z, Cui Y, Liu X, Yi H, Ji J, Yu Z (1998) Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. J Invertebr Pathol 72:73–81

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Steinke K, Guertler P, Paul V, Wiedemann S, Ettle T, Albrecht C, Meyer HHD, Spiekers H, Schwarz FJ (2010) Effects of long-term feeding of genetically modified corn (event MON810) on the performance of lactating dairy cows. J Anim Physiol Anim Nutr 94(5):185–193

Storer NP, Babcock JM, Schlenz M, Meade T, Thompson GD, Bing JW, Huckaba RM (2010) Discovery and characterization of field resistance to *Bt* maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J Econ Entomol 103:1031–1038

Székács A, Darvas B (2012) Forty years with glyphosate. In: Hasaneen MNAE-G (ed) Herbicides – properties, synthesis and control of weeds. InTech, Rijeka, Croatia. pp 247–284. (http://www.intechopen.com/articles/show/title/forty-years-with-glyphosate)

Székács A, Juracsek J, Polgár LA, Darvas B (2005) Levels of expressed Cry1Ab toxin in genetically modified corn DK-440-BTY (YieldGard) and stubble. FEBS J 272(Suppl 1):508

Székács A, Lauber É, Juracsek J, Darvas B (2010a) Cry1Ab toxin production of *MON 810* transgenic maize. Environ Toxicol Chem 29:182–190

Székács A, Lauber É, Takács E, Darvas B (2010b) Detection of Cry1Ab toxin in the leaves of *MON 810* transgenic maize. Anal Bioanal Chem 396:2203–2211

Székács A, Weiss G, Quist D, Takács E, Darvas B, Meier M, Swain T, Hilbeck A (2012) Inter-laboratory comparison of Cry1Ab toxin quantification in *MON 810* maize by enzyme-immunoassay. Food Agric Immunol 23:99–121

Tabashnik BE, Gassmann AJ, Crowder DW, Carrière Y (2008) Insect resistance to *Bt* crops: evidence versus theory. Nat Biotechnol 26:199–202

Tabashnik BE, van Rensburg JBJ, Carriére Y (2009a) Field-evolved insect resistance to *Bt* crops: definition, theory, and data. J Econ Entomol 102:2011–2025

Tabashnik BE, Unnithan GC, Masson L, Crowder DW, Li X, Carrière Y (2009b) Asymmetrical cross-resistance between *Bacillus thuringiensis* toxins Cry1Ac and Cry2Ab in pink bollworm. Proc Natl Acad Sci USA 106:11889–11894

Takács E, Fónagy A, Juracsek J, Kugler N, Székács A (2011) Characterisation of tritrophic effects of *DAS-59122-7* maize on seven-spotted ladybird (*Coccinella septempunctata*) feeding on the bird cherry-oat aphid (*Rhopalosiphum padi*). IOBC/WPRS Bull 73:121–134

Tan FX, Wang JW, Feng YJ, Chi GL, Kong HL, Qiu HF, Wei SL (2010) *Bt* corn plants and their straw have no apparent impact on soil microbial communities. Plant Soil 329:349–364

Tank JL, Rosi-Marshall EJ, Royer TV, Whiles MR, Griffiths NA, Frauendorf TC, Treering DJ (2010) Occurrence of maize detritus and a transgenic insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. Proc Natl Acad Sci USA 107:17645–17650

Tapp H, Stotzky G (1998) Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* in soil. Soil Biol Biochem 30:471–476

Turrini A, Sbrana C, Nuti MP, Pietrangeli B, Giovannetti M (2004) Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi. Plant Soil 266:69–75

US National Research Council (2010) The impact of genetically engineered crops on farm sustainability in the United States. National Academies Press, Washington, DC

van de Wiel CCM, Lotz LAP (2006) Coexistence of genetically modified with unmodified crops. NJAS 54(1):17–35

van Frankenhuyzen K (1993) The challenge of *Bacillus thuringiensis*. In: Entwistle PF, Cory JS, Bailey MJ, Higgs S (eds) *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Wiley, Chichester, pp 1–36

van Frankenhuyzen K (2009) Insecticidal activity of *Bacillus thuringiensis* insecticidal proteins. J Invertebr Pathol 101:1–16

Vazquez-Padron RI, de la Riva G, Agüero G, Silva Y, Pham SM, Soberón M, Bravo A, Aïtouche A (2004) Cryptic endotoxic nature of *Bacillus thuringiensis* Cry1Ab insecticidal crystal protein. FEBS Lett 570:30–36

Visser B, Bosch D, Honée G (1993) Domain-function studies of *Bacillus thuringiensis* crystal proteins: a genetic approach. In: Entwistle PF, Cory JS, Bailey MJ, Higgs S (eds) *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. Wiley, New York, pp 71–88

Volpe G, Ammid NH, Moscone D, Occhigrossi L, Palleschi G (2006) Development of an immunomagnetic electrochemical sensor for detection of BT-CRY1AB/CRY1AC proteins in genetically modified corn samples. Anal Lett 39:1599–1609

Winkler VW, Hansen GD, Yoder JM (1971) Immunochemical analysis of parasporal crystal digests of *Bacillus thuringiensis* as an index of insecticidal activity. J Invertebr Pathol 18:378–382

Xie X, Shu Q (2001) Studies on rapid quantitative analysis of *Bt* toxin by using Envirologix kits in transgenic rice. Sci Agric Sinic 34:465–468

Zeilinger AR, Andow DA, Zwahlen C, Stotzky G (2010) Earthworm populations in a northern U.S. cornbelt soil are not affected by long-term cultivation of *Bt* maize expressing Cry1Ab and Cry3Bb1 proteins. Soil Biol Biochem 42:1284–1292

Zhao J-Z, Cao J, Collins HL, Bates SL, Roush RT, Earle ED, Shelton AM (2005) Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. Proc Natl Acad Sci USA 102:8426–8430

Zwahlen C, Hilbeck A, Gugerli P, Nentwig W (2003) Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Mol Ecol 12:765–775