Environ. Biosafety Res. 10 (2011) 27–49

DOI: 10.1051/ebr/2012002

Available online at:
www.ebr-journal.org

A review of the environmental safety of the Cry1Ac protein

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May 26, 2010

Keywords: Cry1Ac / Bacillus thuringiensis / insect resistance / genetically engineered / environmental risk assessment

INTRODUCTION

This document provides a comprehensive review of information and data relevant to the environmental risk assessment of Cry1Ac and presents a summary statement about the environmental safety of this protein. All sources of information reviewed herein were publically available and included: 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; Codex, 2003a, 2003b; EFSA, 2006; NRC, 1989; OECD, 1992). 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 likelihood and potential consequence.

Regulatory approvals for environmental release of GE plants expressing Cry1Ac have been issued in eleven countries and include three species of plants (CERA, 2010; CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002b, 2003a, 2003c, 2006a, 2006c; USDA APHIS, 1995, 1997a, 1997d, 2001, 2004) (Tab. 1). One event[1] each for maize (*Zea mays*) and tomato (*Lycopersicon esculentu*)[2] has received approval while 12 lines of cotton[3] (*Gossypium hirsutum*) have received approval in at least one country. These regulatory reviews have generally considered the potential for Cry1Ac to adversely affect non-target organisms, the potential for Cry1Ac expression to affect the weediness potential of the modified plant, and the potential for gene flow to impact the weediness of wild relatives (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002b, 2003a, 2003c, 2006a, 2006c; USDA APHIS, 1995, 1997a, 1997d, 2001, 2004).

ORIGIN AND FUNCTION OF CRY1AC

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;

[1] Event refers to a single transformation event: the incorporation of a transgene into a plant genome. A single transformation event can be crossed into multiple lines.

[2] Tomato Event 5345 expressing Cry1Ac was deregulated by USDA APHIS but never received registration as a pesticide with the USEPA and was not commercialized anywhere in the world. It will not be considered elsewhere in this paper.

[3] This includes approvals for lines generated through breeding and transformation with additional transgenes.

Table 1. Regulatory approvals for the environmental release of GE plants containing Cry1Ac.

Species	Event Name	Also Known As	United States	Argentina	Australia	Brazil	Burkina Faso	Canada	Colombia	India	Japan	Mexico	South Africa
Gossypium hirsutum (cotton)	MON-15985-7	MON 15985	X		X	X	X			X			X
	DAS-21023-5	3006-210-23	X										
	31807/31808		X								X		
	DAS-21023-5 x DAS-24236-5		X			X							
	DAS-21023-5 x DAS-24236-5 x MON-01445-2		*										
	DAS-21023-5 x DAS-24236-5 x MON88913		*										
	Event-1									X			
	ACS-GH001-3, MON-15985-7	LLCotton25 x MON15985	*								X		
	MON-15985-7 x MON-01445-2		*		X								
	MON-00531-6 x MON-01445-2		*	X	X	X							X
	MON-15985-7, MON-88913-8	MON15985 x MON88913	*		X								X
	MON-00531-6, MON-00757-7	MON531/757/1076	X	X	X	X			X	X	X	X	X
Lycopersicon esculentum (tomato)	5345		X										
Zea mays (maize)	DKB-89614-9	DBT418	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 are dependent on pesticide registrations which require period renewal.

Schnepf et al., 1998; OECD, 2007). There is tremendous variation within the species with regard to production of a range 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[4], 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) are widely toxic (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 Cry1Ac 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, 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

[4] also called thuringiensin

proteins was proposed in 1989 and widely adopted (Hofte and Whiteley, 1989; OECD, 2007). This system has been subsequently updated to account for additional Cry proteins and expanding knowledge of their molecular function and relatedness, leading to some minor discrepancies in naming with earlier literature (Crickmore et al., 1998; Crickmore et al., 2005; OECD, 2007). This document uses the most recent nomenclature (Cry1Ac for the protein, *cry1Ac* for the gene) but the protein in question is synonymous with the older nomenclature CryIA(c).

All of the Cry1 proteins are closely related based on sequence and the proteins designated Cry1A (including Cy1Aa, Cry1Ab and Cry1Ac) are greater than 85% identical in amino acid sequence (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% (Aronson and Shai, 2001; Bravo et al., 2007; Crickmore et al., 1998; Kumar et al., 1996; OECD, 2007). In the original nomenclature, the Cry proteins were designated based on their insecticidal activity (CryI proteins were those active against lepidopterans), and although the nomenclature is now sequence dependent the target specificity remains largely intact such that proteins designated Cry1 have activity specifically against lepidopterans (Aronson and Shai, 2001; Crickmore et al., 1998; Hofte and Whiteley, 1989; Kumar et al., 1996; OECD, 2007).

Mechanism of Cry1Ac insecticidal activity

Although there is significant variability in amino acid sequence and target range, the general mechanism by which Cry proteins (including Cry1Ac) achieve insecticidal activity is believed to be common across the group (Aronson and Shai, 2001; Bravo et al., 2007; Crickmore et al., 1998, 2005; Hofte and Whiteley, 1989; Kumar et al., 1996; OECD, 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; Bravo et al., 2007; Kumar et al., 1996; 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). These so-called active toxins bind to specific receptors on the plasma membrane of midgut epithelium cells in susceptible insects (Aronson and Shai, 2001; Bravo et al., 2007; Kumar et al., 1996; OECD, 2007). Once bound to receptors, the toxin is able to insert into the plasma membrane and form oligomeric transmembrane pores (Aronson and Shai, 2001; Bravo et al., 2007; 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; Hofte and Whiteley, 1989; Kumar et al., 1996; OECD, 2007). The biochemical process of membrane insertion is not completely understood. 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 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; Zhang et al., 2006).

EXPRESSION OF CRY1AC IN INSECT RESISTANT GE PLANTS

The level of expression of Cry1Ac 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 Cry1Ac in GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents (CFIA, 1996, 1997, 2004, 2005; CTNBio, 2005, 2009; OGTR, 2003, 2006; USDA APHIS, 1994, 1996, 1997a, 1997b, 2003, 2000; USEPA, 2001; USFDA, 1997). Tissue types and collection methods differed between studies but all used an enzyme-linked immunosorbent assay (ELISA) to quantify the amount of Cry1Ac 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 Cry1Ac 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 Cry1Ac protein per gram of fresh weight) (CFIA, 1996, 1997, 2004, 2005; CTNBio, 2005, 2009; OGTR, 2003, 2006; USDA APHIS, 1994, 1996, 1997a, 1997b, 2003, 2000; USEPA, 2001; USFDA, 1997). 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

Table 2. Highest reported protein concentrations of Cry1Ac in GE plant tissue.

Species	Events	Tissue With Highest Expression	Range	Citation
Gossypium hirsutum	MON-15985-7	Seed	3.35±0.63[1] µg/g FW[2]	OGTR, 2002
	DAS-21023-5	Young Leaf	0.46-3.5 µg/g DW[3]	USDA, 2003
	DAS-21023-5 X DAS-24236-5	Flower	1.83 µg/g DW	FSANZ, 2004
	31807/31808	Seed[4]	2.5 µg/g FW	FDA, 1997
	MON-00531-6	Young Leaf	5.00 ± 1.84[1] µg/g FW	OGTR, 2002
Zea mays	DKB-89614-9	Harvest Leaf	626.8 ± 141.62[5] ng/g FW	USDA, 1996

[1] Standard Deviation
[2] FW = fresh weight
[3] DW = dry weight.
[4] Only tissue reported.
[5] Standard Error.

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. (CFIA, 1996, 1997, 2004, 2005; CTNBio, 2005, 2009; OGTR, 2003, 2006; USDA APHIS, 1994, 1996, 1997a, 1997b, 2003, 2000; USEPA, 2001; USFDA, 1997).

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 Cry1Ac 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 Cry1Ac expressing GE plants where data were available. Additional information about expression of Cry1Ac is contained in Annex I.

NON-TARGET ORGANISM (NTO) TESTING AND IMPACTS OF EXPOSURE TO CRY1AC PROTEIN

The Cry1Ac protein has insecticidal properties against certain lepidopteran insects when they feed on a substrate containing the Bt protein (Crickmore et al., 1998, 2005; Hofte and Whiteley, 1989; OECD, 2007). The objective of inserting the *cry1*Ac gene into a crop is to provide protection from feeding damage by such pests. Other organisms that are not pests in the agricultural system may also be exposed to the Cry1Ac protein, and are considered "non-target organisms" (NTOs). Such exposure could be direct, from deliberate or incidental feeding on crop tissues such as pollen or decaying leaf material, or be indirect, from feeding on other herbivores that feed on the crop. Because Cry1Ac has a demonstrated pesticidal activity, the potential for harm to NTOs has been considered as a part of regulatory risk assessments for GE plants that express Cry1Ac, with special consideration to beneficial NTOs that perform valuable functions as well as threatened, endangered and charismatic species (CFIA, 1996, 1997, 2004, 2005; CTNBio, 2005, 2009; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003; 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 in general the level of realism and complexity of tests rise through the tiers (EFSA, 2006; Romeis et al., 2008; Rose, 2007; USEPA, 2007; USEPA, 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; USEPA, 2007). If no effects are observed, additional testing at higher tiers is generally not required (Romeis et al., 2008; Rose, 2007; USEPA, 2010; USEPA, 2007). If adverse effects are observed in early tier tests or unacceptable uncertainty exists, additional testing will progress as necessary through later tiers in order to reduce uncertainty to an acceptable level for decision making (EFSA, 2006; Romeis et al., 2008; USEPA, 2010; USEPA, 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 Cry1Ac protein: exposure to pollen containing Cry1Ac, exposure to Cry1Ac deposited in the soil by decomposing plant material, and tritrophic exposure *via* feeding on herbivores on the GE plant (Japan BCH, 1999; OGTR, 2003; USEPA, 2001).

Table 3. Summary of ecotoxicological tests of Cry1Ac on non-lepidopteran non-target organisms.

Species	Method of Exposure	Duration	Results of Observation
Apis mellifera (honeybee) larvae	Single injection of purified protein solution into cells with developing larvae	1-3 days after hatching until adult emergence	NOEL 20 ppm[1]
Apis mellifera (honeybee) adult	Feeding purified protein in a honey water solution	NA	NOEL 20 ppm[1]
Nasonia vitripennis	Purified protein in a honey water diet	23 days	NOEL 20 ppm[1]
Hippodamia convergens (ladybird beetle)	Purified protein in honey water diet	30 days	NOEL 20 ppm[1]
*Chrysoperla carnea (*green Lacewing*)* larvae	Purified protein mixed in a paste of Sitotroga eggs	11 days	NOEL 20 ppm[1]
Folsomia candida (springtail)	Purified protein in artificial diet	21 days	NOEL > 200ppm[1]
Xenylla grisea (springtail)	Purified protein in artificial diet	21 days	NOEL > 200ppm[1]
Mus musculum (mouse)	Single dose oral gavage >3280mg Cry1Ac/kg body weight	14 days	No observed effects[1]

[1] Data reported in US EPA (2001) and ANZFA (2002).

Exposure through pollen is limited by the generally low expression levels of Cry1Ac 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 (CFIA, 1997; FSANZ, 2004; OGTR, 2002a, 2003b, 2003c, 2005, 2006b, 2008; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003; 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 Cry1Ac on representative pollinator species (*i.e.*, honeybee) (EU SCP, 1998; Japan BCH, 1999; OGTR, 2002b, 2003a, 2003c, 2006a, 2006c; USEPA, 2001). Similarly, the specificity of Cry1Ac toxicity to Lepidoptera and evidence suggesting low exposure through soil has led regulators to require testing for only representative soil dwelling arthropod species (EU SCP, 1998; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; 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 (Crecchio and Stotsky, 1997; Koskella and Stotzky, 1997; 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 Cry1Ac with a half life of approximately 9-40 days (Accinelli et al., 2008; Marchetti et al., 2007). In at least one field experiment, Cry1Ac was not detected by ELISA or bioassay in agricultural fields where Cry1Ac expressing cotton (MON-00531-6) had been grown and tilled into soils for three to six consecutive years (Head et al., 2002). Regulatory approvals of Cry1Ac 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 Cry1Ac (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003; USEPA, 2001). Potential bitrophic and tritrophic exposures are addressed using ecotoxicological testing.

Ecotoxicological testing of Cry1Ac on non-Lepidopteran NTOs

NTO testing of purified Cry1Ac has been conducted on a variety of non-lepidopteran species for regulatory submissions related to Cry1Ac producing GE plants (ANZFA, 2002; OECD, 2007; 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 *Nasonia vitripennis*, as well as soil dwelling Collembola (springtail) species *Folsomia candida* and *Xanylla grisea*. None of these organisms showed a significant response to Cry1Ac at the test concentrations resulting in observations of a No Observed Effects Level (NOEL). Additionally, acute mammalian toxicological testing has been conducted on mouse (*Mus musculus*) (ANZFA, 2002; USEPA, 2001). The results of all of these studies are summarized in Table 3.

Ecotoxicological testing of Cry1Ac on the non-target Lepidopteran *Danaus plexippus* L. (Monarch butterfly)

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 Lepidoptera that might be exposed incidentally to Cry proteins. Most of the investigations have centered on the Monarch butterfly (*Danaus plexippus*), a well known and valued charismatic species in North America. Early monarch butterfly studies (Jesse and Obrycki, 2000; Losey et al., 1999) did not assess Cry1Ac plant material, however subsequent research has examined the toxicity of Cry1Ac on monarch larvae in both Tier I studies with purified proteins in an artificial diet and Tier II studies simulating exposure through pollen from maize event DKB-89416-9 (Hellmich et al., 2001). These studies suggest that monarch larvae are sensitive to Cry1Ac and exposure under laboratory conditions can cause delayed development and mortality to monarch larvae. However, exposure to pollen from Cry1Ac expressing maize (event DKB-89416-9) at concentrations > 1600 pollen grains/cm^2 of milkweed leaf does not affect growth or survival (Hellmich et al., 2001). 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, 2003; Pleasants et al., 2001). 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 Cry1Ac (CFIA, 1997; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003; USEPA, 2001). The results of these studies are summarized in Table 4.

Table 4. Summary of ecotoxicological testing of monarch butterfly (*D. plexippus*)[1].

Species	Method of Exposure	Duration	Result
Danaus plexippus 1st instar larvae	Purified Protein Incorporated in test diet	7 days	LC_{50} = 13.8ng/mL artificial diet[2] EC_{50} = 0.9 ng/mL artificial diet[3]
Danaus plexippus	Pollen Grains from DBT418 dusted on milk-weed leaves as food substrate (100 - >1600 pollen grains/ cm^2)	4 days	No effects observed

[1] Data from Hellmich et al. (2001).
[2] LC_{50} = Concentration at which 50% of larval mortality is expected (Lethal Concentration).
[3] EC_{50} = Concentration expected to produce 50% growth inhibition by calculation.

Field studies of Cry1Ac on non-target organisms

A number of reviews and meta-analyses have analyzed the net results of much of the available literature regarding NTO field studies (Romeis et al., 2006). A database[5] compiling this information has been created to facilitate continuing study (Duan et al., 2008, 2010; Marvier et al., 2007; Naranjo, 2009; Wolfenbarger et al., 2008). When GE plants that express Cry proteins, including Cry1Ac cotton, were compared to control plants that were not treated with chemical insecticide there was a reduction in arthropod abundance, but when control plants are treated with insecticide arthropod abundance is significantly higher in GE plants expressing Cry proteins (Marvier et al., 2007; Naranjo, 2009 Wolfenbarger et al., 2008). When comparisons were made between GE plants expressing Cry proteins and controls where insecticide sprays are applied to both, no significant differences were seen (Marvier et al., 2007). Meta-analysis of Cry1Ac cotton data suggest that the reduction in non-target arthropod abundance when compared to unsprayed control was primarily driven by a reduction in Lepidoptera, but smaller reductions in the number of Coeleoptera and Hemiptera were seen as well (Marvier et al., 2007). When arthropods were grouped by functional guilds (Predator, Parasitoid, Mixed, Herbivore, Omnivore, Detritivore) significant reductions in Predators are seen in Cry1Ac cotton as compared to unsprayed control (Naranjo, 2009; Wolfenbarger et al., 2008). This was a consequence of reductions in two families (Nabidae and Coccinellidae) rather than a uniform reduction (Naranjo, 2009; Wolfenbarger et al., 2008). This reduction has been

[5] 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.

shown to be inconsequential for biological control of non-target pests (Naranjo, 2005a, 2005b).

ESTABLISHMENT AND PERSISTENCE OF CRY1AC-EXPRESSING PLANTS IN THE ENVIRONMENT

Biology of the plant species

Familiarity with the biology of the nontransformed 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.*, Cry1Ac) 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 Cry1Ac 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) (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). Because the Cry1Ac 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) (USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). Statistically significant differences were seen between GE plants expressing Cry1Ac and controls in many cases, but these differences were small and fell within the reported range for the crop species (USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). Collectively, the phenotypic data showed no pattern of changes that would support the hypothesis that the introduction of Cry1Ac protein had any unintended impact on the gross morphology or phenotypic characteristics of plants, besides conferring insect resistance to Lepidoptera pests. The phenotypic data for GE plants expressing Cry1Ac is summarized in Annex II.

Weediness in agricultural environments

Both maize and cotton have some potential to "volunteer" as weeds in subsequent growing seasons (OECD, 2003; OECD, 2008; OGTR, 2008). 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 (Baker, 1974). There are no data indicating a linkage between Cry1Ac protein expression and any increased survival or over-wintering capacity that would alter the prevalence of volunteer maize or cotton in subsequent growing seasons (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). Following-season volunteers expressing Cry1Ac would not be expected to present any management difficulty and can be dealt with in the same manner as conventional volunteers of maize and cotton.

Weediness in non-agricultural environments

The primary mechanisms by which Cry1Ac 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 Cry1Ac have considered the potential impacts associated with both types of movement (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003).

While all plants can be considered weeds in certain contexts, neither maize nor cotton is considered to be an invasive or aggressive weed outside of agricultural

systems. Maize is severely restricted in ability to establish without human intervention but cotton can persist under favorable conditions and may at times require management (OECD, 2003; OECD, 2008; OGTR, 2008). Agronomic data show that Cry1Ac does not have a significant impact on traits associated with weediness (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). 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; Mack, 1996; Mason et al., 2004) most regulatory decisions have agreed that it is unlikely that the addition of resistance to Lepidopteran pests would allow cotton expressing Cry1Ac to become invasive of non-agricultural environments (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). Regulatory decisions in Australia prior to 2006 restricted the release of Cry1Ac cotton in Northern Australia because of uncertainty about the impact of insect- resistance on the ability of cotton to persist. Subsequent studies, however, indicated that lepidopteran herbivory was not significant in limiting the spread of cotton in Northern Australia and the restriction was lifted (OGTR, 2002a, 2003b, 2003c, 2005, 2006b, 2006c).

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. Neither maize nor cotton has wild relatives that are considered invasive of ecosystems or broadly distributed, agriculturally important weeds for which hybridization is a concern (OECD, 2003; OECD, 2008; OGTR, 2008). Maize freely hybridizes with wild teosintes, but gene introgression is thought to be limited (Baltazar et al., 2005; OECD, 2003; Serratos et al., 1995). 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). Cotton has several wild relatives with which it might potentially hybridize (OECD, 2008; OGTR, 2008). The USEPA has restricted the release of Cry1Ac expressing cotton in Hawaii due to uncertainty about the effects on populations of *G. tomentosum* (USEPA, 2001). USEPA has also restricted release in Southern Florida because of uncertainty about the impact of gene flow to naturalized *G. hirsutum* with respect to the development of insect resistance (USEPA, 2001). In Australia, uncertainty about the impact of gene flow to naturalized populations of *G. hirsutum* and *G. barbadense* led to restriction on the planting of Cry1Ac cotton in Northern Australia until 2006, when studies established that Lepidoptera predation was not significant in controlling these populations (OGTR, 2002a, 2003b, 2003c, 2005, 2006b, 2006c). Brazil has established an exclusion zone for the growth of Cry1Ac cotton as well, to prevent potential gene flow to wild species in northwestern Brazil (CTNBio, 2005).

COMPOSITIONAL ANALYSIS OF CRY1AC 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 (Codex, 2003a, 2003b; EFSA, 2006A; FAO/WHO, 1996; OECD, 1992; WHO, 1995). The choice of analyses conducted depends on the nature of the product and its intended uses. Insect resistant GE crops expressing Cry1Ac have typically undergone proximate analysis (crude protein, crude fat, fiber, moisture and ash) (ANZFA, 2002; Berberich et al., 1996; CFIA, 1996, 1997, 2002, 2003, 2005; CTNBio, 2005; Hamilton et al., 2004; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). 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 properties (e.g gossypol in cotton) (ANZFA, 2002; Berberich et al., 1996; CFIA, 1996, 1997, 2002, 2003, 2005; CTNBio, 2005; Hamilton et al., 2004; Japan BCH, 1997, 1999, 2007; OGTR, 2002a, 2003b, 2003c, 2005, 2006b; USDA APHIS, 1994, 1996, 1997a, 1997b, 1997c, 2000, 2003). The data collected can be useful as indicators of unintended changes to the transformed plant (Codex, 2003a, 2003b; Nickson and McKee, 2002).

Data from publicly available compositional analyses are summarized in Annex III. Although some statistically significant compositional differences were observed the composition of GE plants expressing Cry1Ac was found to fall within the normal range observed in the crop species (ANZFA, 2002; Berberich et al., 1996; Hamilton et al., 2004; USDA APHIS, 1996, 1997c, 2000, 2003). Subsequent regulatory analyses did not consider these differences to be meaningful in the context of

environmental safety (CFIA, 1997; CTNBio, 2005; Japan BCH, 1997, 1999, 2007; OGTR, 2002b, 2003a, 2003c, 2006a, 2006c; USDA APHIS, 1995, 1997d, 2001, 2004).

Considering data across approved events, there have been no patterns of consistent or reliable changes in proximate composition in plants expressing Cry1Ac. This indicates that the expression of Cry1Ac does not have any biologically significant effect on the gross metabolism of the transformed plants.

CONCLUSION

The Cry1Ac 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 Cry1Ac. Meta analyses of field studies suggest that cultivation of GE cotton plants expressing Cry1Ac slightly reduced the abundance of non-target arthropods when compared to unsprayed cotton, increased arthropod abundance when compared to cotton sprayed with insecticides and had no discernable effect when both the GE plants and controls were treated with insecticide consistent with conventional insect management practices. Cry1Ac 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 Cry1Ac expression in approved cotton and 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 CRY1AC 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. Expression of Cry1Ac in Zea mays event DBT418 (USDA APHIS, 1996)[1].

Tissue	Genotype	V6-V7 growth stage		Pollen Shed		Dough		Harvest		Senescence	
		Mean	SE[5]	Mean	SE[5]	Mean	SE[5]	Mean	SE[5]	Mean	SE[5]
Leaf	Inbred[2]	33.6	7.12	88.1	19.7	NA	NA	240.4	52.22	NA	NA
	Het.[3]	27.4	6.5	24.6	1.94	NA	NA	324.6	44.14	NA	NA
	Hybrid[4]	44.6	5.66	45.8	9.65	NA	NA	626.8	141.62	NA	NA
Stalk	Inbred[2]	NA	NA	5.7	0.84	NA	NA	36.7	13.88	NA	NA
	Het.[3]	NA	NA	BLD7	BLD	NA	NA	12.1	2.18	NA	NA
	Hybrid[4]	NA	NA	BLD	BLD	NA	NA	34.2	7.48	NA	NA
Root Ball	Inbred[2]	7.0	1.75	11.1	1.78	NA	NA	10.8	1.87	NA	NA
	Het.[3]	5.1	0.97	8.2	2.31	NA	NA	10.7	1.51	NA	NA
	Hybrid[4]	11.9	1.72	10.2	2.92	NA	NA	23.1	3.12	NA	NA
Kernel[6]	Inbred[2]	NA	NA	NA	NA	NA	NA	42.8[6]	16.60	NA	NA
	Het.[3]	NA	NA	NA	NA	NA	NA	37.1[6]	3.97[6]	NA	NA
	Hybrid[4]	NA	NA	NA	NA	NA	NA	36.0[6]	8.14[6]	NA	NA
Silk	Inbred[2]	NA	NA	BLD	BLD	NA	NA	NA	NA	NA	NA
	Het.[3]	NA	NA	14.1	1.37	NA	NA	NA	NA	NA	NA
	Hybrid[4]	NA	NA	BLD	BLD	NA	NA	NA	NA	NA	NA
Pollen	Inbred[2]	NA	NA	BLD	BLD	NA	NA	NA	NA	NA	NA
	Het.[3]	NA	NA	BLD	BLD	NA	NA	NA	NA	NA	NA
	Hybrid[4]	NA	NA	BLD	BLD	NA	NA	NA	NA	NA	NA
Whole Plant	Inbred[2]	NA	NA	27.6	9	NA	NA	NA	NA	124.2[6]	16.47[6]
	Het.[3]	NA	NA	6.7	1.02	NA	NA	NA	NA	41.2[6]	6.42[6]
	Hybrid[4]	NA	NA	14.1	2.82	NA	NA	NA	NA	69.9[6]	17.76[6]
Whole Plant no Root Ball	Inbred[2]	NA	NA	NA	NA	97.2	10.66	NA	NA	NA	NA
	Het.[3]	NA	NA	NA	NA	19.4	3.25	NA	NA	NA	NA
	Hybrid[4]	NA	NA	NA	NA	59.5	18.18	NA	NA	NA	NA

[1] Data are shown in ng/g fresh weight unless noted otherwise.
[2] Inbred (2/3 alleles of Cry1Ac).
[3] Commercial hybrid (Heterozygous for Cry1Ac).
[4] Hybrid (Homozygous for Cry1Ac).
[5] Standard error.
[6] ng/g dry weight.
[7] BLD = below limit of detection (51.6 ng/g dry weight).
[8] NA = not available.

Table I.2. Expression of Cry1Ac in *Gossypium hirsutum* event MON-00531-6 (USDA APHIS, 1994)[1].

Tissue	1992		1993		1999	
	Mean	Range	Mean	Range	Mean	SD[2]
Young Leaf	1.56	1.10-2.04	2.59	0.41-5.91	5.00	1.84
Leaf June	1.40	NA[3]	5.12	NA	NA	NA
Leaf July	1.49	NA	3.21	NA	NA	NA
Leaf August	3.55	NA	0.13	NA	NA	NA
Leaf September	1.3	NA	0.23	NA	NA	NA
Seed	0.86	0.49-1.62	2.18	1.13-3.41	4.30	0.86
Pollen	11.5 ng/g	NA	NA	NA	NA	NA
Nectar	BLD[4]	NA	NA	NA	NA	NA
Whole Plant	0.044	NA	NA	NA	NA	NA

[1] Data reported in µg/g fresh weight unless noted otherwise.
[2] Standard deviation.
[3] NA= not available.
[4] BLD= below limit of detection (1.6 ng/g).

Table I.3. Expression of Cry1Ac in *Gossypium hirsutum* event MON-00531-6 (CTNBio, 2005)[1].

Tissue	Mean
Leaves-20 days after planting	2.93
Leaves-130 days after planting	3.02
Seeds	6.88

[1] Data from field trials in Brazil and values are µg/g fresh weight.

Table I.4. Expression of Cry1Ac in *Gossypium hirsutum* event MON-00531-6 (OGTR, 2002)[1].

Tissue	1998		1999	
	Mean	STD	Mean	STD
Leaf	1.95	1.21	2.05	0.71
Seed	3.22	0.77	2.64	0.63
Whole Plant	0.13	0.04	<0.07	NA[2]
Pollen	0.04	0.01	0.01	<0.01

[1] Data are reported in µg/g fresh weight (this data comes from the regulatory submission for event 15985).
[2] NA= not available.

Table I.5. Expression of Cry1Ac in *Gossypium hirsutum* event MON-15985-7 (OGTR, 2002)[1].

Tissue	1998		1999	
	Mean	SD[2]	Mean	SD
Leaf	2.75	1.32	2.07	0.61
Seed	3.35	0.63	2.6	0.66
Whole Plant	0.17	0.08	0.08	0.01
Pollen	0.02	0.01	0.05	0.07

[1] MON-15985-7 is a re-transformation of MON-00531-6 with an additional Cry protein. This data can be considered additional information for event MON-00531-6. Data are reported as µg/g fresh weight.
[2] SD= standard deviation.

Table I.6. Expression of Cry1Ac in *Gossypium hirsutum* event DAS 21023 (USDA APHIS, 2003)[1].

Tissue	Mean	Range
Young Leaf (3-6 week)	1.92	0.46-3.5
Terminal Leaf	1.44	0.24-2.4
Flower	1.92	1.3-2.4
Square	1.84	1.0-3.1
Boll (early)	0.77	0.46-1.1
Whole Plant (seedling)	1.59	0.8-2.2
Whole Plant (pollination)	1.15	0.57-2.1
Whole Plant (defoliation)	0.81	0.31-1.3
Root (seedling)	0.20	0.09[2]-0.44
Root (pollination)	0.10	ND-0.23
Root (defoliation)	0.05	ND-0.11
Pollen[3]	1.44	0.9-2.4
Seed[3]	0.57	0.33[2]-0.78

[1] Values are expressed in µg/g dry weight unless otherwise noted.
[2] Below the limit of quantification for the method (0.001 to 0.375 ng/mg depending on the matrix).
[3] Values are µg/g fresh weight.

Table I.7. Expression of Cry1Ac in expression of Cry1Ac in *Gossypium hirsutum* event DAS-21023-5 X DAS-24236-5 (FSANZ, 2004)[1].

Tissue	Mean
Young Leaf (3-6 week)	1.82
Terminal Leaf	1.31
Flower	1.83
Square	1.82
Boll (early)	0.64
Whole plant (seedling)	1.37
Whole plant (pollination)	1.05
Whole plant (defoliation)	0.6
Root (seedling)	0.17
Root (pollination)	0.07[2]
Root (defoliation)	ND
Pollen[3]	1.45
Seed[3]	0.55

[1] Data are reported as µg/g dry weight unless noted otherwise.
[2] Data are calculated including some values that are below the limit of quantification of the method (0.001 to 0.375 ng/mg depending on the matrix).
[3] Values reported as µg/g fresh weight.

Table I.8. Expression of Cry1Ac in *Gossypium hirsutum* event 31807/31808 (FDA, 2007).

Tissue	Maximum Expression
Seed	2.5 ppm[1]

[1] Equivalent to 2.5 µg/g fresh weight.

ANNEX II: SUMMARY OF PHENOTYPIC ANALYSES OF GE PLANTS EXPRESSING CRY1AC

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. Summary of phenotypic analysis of event MON-00531-6 (USDA APHIS, 1996)[1].

Phenotypic Characteristic	Reported Result	Observations of differences
Weediness	No significant differences[2]	
Emergence	No significant differences[2]	Increased emergence at one location
Seedling Vigor	No significant differences[2]	Increased vigor at one location
Dormancy	No significant differences[2]	Some dormancy at one location reportedly due to greenhouse produced seed
Germination	No significant differences[2]	
Morphology	No significant differences[2]	
Time to Flowering	No significant differences[2]	
Fruiting	No significant differences[2]	
Boll Formation	No significant differences[2]	
Boll Development	No significant differences[2]	
Yield	No significant differences[2]	
Disease Susceptibility	No significant differences[2]	
Volunteerism	No significant differences[2]	

[1] Summarized from descriptive text in USDA APHIS (1996).
[2] Significant here does not refer to statistical significance. Information on statistical analysis is not provided.

Table II.2. Summary of mean emergence, flowering, and harvest dates for event MON-15985-7 and MON-00531-6 (USDA APHIS, 2000)[1].

Site	Event or Line #	Percent Seedlings Emerged (7 days)	Percent Seedlings Emerged (14 days)	First White Flower Observed	Date of First Cracked Boll Counts	Harvest Date
Arizona 1	15985	71	82	7/21/1998	9/8/1998	10/16/1998
	DP50B[2]	80	85	7/21/1998	9/8/1998	10/16/1998
	DP50[3]	70	73	7/21/1998	9/8/1998	10/16/1998
Arizona 2	15985	51	72	7/27/1998	9/8/1998	11/18/1998
	DP50B	70	76	7/23/1998	9/8/1998	11/18/1998
	DP50	49	62	7/23/1998	9/8/1998	11/18/1998
Louisiana 1	15985	52	68	8/2/1998	9/21/1998	10/27/1998
	DP50B	48	56	7/31/1998	9/21/1998	10/27/1998
	DP50	48	67	7/31/1998	9/21/1998	10/27/1998
Louisiana 2	15985	73	82	7/20/1998	8/31/1998	10/14/1998
	DP50B	68	71	7/20/1998	8/31/1998	10/14/1998
	DP50	54	56	7/20/1998	8/31/1998	10/14/1998
Mississippi 1	15985	70	75	7/30/1998	9/4/1998	10/19/1998
	DP50B	75	76	7/30/1998	9/4/1998	10/19/1998
	DP50	78	62	7/30/1998	9/4/1998	10/19/1998
Mississippi 2	15985	63	78	7/20/1998	9/8/1998	10/5/1998
	DP50B	76	73	7/20/1998	9/8/1998	10/5/1998
	DP50	74	69	7/20/1998	9/8/1998	10/5/1998
South Carolina	15985	88	94	7/23/1998	9/9/1998	10/27/1998
	DP50B	77	87	7/23/1998	9/9/1998	10/27/1998
	DP50	60	78	7/23/1998	9/9/1998	10/27/1998
Texas[4]	15985	73	93	7/23/1998	9/2/1998	9/28/1998
	DP50B	83	83	7/20/1998	9/2/1998	9/28/1998
	DP50	64	69	7/23/1998	9/2/1998	10/9/1998

[1] USDA APHIS 2000 is a regulatory submission for MON-15985-7 which contains information about MON-00531-6 as a GE parental control.
[2] DP50B = the transgenic parent of 15985 (event 531).
[3] DP50 = the non-transgenic parent of DP50.
[4] Harvest dates at the Texas site were different due to excessive moisture which would have increased boll rot.

Table II.3. Summary of mean height:node ration, days to peak bloom and total cracked boll counts for event MON-15985-7, and MON-00531-6 (USDA APHIS, 2000).

Event or Line #	Height:Node Ration	Mean Days to Peak Bloom	Mean Total Number of Cracked Bolls / plot
15985	1.70	15.29	407
DP50B[2]	1.77	15.03	431
DP50[3]	1.72	15.77	284

[1] USDA APHIS (2000) is a regulatory submission for MON-15985-7 which contains information about MON-00531-6 as a GE parental control.
[2] DP50B = the transgenic parent of 15985.
[3] DP50 = the non-transgenic parent of DP50.

Table II.4. Germination and seedling vigor tests on seed from two locations for MON-15985-7 (USDA APHIS, 2000)[1].

Event or Line #	% Germination Day 4	% Germination Day 9	% Cool Germination at 18° C Day 7
15985[2]	76	77	72
DP50B[3]	83	83	80
DP50[4]	88	89	82

[1] USDA APHIS (2000) is a regulatory submission for MON-15985-7 which contains information about MON-00531-6 as a GE parental control.
[2] 15985 = MON-15985-7.
[3] DP50B = the transgenic parent of 15985, MON-00531-6.
[4] DP50 = the non-transgenic parent of DP50.

Table II.5. Germination and dormancy results from seed harvested in three locations in 1999 for event MON-15985-7 and MON-00531-6 (USDA APHIS, 2000)[1].

Temperature (°C)	Variety	Mean pvhs[3] (Dormant) (%)	Mean pgerm[4] (%)	Mean pfms[5] (%)	Mean pdegen[6] (%)
5	1598 DP50B[2] Ref. Range	1.2 0.0 (0-41)	0.0 0.0 (0-1)	95.1 95.2 (53-99)	4.1 5.4 (1-20)
10	1598 DP50B[1] Ref. Range	0.0 0.0 (0-28)	1.2 1.3 (0-3)	73.9[6] 78.5 (38-91)	26.4[6] 21.7 (9-62)
20	1598 DP50B[1] Ref. Range	0.0 0.0 (0-6)	95.4 97.4 (74-100)	0.0 0.0 (0-13)	5.4[6] 3.1 (0-26)
30	1598 DP50B[1] Ref. Range	0.0 0.0 (0-0)	93.9[6] 98.6 (83-100)	0.0 0.0 (0-0)	6.6[6] 2.2 (0-17)
40	1598 DP50B[1] Ref. Range	0.0 0.0 (0-0)	85.9 89.3 (70-96)	0.0 0.0 (0-0)	14.9 11.1 (4-30)
5/20	1598 DP50B[1] Ref. Range	0.0 0.1 (0-29)	NC[7] NC[7] NC[7]	NC[7] NC[7] NC[7]	NC[7] NC[7] NC[7]
10/20	1598 DP50B[1] Ref. Range	0.0 0.0 (0-18)	NC[7] NC[7] NC[7]	1.9 1.2 (0-79)	7.5 5.8 (1-31)
20/30	1598 DP50B[1] Ref. Range	0.0 0.0 (0-2)	NC[7] NC[7] NC[7]	0.0 0.0 (0-1)	5.1 3.7 (0-17)

[1] USDA APHIS (2000) is a regulatory submission for MON-15985-7 which contains information about MON-00531-6 as a GE parental control.
[2] DP50B = The transgenic parent of 15985, MON-00531-6.
[3] pvhs = percent viable hard seed.
[4] pgerm = percent germinated seed.
[5] pfms = percent viable firm-swollen seed.
[6] pdegen = percent degenerated seed.
[7] Indicates a significant difference from DP50B at $P \leq 0.05$.
[8] NC = no comparison of combined means possible due to significant variety by site interaction at $P \leq 0.05$.
Additional anecdotal reports: disease susceptibility in MON-15985-7 is reported similar to control.

Table II.6. Agronomic characteristics of event DAS-21023-5 lines expressing Cry1Ac protein in comparison to parent variety PSC355 (USDA APHIS, 2003).

Variable	Units	3006-210-3 (Cry1Ac)	PSC355 (Null)	Number of Locations
Growth Habit				
Plant Height	Inches	39.9	41.5	17
Total Nodes	Number per plant	17.4	17.6	16
Hieght:Node Ratio	Inches per plant	2.29	2.35	17
Node of the 1st Fruiting Branch	Node	6.7	6.6	17
Fruiting Branches	Number per plant	11.7	12.1	16
Total Fruiting Position	Number per plant	25.6	26.6	17
Vegetative Bolts	Number per plant	2.3	1.6	16
Germination and Emergence				
Field Emergence	%	63.6	82.3	19
Cool Vigor	%	32	38	20
4 Day Warm	%	64	65	20
7 Day Warm	%	80	82	20
Total Germination	%	85	87	20
Dormant Seed	%	0.6	0.3	20
Vegetative Vigor				
Vegetative branches	Number per plant	2.9	2.6	16
Flowering Period				
Days to First Flower	Days	61.9	60.6	18
Node of White Flower – 15 days	Node	12.9	12.9	17
Node of White Flower – 30 days	Node	17.0	16.8	15
Reproductive Potential				
Percent Retention – total	%	49.0	44.4	16
Percent Retention – 1st position	%	58.5	54.3	16
Percent Open Bolls	% per plant	73.5	75.4	17
Seed Cotton Weight per Boll	Grams per boll	5.5	5.1	19
Lint Percent	%	37.9	37.3	19
Seed Index (fuzzy)	Grams per 100 seeds	11.0	10.7	17
Lint per Acre	Pounds per acre	1005	993	17
Fiber Quality				
Length	Inches	1.160	1.147	19
Strength	Grams per tex	31.9	32.6	19
Micronaire	Micronaire units	4.51	4.96	19
Length Uniformity	%	85.8	85.7	19
Reflectance	%	76.0	74.6	19
Yellowness	Hunter's +b scale	8.3	8.4	19

Additional anecdotal reports: disease susceptibility (no difference).

Table II.7. Examples of field performance characteristics of events 31807 and 31808 compared to a commercial variety used as a control (USDA APHIS, 1997c).

Evaluation parameter	Control (e.g. Coker 130)	Event 31807	Event 31808
% Fruit Damaged by *Helicoverpa zea* (% transformed to square root of their arcsine)	12.87	1.88	0.61
Squares Damaged by *Heliothis zea*	15.4	2.92	2.92
Crown Gall Incidence	0	0	0
Cauliflower Mosaic Virus Infection	0	0	0
Susceptibility to *Phomopsis*, *Verticillium* and other normal fungal pathogens of cotton	Within expected range	Within expected range	Within expected range
Levels of non-target insect pests such as cotton aphid, tarnished plant bug, spider mite, and boll weevil	Within expected range	Within expected range	Within expected range
Bromoxynil tolerance	No	Yes	Yes
Seed Germination	normal	normal	normal
Plant Morphology	normal	normal	normal
Flowering Period	normal	normal	normal
Yield	normal	normal	normal
Fiber Quality	normal	normal	normal
Incidence of post-season volunteer cotton plants	0	0	0

Table II.8. Mean comparisons of germination percentages for event 31807 (USDA APHIS, 1997c).

Event or Strain	N	Warm Germination Percentage	Cool Germination Percentage
31707[1]	4	99	92
31803[1]	4	98	86
31807 A	3	97	87
31807 C	4	97	89
Coker 130[2]	4	97	92
ST474[2]	4	95	88
LSD (0.05)		2	5
CV (%)		1.6	3.5

[1] GE plants expressing Cry1Ac.
[2] Untransformed varieties.

Table II.9. Agronomic performance of DBT418-converted hybrid (event DK-B89614-9) as compared to the conventional version of the same hybrid (USDA APHIS, 1996)[1].

Trait	Counterpart Unconverted Hybrid	DBT418
Yield (bushels/acre)	130.4	129.5
Grain Moisture (%)	13.9	14.3[2]
Test Weight (lbs.)	55.0	55.0
Final Stand Count	61.2	61.1
Seedling Vigor (1-9 scale)	6.5	6.2[1]
Plant Height (in.)	89.2	88.5
Ear Height (in.)	42.5	41.0
Pollen GDU	1339	1342
Silk GDU	1335	1342[1]
Stay-Green (1-9 scale)	4.2	4.3[1]
Intactness (1-9 scale)	4.1	4.9[1]
Dropped Ears (%)	0.04	0.04
Stalk Lodged (%)	3.1	2.9
Root Lodged (%)	2.9	5.0
Barren Plants (%)	3.4	5.1

[1] Additional anecdotal reports: disease susceptibility (no different).
[2] Statistically different from the control at the P = 0.5 level.

ANNEX III: SUMMARY OF COMPOSITIONAL ANALYSES OF GE PLANTS EXPRESSING CRY1AC

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 III.1. Proximate analysis of grain from *Zea mays* event DBT418 (ANZFA, 2002)[1].

Constituent Analyzed	DBT418[2]		Control[2]		Literature Range
	Mean	Standard Deviation	Mean	Standard Deviation	
Protein	9.02	0.22	8.56	0.16	6.0-12.0
Oil	4.05	0.05	3.92	0.04	3.1-5.7
Fibre	1.96	0.03	2.02	0.03	2.0-5.5
Ash	1.32	0.01	1.30	0.02	1.1-3.9
Moisture	8.14	0.04	8.22	0.04	7-23

[1] Values are % dry weight.
[2] Sample size = 30.

Table III.2. Proximate analysis of forage from *Zea mays* event DBT418 (ANZFA, 2002)[1].

Constituent Analyzed	DBT418[2]		Control[3]		Literature Range
	Mean	Standard Deviation	Mean	Standard Deviation	
Protein	6.81	0.23	7.12	0.29	3.5-15.9
Oil	2.77	0.07	2.82	0.06	0.7-6.7
Fibre	20.56	0.03	20.57	0.38	2.0-5.5
Ash	4.33	0.15	4.28	0.13	1.3-10.5
Moisture	66.68	0.04	66.96	0.04	NA

[1] Values are % dry weight.
[2] Sample size = 24.
[3] Sample size = 30.

Table III.3. Proximate analysis of seed from *Gossypium hirsutum* event MON-00531-6 (Berberich et al.1996)[1].

Characteristic	Coker 312		MON-00531-6		Literature Range
	Mean	Range	Mean	Range	
Protein	27.00	23.3-28.4	27.56	22.8-31.0	12-32
Fat	22.95	19.6-25.1	23.23	22.2	16.1-26.7
Ash	4.63	4.3-5.0	4.53	3.9-4.7	4.1-4.9
Carbohydrate	45.40	42.8-47.6	44.68	42.0-46.7	NA[2]
Calories/100g	496.32	479-508	498.11	495-511	NA
Moisture	12.36	9.6-15.9	13.43	11.2-14.7	5.4-10.1

[1] All values are % dry weight except moisture (% fresh weight) and calories/100g.
[2] NA = not available.

Table III.4. Proximate analysis of seed from *Gossypium hirsutum* event MON-15985-7 (USDA APHIS, 2000)[1].

Component	Event 15985		DP50B (event 531)		DP50 (non transgenic)		Commercial reference range
	Mean	Range	Mean	Range	Mean	Range	
Protein %	26.13	21.45-28.82	26.06	21.93-28.15	25.96	21.76-27.79	21.76-28.15
Fat %	20.52	17.54-27.42	20.37	16.04-23.48	19.74	15.44-23.64	15.44-23.83
Ash %	4.36	3.93-4.81	4.38	4.06-4.67	4.34	3.76-4.85	3.76-4.85
Fiber, crude %	16.83	14.93-17.95	17.17	15.42-19.69	17.19	15.38-19.31	15.38-20.89
Carbohydrate %	49.09	42.97-52.69	49.23	46.85-51.93	49.94	45.64-52.44	45.64-53.62
Calories/100g DW	485.33	468.50-520.01	484.45	463.09-498.71	481.57	457.77-499.84	457.77-500.49
Moisture %	5.99	4.34-7.59	6.05	4.22-7.28	6.03	3.97-7.26	3.97-8.47

[1] MON-15985-7 is MON-00531-6 re-transformed to express an additional Cry protein. These data can be considered additional information on Cry1Ac event MON-00531-6. No statistically significant differences are reported between 15985 and the DP50B parent line.

Table III.5. Proximate analysis of seed from *Gossypium hirsutum* event MON-15985-7 (Hamilton et al., 2004)[1].

Component	MON 15985		DP50		Literature Range
	Mean	Range	Mean	Range	
Ash	4.28	3.85-4.92	4.32	3.76-5.23	3.87-5.29
Calories (Kcal/100 g)	489.65	468.50-520.01	487.11	457.77-501.84	471.39-506.95
Carbohydrates	47.95	42.97-52.69	48.55	43.69-52.44	45.28-53.62
Total Fat	21.33	17.54-27.42	20.85	15.44-24.29	17.37-25.16
Crude Fiber	16.07	13.81-17.95	16.22	13.45-19.31	13.85-17.94
ADF[2]	25.68	21.40-31.95	25.26	21.10-34.80	21.10-34.80
NDF[3]	38.75	34.90-46.20	38.97	34.75-43.13	32.92-45.83
Moisture	4.86	2.32-7.59	4.88	2.91-7.26	2.25-7.49
Protein	26.26	21.45-28.82	26.12	21.76-28.24	24.54-30.83

[1] MON-15985-7 is MON-00531-6 re-transformed to express an additional Cry protein. This data can be considered additional information on Cry1Ac event MON-00531-6. All values are % dry weight except moisture which is % fresh weight. No statistically significant differences reported ($P \leq 0.05$).
[2] ADF = acid detergent fiber.
[3] NDF = neutral detergent fiber.

Table III.6. Proximate analysis of seed from *Gossypium hirsutum* event DAS-21023-5 (USDA APHIS, 2003).

Proximate (%)	Cry1Ac Seed	Control Seed	Literature Values
Ash	4.0	3.9	3.76-4.85[1]
Total Fat	23.3	21.9	15.4-23.8[1]
Moisture	2.8	3.2	3.97-8.47[1]
Protein	27.3	26.7	21.8-28.2[1]
Carbohydrates	42.8	44.3	45.6-53.6[1]
Calories (Kcal/100g)	490	481	NA
Crude Fiber	15.7	17.0	15.4-20.9[1]
Acid Detergent Fiber	22.6	24.4	26[2], 37.5[1]
Neutral Detergent Fiber	34.1	34.7	37[2], 52.6[1]

[1] Reported from OECD Draft Consensus Document, 2002.
[2] Reported from NCPA, Cottonseed Feed Products Guide.

Table III.7. Nutritional fiber analysis of seed from *Gossypium hirsutum* event 31807/31808 (USDA APHIS, 1997c)[1].

Event	Crude Fiber	ADF[2]	NDF[3]
31707	30.2	39.7	49.4
31803	31.8	36.8	45.7
31807	32.1	42.1	48.8
31808	31.4	40.4	47.5
42317	31.4	41.8	48.5
Coker 130	31.8	38.1	46.3
Stv. 474	30.4	40.4	47.6
St. LA887	32.5	42.4	49.0
DPL 51	33.8	41.4	48.6

[1] Expressed as percentage of fuzzy seed by weight.
[2] Acid detergent fiber.
[3] Neutral detergent fiber.

Table III.8. Proximate analysis of cottonseed meal from *Gossypium hirsutum* event 31807/31808 (USDA APHIS, 1997c).[1]

Event	Moisture	Crude Fat/Oil	Protein	Ash
31707	2.98	2.53	49.52	6.82
31803	2.00	2.42	49.41	6.36
31807	1.69	2.14	53.31	7.16
31808	2.38	2.27	51.02	6.55
42317	1.69	1.73	49.17	6.53
Coker 130	3.14	2.39	53.10	7.14
Stv. 474	2.34	2.13	44.92	6.18
St. LA887	3.05	2.45	46.12	6.44
DPL 51	2.74	3.01	45.54	6.92

[1] Values are % by weight.