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Review article

A review of the environmental safety of the Cry1Ab protein

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INTRODUCTION

This document provides a comprehensive review of information and data relevant to the environmental risk assessment of Cry1Ab and presents a summary statement about the environmental safety of this protein. All sources of information reviewed herein are publically available and include: dossiers presented to regulatory authorities; decision summaries prepared by regulatory authorities; peer reviewed literature; and product summaries prepared by product developers.

Environmental risk assessments related to the introduction of genetically engineered (GE) plants are conducted on a case-by-case basis taking into account the biology of the plant, the nature of the transgene and the protein it produces, the phenotype conferred by the transgene as well as the intended use of the plant and the environment where it will be introduced (*i.e.*, the receiving environment). These assessments are comparative by necessity, and typically involve comparisons to an untransformed parent line or closely related isoline (CBD, 2000a, 2000b; NRC, 1989; OECD, 1992; EFSA, 2006; Codex, 2003a, 2003b). The point of these comparisons is to identify potential risks the GE plant might present beyond what is already accepted for like plants in the environment. Any identified risks can then be assessed for their potential consequence.

Regulatory approvals for environmental release of GE plants expressing Cry1Ab have been issued in 9 countries as well as the European Union. This includes six transformation events, approved in 17 lines[1], many of which contain additional GE traits (see Tab. 1).

[1] As of 12/19/2011. This includes approvals for conventional crosses of two GE plants in countries where approval is required.

ORIGIN AND FUNCTION OF CRY1AB

Bacillus thuringiensis and the Cry δ endotoxins

Bacillus thuringiensis is a rod-shaped, gram positive bacterium capable of forming long-lived endospores. It is often referred to as a soil bacterium although it is ubiquitous in the environment (Hofte and Whiteley, 1989; Schnepf et al., 1998; OECD, 2007). There is tremendous variation within the species with regard to the type or class of pesticidal proteins that differ in mode of action, target specificity and mechanism of expression (Hofte and Whiteley, 1989; Schnepf et al., 1998; OECD, 2007). Pesticidal proteins expressed by *B. thuringiensis* strains include antifungal compounds, β exotoxins[2], vegetative insecticidal protein (Vip), and the δ endotoxins which include the Cry (crystalline) proteins and the structurally unrelated Cyt (cytolytic) proteins (Hofte and Whiteley 1989, Schnepf et al., 1998; OECD, 2007). Most of these have been shown to contribute to insect toxicity and some (notably β exotoxins and Cyt proteins) have a wide spectrum of activity (Hofte and Whiteley, 1989; Schnepf et al., 1998; OECD, 2007).

Preparations of natural isolates of *B. thuringiensis* were first used as a commercial insecticide in France in 1938 and *B. thuringiensis* subspecies *kurstaki* (which produces Cry1Ab among other Cry proteins) has been registered with US EPA since 1961 (Kumar et al., 1996; Schnepf et al., 1998; USEPA, 2001). Microbial preparations of *B. thuringiensis* are currently approved for use around the world including in Australia, Canada, the European Union, and the United States (AVPMA,

[2] Also called thuringiensin.

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Table 1. Regulatory approvals for the environmental release of GE plants containing Cry1Ab.

Event	OECD Unique Identifier	United States	Canada	Argetina	European Union	Japan	Brazil	Colombia	Philippines	South Africa	Uruguay
Bt176, 1786	SYN-EV176-96	x	x	x	x	x					
BT11; X4334CBr, X4734CBR	SYN-BT011-1	x	x	x		x	x	x	x	x	x
MON80100		x									
MON802		x	x			x					
MON809		x	x			x					
MON810	MON-00810-6	x	x	x	x	x	x		x	x	x
BT11 x MIR162	SYN-BT011-1, SYN-IR162-4	x									
BT11 x MIR162 x MIR604	SYN-BT011-1, SYN-IR162-4, SYN-IR604-5	x									
BT11 x MIR604	SYN-BT011-1, SYN-IR604-5	*	x								
BT11 x MIR604 x GA21	SYN-BT011-1, SYN-IR604-5, MON-00021-9	*	x								
MON810 x LY038	MON-00810-8, REN-00038-3	*				x					
MON810 x MON88017	MON-00810-8, MON-88017-3	*	x			x					
MON810 x MON863	MON-00810-8, MON-00863-5	*				x					
MON810 x MON863 x NK603	MON-00810-8, MON-00863-5, MON-00603-6	*	x			x					
MON810 x NK603	MON-00810-8, MON-00603-6	*	x	x		x	x		x	x	

X indicates a regulatory approval.
* Stacked events that may be considered approved for environmental release based on existing approvals for the GE parent lines from which they are derived. Approvals require periodic renewal by regulation on pesticide registrations. Current status of registrations can be found at: http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm.

2010; EU DG SANCO, 2010; PMRA, 2008; USEPA, 2001). These preparations contain a mixture of microbial pesticides including Cry proteins that interact extensively with each other to influence toxicity and insect specificity (Schnepf et al., 1998; OECD, 2007). Although it may be possible to extrapolate some information about the environmental safety of Cry proteins from experience with these bacterial preparations, it should be kept in mind that the activity of bacterial foliar sprays is due to a combination of multiple δ endotoxins as well as other toxins and qualities of the spore itself that can have an impact on selectivity and host range (Schnepf et al., 1998; Tabashnik et al., 1992). Similarly, the exposure profile for foliar sprays of bacterial preparations differs from expression of Cry proteins in a GE plant (OECD, 2007).

The Cry protein δ endotoxins are so named because they are the primary component of the protein parasporal crystals that are characteristic of spore formation in *B. thuringiensis* (Hofte and Whiteley 1989, Kumar et al., 1996; Schnepf et al., 1998; OECD, 2007). A systematic nomenclature for identifying and differentiating Cry proteins was proposed in 1989 and widely adopted (Hofte and Whiteley, 1989; OECD, 2007). Under this nomenclature, the Cry proteins were grouped into four initial classes I, II, III, and IV based on their toxicity to particular orders of insect. CryI proteins were those toxic to only Lepidoptera, CryII proteins were those toxic to Lepidoptera and Diptera, CryIII proteins were toxic to Coleoptera and CryIV proteins were those toxic to Diptera. This system has been subsequently updated to account for additional Cry proteins and expanding knowledge of their molecular structure and function and relatedness, leading to some minor discrepancies in naming relative to earlier literature (Crickmore et al., 1998, 2005; OECD, 2007). This document uses the most recent nomenclature (Cry1Ab for the protein, *cry1Ab* for the gene) but the protein in question is synonymous with the older nomenclature CryIA(b).

The Cry1 proteins are classified based on amino acid sequence and the proteins designated Cry1A (including Cry1Aa, Cry1Ab and Cry1Ac) are greater than 85% identical in amino acid sequence (Hofte and Whitely, 1989; Crickmore et al., 1998). The crystal structure of Cry1Aa has been determined and shows a high degree of structural similarity to other known Cry protein structures (Cry3A, Cry2A, Cry4A, and Cry4B) despite sequence identities that can fall below 30% (Crickmore

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et al., 1998; Aronson and Shai, 2001; Kumar et al., 1996; OECD, 2007; Bravo et al., 2007).

Mechanism of Cry1Ab Insecticidal activity

Although there is significant variability in amino acid sequence and target range, the general mechanism by which Cry proteins (including Cry1Ab) achieve insecticidal activity is believed to be common across the group (Hofte and Whiteley 1989, Crickmore et al., 1998, 2005; OECD, 2007; Aronson and Shai, 2001; Kumar et al., 1996; Bravo et al., 2007). The Cry1 proteins are produced in the form of protoxins of 130–140 kDa in size containing 1100–1200 amino acid residues (Aronson and Shai, 2001; Kumar et al., 1996; Bravo et al., 2007; OECD, 2007). For Cry1A these protoxins are cleaved to generate active toxins consisting of 60–70 kDa fragments from the N terminal portion of the protein (Knowles, 1994; Kumar et al., 1996; OECD, 2007; Soberon, 2009). There are multiple theories about how these active toxins cause cell death, however there is general agreement that the first step is binding of specific receptors on the plasma membrane of midgut epithelium cells in susceptible insects (Aronson and Shai 2001, Kumar et al., 1996; Bravo et al., 2007, 2011; Ibrahim et al., 2010; OECD, 2007; Soberon et al., 2009; Zhang et al., 2006, 2008). The most popular theory holds that, once bound to receptors, the toxin is able to insert into the plasma membrane through the formation of oligomeric transmembrane pores (Aronson and Shai, 2001; Bravo et al., 2007, 2010; Kumar et al., 1996; OECD, 2007). It is believed that these pores form ion channels that disrupt the transmembrane potential, causing osmotic lysis (Aronson and Shai, 2001; Bravo et al., 2007, 2011; Hofte and Whiteley, 1989; Kumar et al., 1996; OECD, 2007; Soberon et al., 2009). The biochemical process of membrane insertion is not completely understood, but it is thought to involve the binding of additional cell surface receptors which facilitate oligomerization (Bravo et al., 2007, 2011; Soberon et al., 2009). A competing theory, based on work in cell culture, suggests that binding to specific cell surface receptors is followed by exocytosis and the induction of a G-protein mediated signaling cascade which leads to oncotic cell death without oligomerization of Cry proteins or pore formation. (Bravo et al., 2011; Ibrahim et al., 2010; Zhang et al., 2006, 2008). There is evidence that some Cry proteins have multiple receptors, or may bind to multiple sites on a single receptor and it has been demonstrated that receptor binding is necessary but not sufficient for toxicity (Aronson and Shai, 2001; Jenkins et al., 1999; OECD, 2007). There is also some evidence based partly on experiments using sublethal concentrations, that there may be other relevant interactions between Cry proteins and their insect targets (Aronson and Shai, 2001).

EXPRESSION OF CRY1AB IN INSECT RESISTANT GE PLANTS

The level of expression of Cry1Ab in GE plants is determined by several factors related to the types of promoter and terminating sequences and the gene insert site(s). Each transformation event therefore results in a different expression profile. Data for the level of expression of Cry1Ab in GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998, EC 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995a, 1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d, 1997; USEPA, 2001). Tissue types and collection methods differed between studies but all used an enzyme-linked immunosorbent assay (ELISA) or Western blot to quantify the amount of Cry1Ab protein present in a given sample.

Typically, one or more samples of plant tissue were taken at a field trial site and pooled for analysis. The amount of Cry1Ab was normally determined on a dry weight basis then calculated to provide environmentally relevant values relative to the total fresh weight of the sample and represented in a ratio (*e.g.*, micrograms of Cry1Ab protein per gram of fresh weight) (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998, EC 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995a, 1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d, 1997; USEPA, 2001). Samples were usually collected from several tissue types and at multiple growth stages providing data from plants over time and from multiple locations. In most cases the data were presented as a mean value (normally a mean of means as values were averaged within a field trial and across trials as well) and a range (normally also a range of means representing the average expression at a trial site, although this also varied depending on the individual example). In other data sets, means are provided with the standard deviation or the standard error of means (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998, EC 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d, USDA APHIS 1994, 1995a,

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1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d, 1997; USEPA, 2001).

Variations in methodology for sample collection make direct statistical cross-comparisons of the data inappropriate but the weight of evidence suggests that GE plants expressed Cry1Ab at very low levels relative to the total protein available in the plant (see Annex I and references therein). Table 2 includes the highest reported values of expression in Cry1Ab expressing GE plants where data were available. Additional information about expression of Cry1Ab is contained in Annex I.

Table 2. Highest reported protein concentrations of Cry1Ab in GE plant tissue[1].

Transformation	Tissue	Cry1Ab (ng/g fresh weight)
BT 176	Leaf	3029
MON80100	Whole Plant	1770
Bt-11	Leaf	5300
MON 809	Leaf	1630
MON 810	Leaf	10340[2]
MON 802	Leaf	9550

[1] Values are reported as mean unless otherwise noted.
[2] Value represents highest observed value from a sample of 6 where the mean was 9350 ng/g fresh weight.

Modifications to the *cry1Ab* gene and Cry1Ab protein in GE plants

There are two types of modifications to the *cry1Ab* gene from *Bacillus thuringiensis* that are relevant for its use in GE plants (see Tab. 3). The first type involves modifications to the nucleotide sequence which do not alter the amino acid sequence of the protein (USDA APHIS 1994, 1995a, 1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d). These modifications are primarily to increase the translation of the gene either by modifying codon usage to align with plant preferred codons, or through the insertion of plant introns in order to improve the efficiency of translation (Perlak et al., 1991; USDA APHIS, 1994, 1995a, 1995b, 1995c, 1995d, 1996a, 1996b, 1996c, 1996d).

The second type of modification involves changes to the nucleotide sequence which ultimately affect the amino acid sequence of the resulting protein. In the case of GE plants expressing Cry1Ab protein, only truncations of the protein have been submitted for regulatory approvals (ANZFA, 2000b, 2000c; CFIA, 1996a, 1996b; EC, 1997; Japan BCH, 2007a, 2007b, 2007d; USDA, 1994, 1995, 1995d, 1996a). This means that the protein expressed in plants contains a subset of the amino acids in the native, full length protein from *B. thuringiensis*. However, no other changes to the amino acid sequence were reported. These truncated proteins mimic the "activated" form of the Cry1Ab protein, following protease digestion in the insect midgut. They still require binding to a specific receptor or receptors in the insect midgut and they retain the species specificity found in the full length protein (ANZFA, 2000b, 2000c; CFIA, 1996a, 1996b; EC, 1997; Japan BCH, 2007a, 2007b, 2007d; USDA 1994, 1995, 1995d, 1996a).

NON-TARGET ORGANISM (NTO) TESTING AND IMPACTS OF EXPOSURE TO CRY1AB PROTEIN

The Cry1Ab protein belongs to the Cry1 group of proteins which were initially classified based on their specific insecticidal properties against certain lepidopteran insects (Hofte and Whiteley, 1989; Crickmore et al., 1998, 2005; OECD, 2007). The objective of inserting the *cry1Ab* gene into a crop is to provide protection from feeding damage by lepidopteran pests. Other organisms that are not pests in the agricultural system may also be exposed to the Cry1Ab protein, and are considered "non-target organisms" (NTOs). Such exposure could be direct, from deliberate or incidental feeding on crop tissues such as leaves, ears, silks and pollen or decaying leaf material, or be indirect, from feeding on other herbivores that feed on the crop. Although the potential for harm to NTOs has been considered as a part of regulatory risk assessments for GE plants that express Cry1Ab, with special consideration to beneficial NTOs that perform valuable functions as well as threatened endangered and charismatic species, the long history of use for microbial preparations of *Bacillus thruinginesis*, along with early characterization of the Cry1A proteins as being harmless to vertebrate species and having specific activity against only a subset of lepidopterans has guided regulatory requirements in this regard (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Hofte and Whitely, 1989; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; OECD, 2007; Rose et al., 2007; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Typically, potential exposures are considered and used to determine what organisms might be impacted by the pesticide, and then these organisms or representative surrogate species can be tested for adverse effects. The impact of pesticides on NTOs is normally determined using a sequential series of tests termed Tier I, Tier II, Tier III and Tier IV (USEPA, 2007). The exact nature of each tier of testing is dependent on the specific case, but

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Table 3. Summary of modifications to the *cry1Ab* gene of *B. thuringiensis* in GE plants.

Transformation	Nucleotide Sequence Modifications	Amino Acid Sequence Modifications	Protein Equivilancy Data Submitted for Regulatory Review[1]	Reference
BT 176	Modified for codon usage	Truncated[2] (N Terminal 648 Amino Acids)	Molecular Weight Immunoreactivity Protein sequencing Post translational modification Bioactivity Trypsin Resistance	USDA APHIS 1994
MON80100	Modification for codon usage	None: full length, native sequence	Molecular Weight Immunoreactivity Trypsin Resistance	USDA APHIS 1995b
Bt-11	Modification for codon usage, intron insertion	Truncated[2]	Molecular Weight Immunoreactivity Trypsin Resistance Amino acid sequencing Glycosylation Bioactivity	USDA APHIS 1995d
MON 809[3]	Modification for codon usage, intron insertion	None: Full length, native sequence	None reported	USDA APHIS 1996b
MON 810	Modification for codon usage, intron insertion	None: Full length, native sequence	None reported	USDA APHIS 1996b
MON 802	Modification for codon usage, intron insertion	None: Full length, native sequence	None reported	USDA APHIS 1996d

All studies of equivilancy reported are for comparisons to full length Cry1Ab protein originating in *B. thuringiensis* subspecies *kurstaki* and expressed in *E. coli*. For details regarding the specific studies, please see the cited reference.

[2] Truncated indicates that the protein expressed in plants contains a subset of the amino acids in the native, full length protein from *B. thuringiensis*. However, no other changes to the amino acid sequence were reported.

[3] This transformation event also contains a gene fragment insertion of the *cry1Ab* gene which does not produce any detectible protein.

in general the level of realism and complexity of tests rise through the tiers (EFSA, 2006; Romeis et al., 2008; Rose, 2007; USEPA, 2007, 2010). Early tier studies involve highly controlled laboratory environments where NTO or surrogate species are exposed to high concentrations of the pesticide being studied to determine if there are any effects (Romeis et al., 2008; Rose, 2007; USEPA, 2010, 2007). If no effects are observed, additional testing at higher tiers is generally not required (Romeis et al., 2008; Rose, 2007; USEPA, 2010, 2007). If adverse effects are observed in early tier tests or unacceptable uncertainty exists, additional testing will progress as necessary through later tiers as necessary in order to reduce uncertainty to an acceptable level for decision making (EFSA, 2006; Romeis et al., 2008; USEPA, 2010, 2007).

Routes of environmental exposure

Regulatory decisions have generally considered three primary routes of exposure in addition to direct contact with the GE plant expressing the Cry1Ab protein: exposure to pollen containing Cry1Ab and exposure to Cry1Ab deposited in the soil by decomposing plant material, and tritrophic exposure via feeding on herbivores on the GE plant (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Exposure through pollen can occur on the maize or surrounding leaves, but is limited by the generally low expression levels of Cry1Ab in pollen of varieties that have received regulatory approvals (see Annex I for expression level data in pollen of approved varieties) as well as the rapidly decreasing density of pollen deposition with increasing distance from the source plant (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997, USEPA, 2001). Although some biologically significant exposure may occur within a short distance of crop fields, regulatory agencies have generally only requested data for the impacts of Cry1Ab on representative pollinator

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species (*i.e.*, honeybee). Similarly, the specificity of Cry1Ab toxicity to Lepidoptera and evidence suggesting low exposure through soil has led regulators to require testing for only representative soil dwelling arthropod species (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998, EC 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d; USEPA, 2001). Several reports have indicated that Cry proteins from GE plants can bind to clay substrates in soil and that these bound proteins are protected from microbial digestion but retain their insecticidal activity (Koskella and Stotzky, 1997; Crecchio and Stotsky, 1998; OECD, 2007). These studies used very high concentrations of Cry proteins relative to the amount of binding substrate, representing much higher exposure than is likely to occur in an agricultural environment. Subsequent studies under conditions more relevant to agricultural fields have supported earlier conclusions about the degradation of Cry proteins with a half life of approximately 9–40 days (Accinelli et al., 2008; Marchetti et al., 2007). Regulatory approvals of Cry1Ab events have considered information on Cry protein rates of degradation in a range of soil types, but have not required additional soil organism toxicity testing for Cry1Ab (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Potential bitrophic and tritrophic exposures are addressed using ecotoxicological testing.

Ecotoxicological testing of Cry1Ab on non-Lepidopteran NTOs

Cry1Ab is known to be toxic to certain lepidopterans, and NTO testing of purified Cry1Ab has been conducted on a variety of non-lepidopteran species for regulatory submissions related to Cry1Ab producing GE plants (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d; USEPA, 2001). Because the spectrum of activity for Cry1 proteins, and Cry1Ab in particular, has long been known, regulatory analysis has focused on confirmation of this spectrum using well characterized test organisms that are frequently subjected to testing with chemical pesticides (Rose, 2007; ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Test organisms included adult and larval *Apis mellifera* (honeybee), predatory Coleoptera *Hippodamia convergens* (ladybird beetle) and Neuoptera *Chrysoperla carnea* (green lacewing), parasitic Hymenoptera *Brachymeria intermedia*, soil dwelling Collembola (springtail) species *Folsomia candida*, aquatic *Daphnia magna* and soil dwelling earthworms (Rose, 2007; USEPA, 2001). None of these organisms showed a significant response to Cry1Ab at the test concentrations resulting in observations of a No Observed Effects Level (NOEL) at concentrations ranging from 20–200 ppm. This can be compared with worst case scenario exposure estimates based on the highest observed tissue concentrations of Cry1Ab in GE plants ranging from 3–10 ppm (see Tab. 2). Additionally, acute mammalian toxicological testing has been conducted on mouse (*Mus musculus*) (USEPA, 2001). The results of these studies are summarized in Table 4. Many additional studies have been conducted using the Cry1Ab protein and a variety of assays to determine potential effects on a wide number of test organisms, but the subset reviewed here has been widely considered in regulatory analyses (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001).

Ecotoxicological testing of Cry1Ab on the non-target Lepidopteran *Danaus plexippus* L. (Monarch butterfly) and subsequent risk assessment

Cry1 proteins are known to have a toxic effect on certain insects of the order Lepidoptera (Crickmore et al., 1998, 2005; Hofte and Whiteley, 1989; OECD, 2007). Because lepidopterans feeding on the plants engineered to express Cry1 proteins are generally considered pests, studies of non-target organisms have considered impacts to non-pest Lepidoptera that might be exposed incidentally to Cry proteins. Considerable attention has been given to investigations that have centered on the Monarch butterfly (*Danaus plexippus*), a well known and valued charismatic species in North America. A laboratory study reported that pollen from Bt maize expressing Cry1Ab could inhibit growth and cause mortality in Monarch larvae, and that this might have a population effect in the field (Losey et al., 1999). However, this study lacked proper experimentation methods and interpretation and was strongly criticized

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Table 4. Summary of ecotoxicological tests of Cry1Ab on non-lepidopteran non-target organisms reviewed in regulatory decisions.

Species	Method of Exposure	Duration of Exposure	Results
Apis mellifera (honeybee) larvae	Single dose exposure to protein at 20 ppm	single dose	NOEL > 20 ppm
Apis mellifera (honeybee) adult	Single dose exposure at 20 ppm	single dose	No statistically significant difference observed between test and control populations. 16.2% mean mortality occurred in the test group
Chrysoperla carnea (green lacewing) larvae	Exposure at 16.7 ppm	7 days	NOEL > 16.7 ppm
Hippodamia convergens (ladybird beetles)	Single dose exposure at 20 ppm	single dose	NOEL > 20 ppm
Brachymeria intermedia (parasitic hymenoptera)	Single dose exposure at 20 ppm	single dose	NOEL > 20ppm
Folsomia candida (Collembola)	Lyophilized leaf tissue (estimated 50.6 µg Cry1Ab/g)	28 days	NOEL > 50% of the diet
Daphnia magna	Exposure to Cry1Ab in corn pollen at multiple concentrations	48 hours	NOEC > 150 mg/L
Earthworm	Exposure to bacterially derived Cry1Ab protein in an artificial soil substrate	14 days	NOEL > 200 ppm
Mus musculus (mouse)	Acute oral gavage at 3280 mg/kg	single dose	No observed effect

Reported in USEPA 2001.

by the scientific community (Shelton and Sears, 2001). An early field study suggested that pollen deposition on milkweed plants (on which Monarchs feed) in corn fields could reach high enough levels to result in mortality (Jesse and Obrycki, 2000). While it has been long known that Monarch larvae are sensitive to Cry1Ab, the important questions were the concentration of Cry1Ab in pollen from different transformation events and the exposure level to the pollen in the field. Studies (Stanley-Horn et al., 2001; Hellmich et al., 2001) indicated that Bt176 (which has a pollen-specific promoter driving expression) maize pollen caused lethality at low concentrations but Bt 11 and MON 810 pollen showed negligible effects at concentrations up to and exceeding 1000 pollen grains/cm^2. A study of corn pollen deposition on milkweed in and around cornfields determined that less than 1% of milkweed leaves within cornfields during the two weeks of anthesis are expected to have concentrations of pollen greater than 900 grains/cm^2 (OECD, 2007; Pleasants et al., 2001, Hellmich et al., 2001). A risk assessment for Monarch exposure to Cry1Ab corn estimates that realistic exposure levels would lead to 0.05% mortality and "worst case" scenario estimates where all Bt maize planted is assumed to be Bt176 which expresses high levels of Cry1Ab in pollen, at 6.1% mortality[3]. This confirms earlier risk assessments which predicted negligible impacts due to the low exposure of non-target Lepidoptera to pollen or other plant tissue containing Cry1Ab (CFIA, 1996a, 1996b, 1997a, 1997b, 1998; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997).

Field studies of Cry1Ab on non-target organisms

Huge numbers of papers have been published regarding studies of Cry1Ab maize on non-target organisms, and a number of reviews and meta-analyses have analyzed the net results of much of the available literature regarding NTO field and laboratory studies (Romeis et al., 2006; Marvier et al., 2007; Naranjo, 2009; Wolfenbarger et al., 2008; Duan et al., 2008, 2010). A database[4] compiling this

[3] This assessment is not for North America as a whole, but for the state of Iowa, which could be considered a "worst case" state due to the high percentage of cultivated land under maize cultivation, the high use of Bt maize and the high overlap between maize fields and Monarch habitat (Sears et al., 2001).

[4] The Nontarget Effects of Bt Crops Database is maintained by the National Center for Ecological Analysis and Synthesis (NCEAS) http://delphi.nceas.ucsb.edu/btcrops/. Papers must meet the following criteria to be included in the database: (i) involve a field crop species that has been genetically transformed to express one or more *cry* genes derived from *Bacillus thuringiensis*; (ii) measure effects of the transformed crop for one or more groups of non-target invertebrate; (iii) include a comparison to a non-transgenic control or a range of exposure levels to the transgenic plant or plant products (*e.g.*, pollen); and (iv) be written in English.

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information has been created to facilitate continuing study (Marvier et al., 2007; Naranjo, 2009; Wolfenbarger et al., 2008; Duan et al., 2008, 2010). When GE maize plants that express Cry1Ab were compared to control plants that were not treated with chemical insecticide, there was a minor reduction in arthropod abundance, but when control plants are treated with insecticide arthropod abundance is significantly higher in Cry 1Ab maize (Marvier et al., 2007; Naranjo, 2009; Wolfenbarger et al., 2008). Meta-analysis of functional groups in maize expressing Cry1Ab show the overall reduction is due to a reduction in parasitoids when compared to control plants that were not treated with insecticide and this reduction is driven by decreased abundance of a specialist hymenoptera predator for the target arthropod. This reduction can be explained by an absence of prey rather than other effects of the Cry1Ab protein (Wolfenbarger et al., 2008; Naranjo, 2009). This is supported by laboratory studies showing no direct or indirect effects on hymenoptera (Romeis et al., 2006; Wolfenbarger et al., 2008; Naranjo, 2009). Excluding the effects of the specialist predator reveals that there is no significant difference in arthropod numbers between Bt maize and control (Wolfenbarger et al., 2008; Naranjo, 2009). In tri-trophic studies on non-target organisms, reports of harm to non-lepidopteran predators and parasitoids have been attributed to poor host quality effects (Naranjo, 2009). Cry1A resistant prey have been used to avoid these spurious effects (Chen et al., 2008; Li et al., 2009). A recent meta-analysis of field studies differentiating between different taxa of spiders also found no difference between maize expressing Cry1Ab and controls for both total spider species and relative abundance of taxa (Peterson et al., 2011).

ESTABLISHMENT AND PERSISTENCE OF CRY1AB-EXPRESSING PLANTS IN THE ENVIRONMENT

Biology of the plant species

Familiarity with the biology of the non-transformed or host plant species in the receiving environment is typically the starting point for environmental risk assessments of GE plants (OECD, 2006). Information about the biology of the host plant can be used to identify species-specific characteristics that may be affected by the novel trait so as to permit the transgenic plant to become "weedy", invasive of natural habitats, or to be otherwise harmful to the environment. It can also provide details on significant interactions between the plant and other organisms that may be important when considering potential harms. By considering the biology of the host plant, a risk assessor can identify potential hazards that may be associated with the expression of the novel protein (*e.g.*, Cry1Ab) and then be able to assess the likelihood of these hazards being realized. For example, if the plant species is highly domesticated and requires significant human intervention to grow or reproduce, the assessor can take that into account when assessing the likelihood of the GE plant establishing outside of cultivation.

Phenotypic data

Information about the phenotype of GE plants expressing Cry1Ab is collected from laboratory, greenhouse and field trial studies and is presented in regulatory submissions to: (1) identify any intentional changes to the phenotype that might impact the environmental safety of the plant; and (2) to identify any unintended changes to the biology of the plant that might impact environmental safety. Phenotypic data in regulatory submissions and peer reviewed publications have focused on characteristics of the plant that might contribute to its survival or persistence (*i.e.*, potential weediness), or that negatively affect agricultural performance (*e.g.,* disease susceptibility and yield data) (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Because the Cry1Ab protein is intended to provide resistance to target insect pests, this is taken into account when phenotypic observations are made. Some of the collected data are quantitative (*e.g.*, plant height or % seed germination) while other data are qualitative and observational (*e.g.*, no differences in disease susceptibility) (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Statistically significant differences were seen between GE plants expressing Cry1Ab and controls in many cases, but these differences were small and fell within the reported range for maize (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d; USEPA, 2001). Collectively, the phenotypic data showed no pattern of changes that would support the hypothesis that the introduction of Cry1Ab protein had any unintended impact on the gross morphology or

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phenotypic characteristics of plants, besides conferring insect resistance to Lepidoptera pests.

Weediness in agricultural environments

Maize has some potential to "volunteer" in subsequent growing seasons (OECD, 2003; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997). The characteristics that influence the ability of a plant to volunteer are largely the same as those for weediness in general such as seed dormancy, shattering, and competitiveness, and maize possesses very few of them (Baker, 1974; OECD, 2003; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997). There are no data indicating a linkage between Cry1Ab protein expression and any increased survival or overwintering capacity that would alter the prevalence of volunteer maize in subsequent growing seasons ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001). Following-season volunteers expressing Cry1Ab would not be expected to present any management difficulty and can be dealt with in the same manner as conventional volunteers of maize.

Weediness in non-agricultural environments

The primary mechanisms by which Cry1Ab may be introduced into a non-agricultural environment are movement and establishment of the GE plant outside of cultivated areas, and gene flow from the GE plant to a naturalized population or other sexually compatible relatives (Mallory-Smith and Zapiola, 2008). Risk assessments for GE plants expressing Cry1Ab have considered the potential impacts associated with both types of movement (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997; USEPA, 2001).

While all plants can be considered weeds in certain contexts, maize is not considered to be an invasive or aggressive weed outside of agricultural systems. Maize is severely restricted in ability to establish without human intervention (OECD, 2003; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997). Agronomic data show that Cry1Ab does not have a significant impact on traits associated with weediness (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d; USEPA, 2001). Although release from natural control factors (including insect herbivores) has been offered as a partial explanation for the success of invasive species (Blumenthal, 2005; Keane and Crawley, 2002; Mason et al., 2003; Mack, 1996) most regulatory decisions have agreed that it is unlikely that the addition of resistance to lepidopteran pests would allow maize expressing Cry1Ab to become invasive of non-agricultural environments (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d; USEPA, 2001).

Movement of the transgene to sexually compatible relatives

The movement of transgenes from a GE plant to its wild relatives is pollen mediated and the production of reproductively viable hybrids depends on the physical and temporal proximity of the GE plants to sexually compatible species. Maize does not have relatives that are considered invasive of ecosystems or broadly distributed, agriculturally important weeds for which hybridization is a concern (OECD, 2003). Maize freely hybridizes with wild teosintes, but gene introgression is thought to be limited (OECD, 2003; Serratos et al., 1995, Baltazar et al., 2005). Wild teosinte populations are limited to Mexico, Guatemala and a single population in Nicaragua and while teosinte is considered a serious weed by some farmers in Mexico, it is treated as a beneficial by others (Serratos et al., 1995).

COMPOSITIONAL ANALYSIS OF CRY1AB PLANTS

Detailed compositional analysis is a scientifically rigorous component of the characterization of GE plants and is a regulatory requirement for GE food and feed safety approvals (OECD, 1992; WHO, 1995; FAO/WHO, 1996; EFSA, 2006; Codex, 2003a, 2003b). The choice of analyses conducted depends on the nature of the product and its intended uses. Insect resistant GE crops expressing Cry1Ab have typically undergone proximate analysis (crude protein, crude fat, fiber, moisture and ash) (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d). Detailed analyses of fatty acid and amino acid composition have also been conducted, as well as analyses of important secondary metabolites that have toxic or anti-nutritional

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properties (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d). The data collected can be useful as indicators of unintended changes to the transformed plant (Nickson and McKee, 2002; Codex, 2003a, 2003b).

Data from publicly available compositional analyses are summarized in Annex II. Although some statistically significant compositional differences were observed the composition of GE plants expressing Cry1Ab was found to fall within the normal range observed in the crop species (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998, EC 1997, 1998; USDA APHIS, 1994, 1995b, 1995d, 1996b, 1996d). Subsequent regulatory analyses did not consider these differences to be meaningful in the context of environmental safety (ANZFA, 2000a, 2000b, 2000c; CFIA, 1996a, 1996b, 1997a, 1997b, 1998; EC, 1997, 1998; Japan BCH, 2004a, 2004b, 2004c, 2004d, 2005a, 2005b, 2006, 2007a, 2007b, 2007c, 2007d; USDA APHIS, 1995a, 1995c, 1996a, 1996c, 1997).

Considering data across approved events, there have been no patterns of consistent or reliable changes in proximate composition in plants expressing Cry1Ab. This indicates that the expression of Cry1Ab does not have any biologically significant effect on the gross metabolism of the transformed plants.

CONCLUSION

The Cry1Ab protein expressed in insect resistant GE plants is derived from the common soil bacterium *Bacillus thuringiensis* and is specifically toxic to Lepidoptera. Toxicity testing with a range of representative non-target organisms (NTOs) produced NOEL values at concentrations representing ten-fold or higher the expected environmental concentrations of Cry1Ab. Meta analyses of field studies suggest that cultivation of GE maize plants expressing Cry1Ab does not affect the abundance of non-target arthropods, with the exception of specialist predators of the target pest. Cry1Ab in plants can be toxic to non-target Lepidoptera, but regulatory risk assessments for approved products have concluded that the low likelihood of exposure results in negligible additional risk compared to other agricultural practices. The weight of evidence from analyses of phenotypic and compositional data demonstrates that Cry1Ab expression in approved maize events did not alter the gross physiology of the plant, and that these plants are not more likely to become weedy or invasive than their conventional counterparts.

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ANNEX I: SUMMARY OF CRY1AB PROTEIN EXPRESSION DATA

The tables that follow present summary data from peer-reviewed publications and regulatory submissions. The data is presented in the format in which it is available in the cited document in order to facilitate cross-referencing. Additional information on collection and sampling methodologies can be found in the referenced sources.

Table I.1. Cry1Ab protein levels in Bt176 corn and hybrid lines during development (ELISA) (USDA, 1994; CFIA, 1996; ANZFA, 2000b).

	Stage of Development µg/g Dry Weight (n)			
	Seedling	**Anthesis**	**Seed Maturity**	**Senescence**
Leaves				
Bt176[1]	10.5 (3; 8.57–13.09)	3.04 (3; 2.84–3.43)	1.43(3; 0.46–2.70)	0.10 (2; 0.09–0.10)
176 x 554[2]	4.78 (5; 2.41–5.95)	2.70 (3; 2.22–3.36)	1.65 (3; 0.95–2.90)	0.12 (3; 0.08–0.19)
176 x 564[3]	7.56 (3; 2.56–11.28)	13.37 (2; 7.20–19.54)	1.52 (2; 1.25–1.78)	0.30 (3; 0.06–0.50)
Whole Plant				
Bt176[1]	4.19 (3; 2.45–7.45)	1.44 (5; 0.93–1.94)	0.29 (4; 0.10–0.49)	<0.02 (5)
176 x 554[2]	2.85 (6; 0.75–7.33)	0.20 (3; 0.05–0.44)	0.15 (4; 0.09–0.25)	<0.02 (4)
176 x 564[3]	3.40 (2 3.27–3.52)	0.74 (3; 0.52–0.86)	0.26 (4; 0.17–0.33)	<0.02 (3)
Kernels				
Bt176[1]			<0.01 (4)	<0.01 (5)
176 x 554[2]			<0.01 (3)	<0.01 (3)
176 x 564[3]			<0.01 (2)	<0.01 (3)
Pollen[4]				
Bt176[1]		4.32 (4; 3.70–5.58)		
176 x 554[2]		2.34 (3; 1.77–3.15)		
176 x 564[3]		5.01 (3; 4.76–5.21)		
Roots				
Bt176[1]	<0.1 (2)	<0.04 (4)	<0.04 (4)	na
176 x 554[2]	<0.1 (6)	<0.04 (3)	<0.04 (3)	na
176 x 564[3]	<0.1 (1)	<0.04 (3)	<0.04 (2)	na
Pith				
Bt176[1]	na	<0.07 (4)	<0.04 (4)	na
176 x 554[2]	na	<0.07 (3)	<0.04 (3)	na
176 x 564[3]	na	<0.07 (3)	<0.04 (2)	na

[1] Genotype Bt176 refers to CG00526-176 which is homozygous for the *cry1Ab* gene.
[2] Genotype 176 x 554 refers to the hybrid corn line developed by the cross of CG00526-176 and line CG00554 and is hemizygous for *cry1Ab*.
[3] Genotype 176 x 564 refers to the hybrid corn line developed by the cross of CG00526-176 and line CG00554 and is hemizygous for *cry1Ab*.
[4] Pollen values were determined on dry pollen samples and extrapolated to fresh weight.

Table I.2. Cry1Ab levels in Bt-176 corn hybrids (µg/g total protein as detected by ELISA)[1] (ANZFA, 2000b).

Line	Kernels at Seed Maturity	Whole Plants at Seed Maturity	Whole Plants at Anthesis
Bt 176	<0.093	3.63	14.40
Bt 176 x 554	<0.093	2.14	2.50
Bt176 x 564	<0.103	3.71	7.40

[1] Values were derived by calculation from values in Table 1.
[2] Below the limit of quantification.

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Table I.3. Cry1Ab protein levels in Bt-176 on a fresh weight basis during Bt maize development, summer 1993 (ELISA) (USDA, 1994).

Tissue	Genotype, Maize Line	Mean ng Cry1Ab/g Fresh Weight (N; range)			
		Seedling[1]	Anthesis	Seed Maturity	Senescence
Leaves	+/+ CG00526–176	1159 (3; 892–1506)	735 (3; 657–793)	465 (3; 158–922)	66 (2; 55–77)
	+/– CG00554 x CG00526–176	596 (5; 308–738)	530 (3; 449–614)	471 (3; 266–765)	88 (3; 57–141)
	+/– CG00564 x CG00526–176	839 (3; 285–1253)	3029 (2; 1631–4427)	442 (2; 365–520)	225 (3; 47–379)
Roots	+/+ CG00526–176	<8 (2)	<8 (4)	<8 (4)	na
	+/– CG00554 x CG00526–176	<8 (6)	<8 (3)	<8 (3)	na
	+/– CG00564 x CG00526–176	<8 (1)	<8 (3)	<8 (2)	na
Pith	+/+ CG00526–176	na	<8 (4)	<8 (4)	na
	+/– CG00554 x CG00526–176	na	<8 (3)	<8 (3)	na
	+/– CG00564 x CG00526–176	na	<8 (3)	<8 (2)	na
Pollen[2]	+/+ CG00526–176		2021 (4; 1732–2611)	<5 (4)	
	+/– CG00554 x CG00526–176		1137 (3; 828–1474)	<5 (3	
	+/– CG00564 x CG00526–176		2348 (3; 2226–2438)	<5 (2)	
Kernels	+/+ CG00526–176				<5 (5)
	+/– CG00554 x CG00526–176				<5 (3)
	+/– CG00564 x CG00526–176				<5 (3)
Whole Plant	+/+ CG00526–176	315 (3; 191–532)	182 (5; 159–213)	73 (4; 50–107)	<5 (5)
	+/– CG00554 x CG00526–176	230 (6; 81–556)	44 (3; 14–88)	41 (4; 21–68)	<5 (4)
	+/– CG00564 x CG00526–176	316 (2; 305–328)	144 (3; 102–167)	71 (4; 48–92)	<5 (3)

All samples were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng Cry1Ab/g fresh weight. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue. Plants that were homozygous or Hemizygous for the transgenes were designated "+/+" or "+/–" respectively. na = not analyzed. Blank space means tissue is not available at this developmental stage.
[1] Seedlings were greenhouse grown and analyzed three weeks after planting; all other stages were field-grown.
[2] Values were determined on dried pollen samples and extrapolated to fresh wt. by multiplying µg Cry1Ab/g dry weight pollen by 0.486 (g dry weight pollen/g fresh weight pollen).

Table I.4. Summary of specific protein levels measured in MON 80100 tissues[1] (USDA, 1995b).

Corn Line	Leaf	Grain	Whole Plant[3]	Pollen[3]
MON 80100	1.3	0.57	1.77	N.D.[2]

[1] Values are means calculated across five sites from mean values calculated from the analysis of 3–4 replicate samples per site and are expressed as µg/g fresh weight.
[2] Not detected.
[3] Values are means calculated from four replicate samples from one site.
[4] Determination from duplicate analysis of one pollen sample from one site.

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Table I.5. Specific concentration of Cry1Ab protein in Bt11 transgenic corn tissues during the life cycle[1] (USDA, 1995d).

	Days Post Planting									
Tissue	**5**	**10**	**15**	**20**	**25**	**30**	**37**	**59**	**84**	**119**
Cotyledon	20.5 (0.4)	36 (1.7)								
Roots	22.1 (1.3)	11.7 (0.8)					37 (7)	12 (3.4)	18.2 (4)	2.2 (1.2)
2nd Leaf		106 (4.7)	27.9 (3)	22.4 (0.9)	125 (5)	38 (1.3)	55.6 (4)			
5th Leaf				45.7 (2)	168 (5)	34 (1.3)	54 (3.3)	16.7 (1.2)		
10th Leaf							102 (6)	30 (1.5)	9.4 (1)	
15th Leaf								37.9 (2.2)	10.2 (1.1)	
Stalk Epidermis							36 (3.3)	10.4 (2.6)	12.6 (3.4)	9.0 (2.2)
Stalk Pth							27 (4)	19.2 (3.1)	18.0 (4.8)	8.8 (2.0)
Tassel								8.0 (1.4)	8.8 (2.0)	6.8 (4.2)
Pollen								1.25 (.75)		
Silk								2.4 (0.6)	6.6 (1.8)	5.2 (3.8)
Ear Shank								13.6 (2.3)	27.2 (8.8)	5.2 (1.4)
Husk								24.8 (2.9)	15.4 (5.3)	2.6 (2.6)
Cob								13.0 (3.0)	26.6 (6.4)	16.2 (3.3)
Brace Root								3.2 (1.2)	7.0 (2.1)	4.8 (2.1)
Kernel									8.2 (2.5)	0.4 (0.4)

[1] Values are ng Cry1Ab/mg plant protein and are not corrected for actual extraction efficiency (Standard Error of Mean). Plants were grown in the greenhouse and five replicate plants were extracted for each destructive sample time.

Table I.6. Cry1Ab Protein in tissues of Bt 11 corn plants at R6 stage (USDA, 1995d).

Tissue	**Cry1Ab ug/g Fresh Weight**
Leaves[1]	3.26
Stalk[2]	0.14
Grain	1.40

[1] Leaves include: leaf, leaf shank, and husk.
[2] Stalk includes: stalk, ear shank, tassel, cob, roots, and silk.

Table I.7. Specific concentration of the Cry1Ab protein in Bt-11 deet corn tissues during the life cycle of plants grown in the greenhouse (ELISA)1 (ANZFA, 2000c).

	ng Cry1Ab/mg Plant Protein ± SE				
Tissue	10[2]	25[2]	59[2]	84[2]	119[2]
Roots	11.7 ± 1.7		12 ± 3.4	18.2 ± 4	2.2 ± 1.2
2nd Leaf	106 ± 4.7	125 ± 5			
15th Leaf			37.9 ± 2.2	10.2 ± 1.1	
Pollen			1.25 ± 0.8		
Kernel				8.2 ± 2.5	0.4 ± 0.4

[1] Values are means of samples from 5 replicate plants (n = 5). Data points that are not available at certain developmental stage are left blank.
[2] Days post planting.

Table I.8. Mean levels of the Cry1Ab protein as detected by ELISA (ANZFA, 2000c).

	Mean Levels in Leaf and Kernel (µg/g Fresh Weight)[1]			
	Leaf	**Kernel**	**Husk**	**Stalk**
X4334-CBR	4.3 ± 0.66	1.5 ± 0.21	1.1 ± 0.26	0.71 ± 0.11
X4734-CBR	5.05 ± 0.35	1.30 ± 0.28	0.84 ± 0.18	0.55 ± 0.06
X6534-CBR	5.30 ± 0.90	1.50 ± 0.04	0.79 ± 0.03	0.64 ± 0.04
X7634-CBR	5.24 ± 0.78	1.60 ± 0.13	1.04 ± 0.23	0.53 ± 0.06
Control NK7514	0	0	0	0

[1] n = 4

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Table I.9. Cry1Ab protein levels in tissues from Bt-11 sweet corn hybrids (ELISA)[1] (ANZFA, 2000c).

	Cry1Ab Levels in Bt-11 Tissue (μg/g Fresh Weight)			
	Leaves		Kernel	
	Mean	Range	Mean	Range
Control[2]	0		0	
Hybrid 0943	4.53	3.87–5.18	3.17	2.54–3.80
Hybrid 0937	3.10	2360–3.86	1.59	1.41–1.80
Hybrid 0941	3.31	2.66–3.92	0.78	0.51–1.08

[1] Values are μg/g fresh weight. n = 3 except for hybrid 0943 where n = 2.
[2] Control plant varieties are Jubilee, Bonus and Empire. Control plants had ELISA values corresponding to 0 ng Cry1Ab/g fresh weight.

Table I.10. Cry protein tissue expression in Bt11[1] (USEPA, 2001).

Active Ingredient	Leaf	Root	Pollen	Seed
Cry1Ab – Bt11 (006444)	3.3 ng/mg	2.2–37.0 ng/mg protein	< 90 ng Cry1Ab/g dry weight pollen	1.4 ng/mg (kernel)

[1] Values indicate fresh tissue weight unless otherwise noted.

Table I.11. Summary of specific protein levels measured in tissues of YieldGard corn line MON 809 (μg/g fresh weight)[1] (USDA, 1996b; CFIA, 1997a).

Corn Line	Leaf	Grain	Whole Plant[2,3]	Pollen[2]
MON 809	1.63	0.55	1.23	N.D.4

[1] Values are means calculated from the analyses of six plant samples, one from each of six field sites, unless noted otherwise.
[2] The mean was calculated from the analyses of plant sample(s) from one site.
[3] Values are means calculated from the analyses of two replicate plant samples from one site.
[4] Not detected.

Table I.12. Protein expression levels in the insect-protected corn lines as determined by ELISA analysis (ANZFA, 2000a; USDA, 1996b; CFIA, 1997b).

	Mean Expression Levels and Ranges (μg/g Fresh Weight)[1]							
	Leaf		Grain		Whole Plant[2]		Pollen[3]	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Cry1Ab	9.35	7.93–10.34	0.31	0.19–0.39	4.15	3.65–4.65	0.09	na

[1] Values are means from six plant samples (n = 6). One plant is taken from each site unless otherwise noted.
[2] Values are means from sample(s) from replicate plant samples.
[3] Values are means from samples(s) from one site only (n = 6).
na = not assayed.

Table I.13. Cry protein tissue expression[1] (USEPA, 2001).

Active Ingredient	Leaf	Pollen	Seed	Whole Plant
Cry1Ab – MON810 (006430)[2]	10.34 ng/mg	< 90 ng Cry1Ab/g dry weight pollen	0.19–0.39 ng/mg (grain)	4.65 ng/mg

[1] Values indicate fresh tissue weight unless otherwise noted.
[2] 1994 field data.

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Table I.14. Summary of specific protein levels measured in tissues of MON 802 corn plants (USDA, 1996d; CFIA, 1998).

Corn Line	Leaf[1]	Grain[1]	Whole Plant[2]
MON 802	9.55	3.20	1.35

[1] Values are means calculated across six sites.
[2] Values are means calculated from the analysis of two replicate plant samples from one site.

ANNEX II: SUMMARY OF COMPOSITIONAL ANALYSES OF GE PLANTS EXPRESSING CRY1AB.

The tables that follow present summary data from peer-reviewed publications and regulatory submissions. The data is presented in the format in which it is available in the cited document in order to facilitate cross-referencing. Additional information on collection and sampling methodologies can be found in the referenced sources.

Table II.1. Proximate analyses of kernels from control and Bt maize (Bt 176) (USDA, 1994; ANZFA, 2000b).

	Days Post Planting						
Genotype[1]	N[2]	Ash	Fiber	Fat	Moisture[3]	Protein	Starch
526	5	1.44 ± 0.03 (1.38–1.46)	1.95 ± 0.08 (1.85–2.05)	4.74 ± 0.8 (3.84–5.65)	11.94 ± 0.44 (11.28–12.49)	12.21 ± 0.43 (11.60–12.74)	65.85 ± 4.17 (60.86–72.08)
526–176	5	1.41 ± 0.04 (1.35–1.45)	1.86 ± 0.13 (1.69–2.03)	4.07 ± 0.80 (3.30–5.38)	12.38 ± 0.34 (12.02–12.82)	11.71 ± 0.35 (11.23–12.05)	65.95 ± 0.88 (68.69–70.84)
554x526	5	1.30 ± 0.05 (1.26–1.36)	1.50 ± 0.13 (1.30–1.65)	2.55 ± 1.14 (0.95–3.90)	9.64 ± 0.40 (9.29–10.27)	11.96 ± 0.35 (11.43–12.33)	68.29 ± 10.06 (57.28–79.51)
554x526–176	5	1.27 ± 0.03 (1.22–1.30)	1.41 ± 0.12 (1.20–1.50)	4.21 ± 0.794 (3.35–5.28)	12.23 ± 0.304 (11.98–12.72)	10.88 ± 0.174 (10.70–11.11)	72.19 ± 2.56 (68.25–74.64)
637x526	5	1.63 ± 0.25 (1.26–1.90)	1.97 ± 0.10 (1.90–2.14)	4.07 ± 1.12 (2.64–5.08)	12.17 ± 0.49 (11.55–12.72)	12.13 ± 0.48 (11.53–12.72)	66.84 ± 2.97 (63.88–71.09)
637x526–176	5	1.68 ± 0.23 (1.31–1.90)	1.77 ± 0.32 (1.20–2.00)	3.49 ± 1.62 (1.01–5.54)	10.24 ± 1.88 (7.87–11.97)	13.62 ± 0.4 (13.14–14.14)	68.85 ± 2.29 (66.81–72.06)
684x526	5	1.73 ± 0.16 (1.45–1.84)	1.56 ± 0.38 (0.90–1.80)	3.66 ± 0.96 (2.35–5.04)	12.14 ± 0.28 (11.93–12.46)	12.85 ± 0.39 (12.51–13.48)	58.23 ± 7.19 (50.37–67.51)
684x526–176	5	1.63 ± 0.16 (1.36–1.74)	1.61 ± 0.16 (1.45–1.80)	2.04 ± 0.60 (1.45–2.89)	9.01 ± 1.274 (6.97–10.30)	13.32 ± 0.37 (12.85–13.87)	68.07 ± 3.014 (64.54–71.82)
615	2	1.73 ± 0.21 (1.58–1.87)	1.84 ± 0.08 (1.78–1.90)	4.67 ± 0.59 (4.25–5.08)	10.82 ± 0.26 (10.63–11.00)	10.07 ± 0.15 (9.96–10.17)	63.16 ± 0.93 (62.50–63.82)
615–176	2	1.82 ± 0.01 (1.81–1.83)	1.70 ± 0.22 (1.54–1.85)	4.34 ± 0.13 (4.24–4.43)	12.38 ± 0.04 (12.35–12.41)	11.79 ± 0.074 (11.74–11.84)	59.14 ± 0.98 (58.45–59.83)
635x615	2	1.93 ± 0.08 (1.87–1.99)	1.74 ± 0.06 (1.69–1.78)	4.14 ± 0.10 (4.07–4.21)	13.22 ± 0.27 (13.03–13.41)	11.17 ± 0.62 (10.73–11.60)	61.51 ± 0.75 (60.96–62.04)
635x615–176	2	1.81 ± 0.01 (1.80–1.82	1.92 ± 0.23 (1.75–2.08)	4.05 ± 0.21 (3.90–4.19)	12.06 ± 0.10 (11.99–12.13)	11.38 ± 0.33 (11.14–11.61)	61.04 ± 1.82 (59.75–62.32)

[1] Abbreviations: 526 = CG00526 inbred; 554 x 526 = CG00554 x CG00526 hybrid, *etc*. CG numbers designate proprietary seed lines. The suffix "–176" indicates the transgenic Bt line/hybrid.
[2] Number of replicate samples analyzed from a pooled sample of kernels representing multiple plants. Where N = 5, samples were analyzed once. Where N = 2, each sample was analyzed twice.
[3] Moisture is given as a percentage of sample weight (prior to drying).
[4] Indicates significantly different from the corresponding control mean ($p \leq 0.05$).

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Table II.2. Compositional analysis of kernels from inbred CG00526–176 corn[1] (Bt 176) (ANZFA, 2000b).

	Control CG00526		Bt CG00526–176		
Component	**Mean ± Standard Deviation**	**Range**	**Mean ± Standard Deviation**	**Range**	**Literature Range[2](%)**
Protein %	12.21 ± 0.43	11.60–12.74	11.71 ± 0.35	11.23–12.05	6–12
Total Fat %	4.74 ± 0.80	3.84–5.65	4.07 ± 0.80	3.30–5.38	3.1–5.7
Ash %	1.44 ± 0.03	1.38–1.46	1.41 ± 0.04	1.35–1.45	1.1–3.9
Starch %	65.85 ± 4.17	60.86–72.08	69.05 ± 0.88	568.69–70.84	65.3–83
Fibre %	1.95 ± 0.08	1.85–2.05	1.86 ± 0.13	1.69–2.03	2.5[3]
Moisture	11.94 ± 0.44		12.38 ± 0.34		7–23

[1] Values are means from six plant samples (n = 6). One plant is taken from each site unless otherwise noted.
[2] Values are means from sample(s) from replicate plant samples.
[3] Values are means from samples(s) from one site only (n = 6).
na = not assayed.

Table II.3. Compositional analysis of kernels from inbred CG00526–176 corn[1] (Bt 176) (ANZFA, 2000b).

Component	MON 80100	MON 80080
Protein	13.12	12.0
Fat	4.0	4.0
Ash	1.6	1.6
Fiber	2.3	2.2
Carbohydrates	81.32	82.2
Moisture	14.8	14.6

[1] Analyses of data from seed samples from 5 sites.
[2] Values are significantly different at the 95% confidence level from the MON 80080 control line.

Table II.4. Summary of compositional analysis for Bt-11 and control corn plants[1] (ANZFA, 2000c).

	Inbred Line H8540-Bt	Isogenic Control H8540	Hybrid Bt+/Bt-	Control Hybrid	Normal Range[2]
Total Nitrogen[3]	13.18 ± 0.07	12.35 ± 0.06	12.28 ± 0.03	12.30 ± 0.0	7.7–10[4]
Moisture	12.3	12.6	12.6	13.3	7–23
Ash	1.47 ± 0.04	1.79 ± 0.007	1.70 ± 0.02	1.6 ± 0.02	1.1–3.9
Starch	68.02 ± 0.4	67.57 ± 0.4	70.83 ± 0.81	70.25 ± 0.48	61–78
Cellulose	2.99 ± 0.007	2.9 ± 0.05	2.67 ± 0.28	2.92 ± 0.05	3.3–4.3 1.93–2.5[4]
Xanthophylls	24.2	21.0	21.6	19.1	19.2–33.1[4]

[1] Samples are 500g of kernels from: Bt+/Bt+ H8540 ears n = 54, Control H8540 n = 56, Bt+/Bt- hybrid n = 50, Control hybrid ears n = 45. Each data point represents the mean of two replicate analyses made with the 500g sample. Data from AGPM. All data except moisture (% H_2O) and xanthophyll (mg/kg dry weight basis) are presented on a % dry weight basis.
[2] Wright, 1987 in Corn chemistry and technology, 1987, Watson S. A and Ramstad P.E. (eds), American Association of Cereal Chemists, St. Paul, Minesota, USA.
[3] All values from control and genetically modified lines are significantly different to range.
[4] Data from AGPM.

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Table II.5. Summary of compositional analysis for Bt-11 and control corn plants[1] (ANZFA, 2000c).

	X6534CBR	Isogenic Control X6514	X7634CBR	Isogenic Control X7514	Normal Range
Protein	9.89 (9.40–10.60)	9.96 (9.10–11.40)	10.55 (10.24–11.00)	9.68 (8.90–10.94)	6–12
Oil	4.09 (4.004.16)	4.11 (4.10–4.13)	4.02 (4.00–4.02)	4.07 (3.80–4.31)	3.1–5.7
Starch	70.09 (68.80–71.07	70.19 (67.80–71.50)	69.32 (68.60–70.36)	70.36 (69.07–71.40)	61–78
Fibre	2.95 (2.86–3.00)	2.97 (2.92–3.00)	2.93 (2.89–3.0)	2.91 (2.90–2.92)	2.54

[1] Values presented as % dry weight. Values are means of 3 samples taken from 3 locations (*i.e.*, 1 sample/location), ranges are given in brackets. Genetically modified corn lines are denoted CBR and are isogenic to their controls except for the presence of the novel genes.

Table II.6. Summary of compositional analysis for Bt-11 and control corn plants[1] (ANZFA, 2000c).

Northern/Early	**X4334CBR**	**Control N4242**	**X4734CBR**	**Control N4640**	**Normal Range[2]**
Protein	8.65[3] (8.03–9.11)	9.25 (8.63–9.63)	8.19[4] (7.74–9.16)	8.96 (8.28–9.53)	6–12
Oil	3.17 (2.81–3.73)	3.23 (3.04–3.50)	3.34 (3.36–3.48)	3.30 (3.12–3.68)	3.1–5.7
Starch	72.93 (71.8–73.2)	72.57 (71.7–73.4)	72.73 (71.5–73.7)	72.62 (71.3–73.2)	61–78
Fibre	2.69 (2.66–2.83)	2.75 (2.67–2.93)	2.77 (2.68–2.83)	2.77 (2.69–2.83)	2.5[5]
Southern/Late	**X6534CBR**	**X6514**	**X7634CBR**	**X7514**	
Protein	9.52 (8.35–10.60)	9.93 (9.10–11.40)	9.85 (8.63–11.0)	9.87 (8.67–10.94)	6–12
Oil	3.80 (3.63–4.16)	3.93 (3.27–4.13)	3.37 (2.59–4.00)	3.48 (2.70–4.31)	3.1–5.7
Starch	70.77 (68.8–72.5)	71.07 (67.8–72.7)	71.33 (68.6–74.3)	71.12 (69.1–73.9)	61–78
Fibre	2.78 (2.55–3.00)	2.80 (2.61–3.0)	2.74 (2.53–3.00)	2.72 (2.46–2.92)	2.5[5]

[1] Values presented as % dry weight. Values are means of a total of 6 samples taken from 2 sites in 3 locations (*i.e.* two distinct samples from each of the 3 locations), ranges are given in brackets.
[2] From Corn Chemistry and technology, 1987, Watson S.A. and Ranstad P. E (eds.), American Association of Cereal Chemists, St. Paul, Minnesota, USA.
[3] Values are significantly different to that of control value at 5% level of probability.
[4] Values are significantly different to that of control value at 1% level of probability.
[5] Average value.

Table II.7. Summary of proximate analysis of grain from corn lines MON 809, MON 810, and 818 (control)[1] (USDA, 1996b).

Characteristic	**MON 818**	**MON 809**	**MON 810**	**Reported Ranges[3]**
Protein[2]	12.8	13.1	13.1	6.0–12.0 9.7–16.1 6.8–13.4 10.0–14.1
Fat[2]	2.9	2.6	3.0	3.1–5.7 2.9–6.1 2.0–5.9 1.0–5.7
Ash[2]	1.5	1.5	1.6	1.1–3.9
Carbohydrates[2]	82.7	82.8	82.4	Not reported
Calories/100g[2]	409	407	408	Not reported
Moisture	12.0	13.2	12.4	7–23

[1] Values reported are a mean of six samples, on sample from each field site.
[2] Percent dry weight of sample.
[3] Ranges from different literature sources. For references see the original documentation (USDA APHIS, 1996b).

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Table II.8. Mean values and ranges of proximate analyses for corn trials (MON 810) (ANZFA, 2000a).

	Control[2]		MON 810[2]	
	Mean	**Range**	**Mean**	**Range**
Protein[1]	12.8	11.7–13.6	13.1	12.7–13.6
Fat[1]	2.9	2.6–3.2	3.0	2.6–3.3
Ash[1]	1.5	1.5–1.6	1.6	1.5–1.7
Carbohydrate[1]	82.7	81.7–83.8	82.4	81.8–82.9
Calories Kcal/100g[1]	409	406–410	408	407–410
Moisture	12	10.6–14.2	12.4	11.0–14.4

[1] Data as a percentage of dry weight.
[2] Value is the mean of six samples (n = 6), one from each of six sites.

Table II.9. Summary of proximate, calcium and phosphorous analysis of corn grain from line MON 802[1] (USDA, 1996d).

Characteristic	MON 818 Control[2]	MON 802[2]	Literature Range[3]
Protein	12.8 (11.7–13.6)	12.9 (11.8–13.7)	(6.0–12.0) (9.7–16.1)
Fat	2.9 (2.6–3.2)	3.1 (2.8–3.2)	(3.1–5.7) (2.9–6.1)
Ash	1.5 (1.5–1.6)	1.6 (1.5–1.8)	(1.1–3.9)
Crude fiber	2.4 (2.3–2.5)	2.4 (2.1–2.6)	(2.0–5.5)
Carbohydrate	82.7 (81.7–83.8)	82.4 (81.5–83.2)	Not reported
Calories/100g	409 (406–410)	409 (409–410)	Not reported
Calcium %	0.003 (0.003–0.004)	0.003 (0.003–0.003)	(0.01–0.1)
Phosphorus %	0.348 (0.327–0.363)	0.336 (0.21–0.356)	(0.26–0.75)
Moisture %	12.0 (10.6–14.2)	12.6 (11.2–14.8)	(7–23)

[1] Percent dry weight of sample, except for moisture.
[2] Mean (range) where the mean is the mean of six samples, one from each field sit and the range denotes the lowest and highest individual for each line.
[3] Literature range from multiple sources. For references see original citation (USDA, 1996d).

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