A Review of the Environmental Safety of the Cry1Ab Protein

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INTRODUCTION

This document provides an updated comprehensive review of information and data relevant to the environmental risk assessment of Cry1Ab, a protein encoded by genes isolated from *Bacillus thuringiensis* (Bt), and presents a summary statement about the environmental safety of this protein when produced in genetically engineered (GE) crops. The original version of this review was published in 2011 (AFSI, 2011). All sources of information reviewed herein are publicly available and include dossiers presented to regulatory authorities, decision documents prepared by regulatory authorities, product descriptions prepared by product developers, and peer reviewed literature.

Environmental risk assessments related to the introduction (cultivation) of GE crops are conducted on a case-by-case basis taking into account the biology of the plant, the characteristics of the transgenes and any encoded proteins, the phenotype conferred by the transgene, the intended uses of the crop, and the nature of the receiving environment into which the plant will be introduced. These assessments, which consider both potential hazards and exposure levels, typically involve comparisons to an untransformed parent line or closely related isoline. These comparisons are meant to identify any modifications in the agricultural or phenotypic characteristics of the GE plant, including the intended modification or any biologically significant unintended modifications, that present a potential risk beyond those already considered acceptable when similar, non-GE plants are grown in the environment. The likelihood and consequences of these potential risks, if any, are then evaluated (CBD, 2000; OECD, 2007, 2023; Craig *et al.*, 2008; EFSA, 2010a; OGTR, 2013; Anderson *et al.*, 2021).

The original version of this review documented regulatory approvals[1] for cultivation (typically assessed for environmental safety) of GE maize plants expressing Cry1Ab in nine countries and the European Union. These approvals included event Bt11 (Agrisure™ CB/LL) and event MON810 (YieldGard™, MaizeGard™) and four other single transformation events[2], and nine lines that are 'stacked events', i.e., 'breeding stacks' derived through conventional breeding with Bt11 or MON810 to combine Cry1Ab insect resistance with other GE traits. Up to the writing of this current version of the review in 2025, according to the ISAAA's GM Crop Approval Database (https://www.isaaa.org/gmapprovaldatabase, Accessed December 5,

1. 'Regulatory approval' should not be interpreted as an indication that the product is in commercial production. There are many examples of products that were assessed for safety and granted 'regulatory approval' but were never commercialized, or if they were, have been subsequently discontinued.

2. A 'single-transformation event' refers to the specific, one-time insertion of foreign DNA into a plant genome, creating a distinct GE plant. A 'single transformation event' can have one or multiple ('stacked') genes inserted, different than 'stacked' events developed through conventional breeding. In this document, 'stacked events' refer to events with multiple genes combined through conventional breeding ('breeding stacks').

KEYWORDS

Cry1Ab, *Bacillus thuringiensis* (Bt), insect resistance, genetically engineered (GE), environmental risk assessment (ERA)

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2025), maize events expressing Cry1Ab have now been approved for cultivation (typically assessed for environmental safety) in as many as 20 countries (Tables 1-3). These approvals include the six single transformation maize events recorded earlier and four additional single transformation events approved in China[3], one in 2019 and three others in 2024 (Table 1). MON810 has been approved for cultivation in 12 countries and the European Union[4], and Bt11 in 10 countries. All these approvals were before 2010.

In addition to the single transformation maize events, there have been 19 breeding stacks with MON810 approved for cultivation in at least one of 13 countries[5] (Table 2) and 30 breeding stacks with Bt11 approved for cultivation in at least one of eleven countries[5] (Table 3). Most approved stacks with MON810 are derived from crosses with glyphosate herbicide tolerant NK603 (Roundup Ready™ 2 Maize) with the *cp4 epsps* gene and TC1507 (Herculex™ I, Herculex™ CB) with a modified cry1F gene for lepidopteran insect resistance and the *pat* gene for glufosinate herbicide tolerance. The most common breeding stacks with Bt11 approved for cultivation have been derived from crosses with GA21 (Roundup Ready™ Maize, Agrisure™GT) modified with the *epsps* gene for glyphosate herbicide tolerance and with MIR162 (Agrisure™ Viptera) modified with a vip3 gene for lepidopteran resistance. Approvals for MON810 breeding stacks were all between 2005–2015, while most of the Bt11 breeding stacks were approved between 2010–2020, with a few countries granting more recent approvals for cultivation including in Argentina, Canada, Japan[6], the Philippines and Singapore. The ISAAA database also lists cultivation approvals in China (in 2024) of two maize breeding stacks that express the Cry1Ab protein, nCX-1 × Ruifeng 125 and nCX-1 × Zheda Ruifeng 8 (not shown in the tables). These were derived from conventional crosses of the herbicide tolerant (*cp4 epsps*) event nCX-1 with event Ruifeng 125 expressing Cry1Ab or with event Zheda Ruifeng 8 that expresses Cry1Ab and Cry2Ab[3].

Table 1: Cry1Ab maize single transformation events with cultivation approvals. Approvals after 2020 are highlighted.

Crop	Event Name*	Argentina	Brazil	Canada	China	Colombia	Egypt	EU	Honduras	Japan	Paraguay	Philippines	South Africa	Uruguay	USA
Zea mays (Maize)	Bt176 (176, 1786)	x		x						x					x
	Bt11 (X4334CBR, X4734CBR)	x	x	x		x				x	x	x	x	x	x
	MON810	x	x	x		x	x	x	x	x	x	x	x	x	x
	MON801														x
	MON802			x											x
	MON809			x											x
	DBN9936				x										
	LP026-2				x										
	WYN041				x										
	BBL2-2				x										

**OECD Unique Identifiers for the event names can be found in the ISAAA GM Crop Approval Database.*

3. Approvals in China are according to the ISAAA GM Crop Approval Database, but also see Zhang and Zhou (2026).

4. MON810 is the only maize event expressing Cry1Ab currently listed in the ISAAA GM Crop Approval Database as approved for cultivation by the European Union. MON810 was approved for cultivation in 2003. Bt176 was approved for cultivation from 1998 to 2005 before it was withdrawn from the European market in 2006 (Bardosa *et al.*, 2004; Devos *et al.*, 2006). EFSA issued a scientific opinion on the safety of maize event Bt11 expressing Cry1Ab for cultivation (See Annex 1), although a final authorization has not been issued by the EC.

5. Some countries, including the US, consider breeding stacks with approved single events as safe for cultivation based on the existing approvals of the transformation events from which the stacks are derived and therefore approvals of breeding stacks may not be recorded in these countries.

6. Japan has approved more maize events with Cry1Ab (3 single transformation events and 28 breeding stacks) for cultivation than any other country. There are two reasons for this: 1) to obtain regulatory approvals, even if only for import, all 'Type I' GM events must complete full safety assessments for food, feed, and the environment, and are thus granted approval for food, feed, and cultivation (https://www.scc-gmbh.de/japan-genetically-modified-crop-compliance); 2) Until 2014, Japan required full safety assessments of every stacked event derived from conventional breeding (Iizuka, 2020). Since 2014, only one approval of a stacked maize event with Cry1Ab (one with seven stacked traits) has been issued for cultivation in Japan.

Not only has Cry1Ab expressing maize been approved, but expression of the Cry1Ab protein has been introduced for insect resistance and approved for cultivation in five other crops (Table 4). Three Cry1Ab expressing single transformation cotton events were approved for cultivation in the US in 2011–2012, and five breeding stacks derived from crosses with cotton event T304-40 have been approved for cultivation in three more countries. Two Cry1Ab expressing sugarcane events and one eucalyptus event[7] and a breeding stack have been approved in Brazil, and one Cry1Ab expressing cowpea has been approved for cultivation in Nigeria and Ghana. China has approved two rice events for cultivation, and another rice event has been approved in Iran.

The global trend in cultivation approvals of GE maize, as demonstrated for the *cry1Ab* gene in Tables 2-3, particularly for Bt insect resistance and herbicide tolerance, has clearly been toward events with stacked genes/traits typically obtained by conventional breeding of two or more GE plant varieties of the same species (EFSA, 2007; Goodwin *et al.*, 2021). This is also the trend for other crops including cotton and soybean. In the US, the adoption rates for stacked varieties of maize and cotton with one or more Bt insect resistant and herbicide tolerant traits have increased steadily since 2000, with more than 80 percent of US acreage of these two crops planted with stacked seeds in 2025 (USDA ERS, 2025). Some regulatory authorities have historically reviewed environmental safety for stacked gene products (EFSA, 2007; US EPA, 2025), particularly regarding non-target organism effects from stacked insecticidal proteins, including Cry1Ab. However, as more experience has been gained with the assessment and adoption of GE crops, many countries have developed simplified procedures for regulation of stacked events, generally considering breeding stacks as safe for cultivation based on the existing approvals of the single transformation events from which the stacks were derived (Kok *et al.*, 2014; Goodwin *et al.*, 2021; Sul *et al.*, 2021; US EPA, 2025).

Table 2: Cry1Ab expressing maize event MON810 breeding stacks with cultivation approvals.

Crop	Event Name*	Argentina	Brazil	Canada	Colombia	Japan	Mexico	Pakistan	Paraguay	Philippines	Singapore	South Africa	South Korea	Uruguay
Zea mays (Maize)	NK603 × MON810	x	x	x	x	x		x		x		x		x
	TC1507 × MON810	x	x		x				x	x		x		
	TC1507 × MON810 × NK603	x	x	x	x	x			x	x		x		
	TC1507 × MON810 × MIR162	x	x			x								
	TC1507 × MON810 × MIR162 × NK603	x	x		x	x								
	TC1507 × MON810 × NK603 × MIR604					x								
	TC1507 × 59122 × MON810 × NK603			x		x								
	TC1507 × 59122 × MON810 × MIR604 × NK603			x		x								
	MON810 × MIR162	x	x											
	MON810 × MIR162 × NK603	x												
	MON810 × MON88017			x		x								
	MON863 × MON810					x				x				
	MON863 × MON810 × NK603			x		x								
	NK603 × MON810 x 4114 × MIR604			x		x								
	DP4114 × MON810 × MIR604 × NK603					x	x							
	GA21 × MON810					x								
	LY038 × MON810					x								
	MON87427 × MON89034 × MON810 × MIR162 × MON87411 × MON87419					x								
	T25 × MON810					x								

**OECD Unique Identifiers for the event names can be found in the ISAAA GM Crop Approval Database.*

7. Eucalyptus single transformation event 1521K059, approved for commercial use in Brazil in 2023 (Avisar *et al.*, 2024), is omitted from the ISAAA GM Crop Approval database in error.

Regulatory submissions, safety assessments, and decisions for many of these GE events expressing the Cry1Ab protein are available from different regulatory agencies around the world. A number of those documents for single transformation events of the six crops are publicly available and are listed in Annex 1. Where regulatory decisions or outcomes of regulatory risk assessments are referred to throughout this monograph, examples of these decisions or assessments can be found in the documents in Annex 1.

Table 3: Cry1Ab expressing maize event Bt11 breeding stacks with cultivation approvals. Approvals after 2020 are highlighted.

Crop	Event Name*	Argentina	Brazil	Canada	Japan	Mexico	Paraguay	Philippines	Singapore	South Africa	Uruguay	Vietnam
Zea mays (Maize)	Bt11 × GA21	x	x	x	x		x	x		x	x	x
	Bt11 × MIR162	x	x		x							
	Bt11 × MIR162 × NK603	x					x					
	Bt11 × MIR162 × GA21	x	x	x	x		x				x	
	Bt11 × MIR162 × MIR604		x						x			
	Bt11 × MIR162 × TC1507 × GA21	x		x	x							
	Bt11 × MIR162 × MIR604 × GA21	x	x	x	x		x					
	Bt11 × MIR162 × MIR604 × MON89034 x 5307 × GA21			x	x							
	Bt11 × MIR162 × MON89034	x	x									
	Bt11 × MIR162 × MON89034 × GA21	x	x					x		x		
	Bt11 × MIR162 × MIR604 × TC1507		x									
	Bt11 × MIR162 × NK603 × TC1507			x								
	Bt11 × MIR162 × TC1507 × GA21	x		x	x							
	Bt11 × MIR604		x	x	x							
	Bt11 × MIR604 × GA21			x	x							
	Bt11 × TC1507	x	x									
	Bt11 × TC1507 × GA21	x						x				
	Bt11 × TC1507 × NK603			x								
	Bt11 × DP4114 × MIR162 × MZIR098 × NK603			x								
	Bt11 × DP4114 × MZIR098 × NK603			x								
	Bt11 × MON89034		x									
	Bt11 × MON89034 × GA21	x										
	Bt11 x 59122 × MIR604 × TC1507 × GA21			x	x							
	Bt11 x 5307		x									
	5307 × MIR604 × Bt11 × TC1507 × GA21			x	x							
	5307 × MIR604 × Bt11 × TC1507 × GA21 × MIR162	x	x	x	x							
	3272 × Bt11 x 59122 × MIR604 × TC1507 × GA21				x							
	3272 × Bt11 × MIR162 × GA21	x	x		x	x						
	3272 × Bt11 × MIR162 × MIR604 × TC1507 x 5307 × GA21				x							
	3272 × Bt11 × MIR604 × TC1507 x 5307 × GA21				x							

**OECD Unique Identifiers for the event names can be found in the ISAAA GM Crop Approval Database.*

Table 4: Cry1Ab cotton, cowpea, eucalyptus, rice, and sugarcane with cultivation approvals. Approvals after 2020 are highlighted.

Crop	Event Name*	Argentina	Australia	Brazil	China	Ghana	Iran	Nigeria	USA
Gossypium hirsutum (Cotton)	COT67B (IR67B)								x
	T303-3								x
	T304-40								x
	GHB614 × T304-40 × GHB119			x					
	T304-40 × GHB119	x		x					x
	T304-40 × GHB119 × COT102			x					
	GHB614 × T304-40 × GHB119 × COT102	x	x	x					
	GHB811 × T304-40 × GHB119 × COT102	x		x					
Vigna unguiculata (Cowpea)	AAT709A					x		x	
Eucalyptus sp. (Eucalyptus)	1521K059			x					
	955S024 x 1521K059 × H421			x					
Oryza sativa (Rice)	Shanyou63				x				
	Huahui-1/TT51-1				x				
	Tarom molail + cry1Ab						x		
Saccharum sp. (Sugarcane)	CTB141175/01-A (CTC175-A)			x					
	CTC20BT			x					

**OECD Unique Identifiers for the event names can be found in the ISAAA GM Crop Approval Database.*

ORIGIN AND FUNCTION OF CRY1AB

Bacillus thuringiensis and the Cry1Ab Protein

Insect resistant GE crops adopted at high rates have predominantly been developed using genes from the bacterium *Bacillus thuringiensis* (Bt) (Bravo *et al.*, 2023; Gassmann & Reisig, 2023). *B. 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 (Martínez & Caballero, 2002; Apaydin *et al.*, 2008; Seifinejad *et al.*, 2008). *B. thuringiensis* has been studied extensively and used commercially for many years due to its ability to synthesize proteins with pesticidal properties (Höfte & Whiteley, 1989; Cannon, 1996; Schnepf *et al.*, 1998; OECD, 2007; van Frankenhuyzen, 2009). The species produces diverse insecticidal proteins with distinct structures and modes of action (Crickmore *et al.*, 2021; Liu *et al.*, 2021; Bravo *et al.*, 2023). Of these, the Cry insecticidal proteins, so named because they are the primary component of the protein parasporal crystals that are characteristic of spore formation in *B. thuringiensis*, have been used most commonly (Höfte & Whiteley, 1989; Kumar *et al.*, 1996; Schnepf *et al.*, 1998; OECD, 2007; Liu *et al.*, 2021; Bravo *et al.*, 2023).

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 (US EPA, 1998). Microbial preparations of *B. thuringiensis* are approved for use around the world including in Africa, Australia, Brazil, Canada, the European Union, and the United States (US EPA, 1998; Health Canada, 2008; Kabaluk *et al.*, 2010; do Nascimento *et al.*, 2022; APVMA, 2023; Ragasruthi *et al.*, 2024; EU Pesticides Database, 2025). Bt preparations are the most widely used microbial insecticides in agriculture and forestry and considered a low-risk option to control Lepidoptera, Diptera, and Coleoptera insects, due to their lack of polluting residues and high specificity to target insect species (Adang *et al.*, 2014; Liu *et al.*, 2021; Ragasruthi *et al.*, 2024).

There have been changes to the nomenclature assigned to the insecticidal proteins from *B. thuringiensis* and from other bacteria over the years (Crickmore *et al.*, 2021; Bravo *et al.*, 2023). A systematic nomenclature for identifying and differentiating Cry proteins proposed in 1989 initially grouped them into four classes I, II, III, and IV based on their toxicity to particular orders of insects (Höfte & Whiteley, 1989; OECD, 2007). CryI proteins were those toxic to Lepidoptera, CryII proteins were those toxic to Lepidoptera and Diptera, CryIII proteins were toxic to Coleoptera and CryIV proteins were those toxic to Diptera. As technology for studying the proteins

advanced, it became apparent that proteins with shared sequence homology had different pesticidal specificities, and the proteins were subsequently reclassified not by pesticidal specificities but according to amino acid sequence similarity (Crickmore *et al.*, 1998). Proteins sharing not more than 45% amino acid sequence similarity were divided into primary groups (Cry1, Cry2, etc.). Within those primary groups, proteins with up to 78% sequence similarity were placed into secondary groups (Cry1A, Cry1B, etc.), and proteins within those secondary groups with at least 95% similarity were placed into tertiary groups (Cry1Aa, Cry1Ab, etc.). A fourth level of grouping divided proteins within the tertiary groups that shared more than 95% sequence identity (Cry1Aa1, Cry1Aa2, etc.) (Crickmore *et al.*, 1998).

This four-level naming system within the Cry proteins has been modified only slightly in recent years, based mainly on structural similarities (Crickmore *et al.*, 2021). The Cry proteins are now clearly designated as separate from other bacterial derived-insecticidal proteins (e.g., Cyt, Vip, SIP) based on structural differences, with the defining structure for the Cry proteins being the classic three-domain structure (Crickmore *et al.*, 2021). To date, according to this system based on amino acid sequence identity, there are 755 different Cry proteins described and classified in at least 57 groups and 166 subgroups (Crickmore *et al.*, 2021; Bravo *et al.*, 2023). The proteins designated as Cry1 share at least 45% amino acid sequence similarity, those designated as Cry1A are at least 76% similar, and Cry1Ab proteins are at least 95% identical in amino acid sequence. The Cry1Ab protein shares up to 94% sequence identity with other Cry1A proteins (including Cry1Aa and Cry1Ac) (Crickmore *et al.*, 2021).

Mechanism of Cry1Ab Insecticidal Activity

The classic model for Cry protein mode of action follows a series of steps (Vachon *et al.*, 2012; Pardo-López *et al.*, 2013; Adang *et al.*, 2014; Bravo *et al.*, 2023). The crystal is produced by the Bt bacterium during sporulation and ingested by the insect. The protein in the crystal, in the form of a 'protoxin', is solubilized in the insect midgut, and the solubilized protoxin is activated by midgut proteases. Two different types of Cry protoxins have been described based on their size, short (~70–73 kDa) protoxins characteristic of the Cry2 and Cry3 proteins and long (~120–130 kDa) protoxins characteristic of the Cry1 proteins (OECD, 2007). After activation (cleavage) by host gut proteases, all Cry protoxins yield a ~55–60 kDa three domain 'activated' (toxic) core (de Maagd *et al.*, 2001; OECD, 2007). Cry1 proteins are cleaved at both the C-terminal and N-terminal regions of the protoxin, while cleavage only occurs at the N-terminal region of the shorter Cry2 and Cry3 protoxin, although precise cleavage sites are not clear (Liu *et al.*, 2020).

This 'activated' form of the protein is believed to interact with specific receptors on the surface of midgut epithelial cells, allowing them to insert into the membrane through the formation of transmembrane pores. These pores lead to increased permeability, osmotic imbalance, and disruption of the membrane. This damage to the cells of the midgut epithelial tissues ultimately leads to the death of the insect. Although there are other models for the insecticidal mechanism of the Cry proteins, this classic 'pore formation' model is the most widely accepted (Bravo *et al.*, 2023). It has also been proposed that the binding to receptors of the active toxin in the insect midgut stimulates an intracellular signaling pathway that leads to necrotic cell death with a minimal role of pore formation, although there is more evidence supporting the importance of pore formation than this 'signaling pathway' theory proposes (Soberón *et al.*, 2009; Vachon *et al.*, 2012; Adang *et al.*, 2014; Khorramnejad *et al.*, 2020; Bravo *et al.*, 2023).

A distinguishing characteristic of the Cry proteins is the three-domain structure that forms the 'active' protein central to the insecticidal mode of action (Crickmore *et al.*, 2021; Bravo *et al.*, 2023). The N-terminal domain I is involved in toxin oligomerization, membrane insertion and pore formation; domain II is involved in binding to Cry receptors and is considered an important specificity domain; domain III binds to larval gut proteins and is also involved in stabilization of the three-domain toxic core against protease action (Bravo *et al.*, 2023). Cry proteins show high levels of specificity for their target organism, relying on the specific interaction with the different midgut membrane proteins of the insect that are the Cry receptors, although a number of other factors are involved in determining specificity (Pardo-López *et al.*, 2013; Adang *et al.*, 2014; Jurat-Fuentes & Crickmore, 2017). While a great deal is understood about the Cry protein mode of action and the different receptors, the details by which solubilization, binding, oligomerization, pore formation, and membrane insertion occur and their role in the interaction of the active toxin with the host receptor in determining the specificity of the Cry proteins to target organisms remain topics of study (Jurat-Fuentes & Crickmore, 2017; Bravo *et al.*, 2023).

Modifications to the Cry1Ab Gene and Cry1Ab Protein in GE Plants

There are two main types of modifications to the *cry* genes from Bt that are common for use in GE plants (Sanahuja *et al.*, 2011; Koch *et al.*, 2015). The first type, typical for any bacterial gene expressed in GE plants, involves modifications to the nucleotide sequence. These modifications are primarily used to increase the translation of the gene by modifying codon usage to align with plant-preferred codons. Because the DNA of plants and bacteria differ in nucleotide content (Perlak *et al.*, 1991, Koziel *et al.*, 1993, Sanahuja *et al.*, 2011), to increase the expression of the Cry protein in the plant to a level sufficient for pest control, the sequences used for GE plants have been modified from that in the bacteria to increase the GC content and frequency of ATTTA sequences (Perlak *et al.*, 1991, Koziel *et al.*, 1993, Sanahuja *et al.*, 2011). The use of strong promoters and inclusion of plant introns in the expression construct (Sanahuja *et al.*, 2011), or other enhancers such as the 5' untranslated region of the small sub-

unit of the RUBISCO gene (SSU UTR5') can further increase expression. Directing the cry gene expression into the chloroplast genome of the plant also significantly increases *cry* gene expression (McBride *et al.*, 1995, Sanahuja *et al.*, 2011).

The second type of modification involves modifications to the amino acid sequence of the resulting protein through deletion of sequences outside of the sequence of the core protein responsible for insecticidal activity (Perlak *et al.*, 1991). In most GE crops expressing the Cry1Ab protein, truncations of the Bt protein have been utilized. The Cry protein expressed in these plants consists of the "activated" form of the Cry protein that still requires binding to a specific receptor in the insect midgut and retains the species specificity of the full-length protein (Perlak *et al.*, 1991). Research on the Cry1 proteins suggests that the active toxin and the full-length protoxin may both have a toxic effect on target insects, via different pathways, raising prospects for future genetic engineering with these Cry proteins (Gomez *et al.*, 2014; Tabashnik *et al.*, 2015).

Most approved GE plants expressing the Cry1Ab protein have been developed using these types of modifications to the *cry1Ab* gene and Cry1Ab protein. These modifications do not alter the structure or function of the toxic core protein to the degree that it affects the safety of these proteins, and the history of safe use of the 'native' Cry proteins as a pesticide applied to crops can still be applied to the assessment of these modified proteins (Koch *et al.*, 2015). For these reasons, regulatory authorities have concluded in their safety assessments that these modifications to the Cry1Ab proteins in Bt crops do not raise safety concerns (See Annex 1).

EXPRESSION OF CRY1AB IN INSECT RESISTANT GE PLANTS

The concentration levels of Cry1Ab in various parts of the GE plants, together with information on interactions of organisms with the plant, can be used to estimate the potential exposure to the protein of these organisms in the environment. Transgene expression levels in a GE plant can be influenced by several factors related to the genetic transformation, including the types of promoter and terminator sequences employed, as well as the chromosomal location of the transgene in the genome. Expression levels may also be influenced by the type of tissue, the age of the plant, and the environmental conditions during plant growth (Siebert *et al.*, 2009; Brune *et al.*, 2021). Each transformation event therefore exhibits a different expression profile. Data on the level of expression of Cry1Ab in GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents (See Annex 1). Tissue types (leaf or whole plant) and collection methods differed between studies, but all used an enzyme-linked immunosorbent assay (ELISA) or Western blot analysis to quantify the amount of Cry1Ab protein present in a sample. Variations in methodology for sample collection make direct statistical cross-comparisons of the data inappropriate, but the weight of evidence from regulatory submissions suggests that Cry1Ab is expressed at very low levels relative to the total protein synthesized by the plant. Some examples of expression data of Cry1Ab and references are presented in Annex 2.

NON-TARGET ORGANISM (NTO) ASSESSMENT FOR CRY1AB PROTEIN

The objective of inserting the *cry1Ab* gene into a crop is to provide protection from feeding damage by lepidopteran pests. Other organisms in the environment that are not pests of the GE crop but are directly or indirectly exposed to the Cry1Ab protein are considered non-target organisms (NTOs). The approach to NTO assessments for the earliest Bt crops expressing insecticidal Cry proteins was modeled after that historically applied to chemical and microbial biopesticides (including Bt pesticides) (Romeis *et al.*, 2008; Carstens *et al.*, 2014; US EPA, 2025). For assessing the toxicity of chemical pesticides to NTOs, the "tiered" approach, using a sequential series of tests termed Tier I, Tier II, Tier III and Tier IV, has been used effectively for many years (US EPA, 2007). Tiered testing has also been determined as appropriate for the assessment of potential impacts of Bt proteins and GE crops on NTOs (Dutton *et al.*, 2003; Garcia-Alonso *et al.*, 2006; US EPA, 2007; Romeis *et al.*, 2008, 2011, 2013; Duan *et al.*, 2010; EFSA, 2010a; Carstens *et al.*, 2014; Roberts *et al.*, 2020).

Tier I studies involve highly controlled laboratory environments where the specific NTO or a surrogate species are exposed to high ("worst-case") concentrations of the pesticidal substance (Cry1Ab) being studied to determine if there are any adverse effects (US EPA, 2007; Romeis *et al.*, 2008, 2011; EFSA, 2010a; Roberts *et al.*, 2020). If no adverse effects are observed, additional testing at higher tiers is generally not required (Romeis *et al.*, 2008, 2011; US EPA, 2007, 2010, 2025). If adverse effects are observed in lower tier tests or unacceptable uncertainty exists, additional testing will progress as necessary through higher tiers in order to reduce uncertainty to an acceptable level for decision making (US EPA, 2007, 2010; Romeis *et al.*, 2008; EFSA, 2010a). 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 rises through the tiers (Garcia-Alonso *et al.*, 2006; US EPA, 2007; Romeis *et al.*, 2008; EFSA, 2010a).

The goal of the NTO assessment for a pesticidal substance in GE crops is to evaluate the risk of harm to organisms in the environment, with a special consideration to beneficial NTOs that perform valuable functions in the environment (functional species) (Sanvido *et al.*, 2012; Garcia-Alonso & Raybould, 2014; Devos *et al.*, 2015). The risk to these 'valued' organisms is assessed based on the potential susceptibility of organisms to the pesticidal substance (Cry1Ab) (hazard) and the potential environmental exposure of the organism to that substance (exposure) (Romeis *et al.*, 2008, 2011; EFSA, 2010a; OGTR, 2013). The long history of use for microbial preparations of *Bacillus thuringiensis*, 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 (Höfte & Whiteley, 1989; OECD, 2007; US EPA, 2007, 2025; Koch *et al.*, 2015).

Routes of Exposure and Exposure Assessment

NTOs subject to direct exposure are those that feed on living crop tissues expressing Cry proteins or on their crop residues, either above or belowground. In addition to direct contact with the GE plant expressing the Cry protein, there are three primary routes of exposure typically considered in NTO assessments of Bt crops: exposure to pollen containing the Cry protein, exposure to Cry proteins deposited in the soil by decomposing plant material, and tritrophic exposure via feeding on herbivores (e.g., insect pests) on the GE plants (OECD, 2007; US EPA, 2007). Other routes of exposure considered in NTO assessments for Bt crops include exposure of aquatic organisms to the Cry proteins deposited in water, and the possibility of exposure to organisms should gene flow from the crop to naturally occurring plant populations result in the expression of the Cry protein (OECD, 2007; EFSA, 2010b; US EPA, 2025). The persistence of the Cry proteins in the environment is of importance to determine levels of exposure, however Cry proteins are generally thought not to persist or to remain active for long periods in the environment (soil, sediment or surface waters) at levels that raise safety concerns (Clark *et al.*, 2005; OECD, 2007; Sanvido *et al.*, 2007; Icoz & Stotzky, 2008; Koch *et al.*, 2015; Anderson *et al.*, 2021; US EPA, 2025).

Direct exposure through feeding on living Bt crop tissue, including pollen, remains the most likely route of exposure for NTOs. Regulators typically consider protein expression data generated from different plant tissues of individual events as part of the exposure assessment (See Annex 1). Examples of Cry1Ab protein expression data included in regulatory submissions are shown in Annex 2. Plant tissues producing little or no Cry protein are unlikely to pose a hazard to NTOs. If exposure is expected to be negligible, NTO testing may not be necessary.

Ecotoxicological Testing of Non-Target Arthropods

Typically, potential exposures to organisms are considered and used to determine which 'valued' organisms might be impacted by a pesticidal substance, and then these organisms or representative surrogate species have been tested for adverse effects (Carstens *et al.*, 2014). Because the spectrum of activity for the Cry proteins, and Cry1Ab in particular, is well known, regulatory analysis has focused on confirmation of this spectrum using well characterized test organisms that are amenable and frequently subjected to testing with chemical and microbial pesticides (US EPA, 2001; US EPA, 2007; Carstens *et al.*, 2014; US EPA, 1998, 2025). Tier 1 testing for toxicity to other organisms has focused on non-target arthropod species with potential exposure and those most closely related to the target taxa that are most likely to be susceptible (US EPA, 2025).

The most relevant assessments for Bt crops have focused on non-target arthropods that serve an ecological function such as pollinators, natural predators and parasitoids, decomposers in the soil, and aquatic insects that are important in aquatic food webs and nutrient cycling, as well as insect species that are threatened and endangered or have some cultural significance (Sanvido *et al.*, 2012; Garcia-Alonso & Raybould 2014; Devos *et al.*, 2015; Roberts *et al.*, 2020; US EPA, 2025). Results of studies on organisms more distantly related to the lepidopteran target pests including aquatic organisms (fish), and acute oral toxicity studies on birds and mammals, have also been submitted for regulatory review (US EPA, 2025). Nearly three decades of experience with Bt crop NTO assessment confirm that testing of these more distantly related organisms is of limited value to support risk assessments, as no biologically relevant effects have been seen outside of insects in laboratory toxicity testing (US EPA, 2025).

Test organisms typically include arthropod species that serve as surrogates representing the primary ecological functional groups (Garcia-Alonso *et al.*, 2006; Carstens *et al.*, 2014; Romeis *et al.*, 2013; Roberts *et al.*, 2020) (See Table 5). Because Cry1Ab is known to be toxic to several lepidopteran species, tests have also been conducted on non-target lepidopteran species that are protected or of local importance, such as the monarch butterfly (Mendelsohn *et al.*, 2003; EFSA, 2010a; Sanvido *et al.*, 2012). (See the section below on NTO assessment of Cry1Ab on non-target lepidoptera.) With the Cry proteins, Tier I laboratory testing has been conducted using established protocols on well characterized test organisms, similar to testing with chemical pesticides (OECD, 2007, 2023; Carstens *et al.*, 2014; US EPA, 2007, 2010, 2025; Roberts *et al.*, 2020).

The test materials selected for each study (plant tissues, Bt, or purified protein) are based on the crop biology, expected routes of exposure, the species being evaluated, and whether more ecologically relevant exposures (plant tissues) can be used without compromising the performance of the study (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2011). Pure proteins have been used in many cases in Tier I testing because ingestion of the material could be ensured using artificial diets containing high levels of Cry protein (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008, 2011; EFSA, 2010a). These tests determine the highest level or concentration of the Cry protein at which there is no adverse effect on the test organism (No-observed effect level, NOEL; No-observed effect concentration, NOEC). NOEL/Cs from tier 1 tests with Cry1Ab on key indicator species are shown in Table 5. These test organisms represent a subset of test organisms that has been widely considered in regulatory analysis. The results of these tests have been used to predict effects of the Cry1Ab protein on similar organisms that are expected to be exposed where the crop will be grown.

Table 5: Examples of ecotoxicological test organisms for non-target assessment of Cry1Ab protein reviewed in regulatory decisions. (Results for Cry1Ab from studies on maize events Bt11 and MON810 (reported in US EPA, 2010))

Functional Group	Class and Order	Species	Method of Exposure	Duration of Exposure	NOEL/C*
Pollinator	Insecta Hymenoptera	Apis mellifera (honeybee) larvae	Single dose exposure to protein at 20 ppm	single dose	> 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
Predator	Insecta Neuroptera	Chrysoperla carnea (green lacewing) larvae	Exposure at 16.7 ppm	7 days	> 16.7 ppm
	Insecta Coleoptera	Hippodamia convergens (ladybird beetles)	Single dose exposure at 20 ppm	single dose	> 20 ppm
Parasitoid	Insecta Hymenoptera	Brachymeria intermedia (parasitic hymenoptera)	Single dose exposure at 20 ppm	single dose	> 20ppm
Decomposer	Insecta Collembola	Folsomia candida (Springtails)	Lyophilized leaf tissue (estimated 50.6 µg Cry1Ab/g)	28 days	> 50% of the diet
	Annelida Opisthopora	Earthworm	Exposure to bacterially derived Cry1Ab protein in an artificial soil substrate	14 days	> 200 ppm
Aquatic	Branchiopoda Anomopoda	Daphnia magna	Exposure to Cry1Ab in corn pollen at multiple concentrations	48 hours	> 150 mg/L
Mammal	Mammalia Rodentia	Mus musculus (mouse)	Acute oral gavage at 3280 mg/kg	single dose	No observed effect

**NOEL /C - No-observed effect level/No-observed effect concentration*

Margins of Exposure

For NTO assessment the highest concentration levels of Cry protein in relevant plant tissues (based on the routes of exposure and the test organism) are used to calculate an estimated environmental dose (EED) or estimated environmental concentration (EEC) representing 'worst-case' exposures to key indicator test species. This can be compared to the NOEL/C for a test organism to determine a 'margin of exposure' (MOE). As an example, for the environmental risk assessment of eucalyptus event 1521K059 approved for cultivation in Brazil (CTNBio, 2023), Tier 1 tests to determine the NOEL/C were conducted following OECD guidelines for the three Cry proteins (Cry1Ab, Cry1Bb and Cry2Aa) expressed in that event. Although the NOEL/C of Cry1Ab is established for key indicator species (see Table 5) and it was not necessary to generate new data, these tests were conducted again with Cry1Ab for the eucalyptus event at the time Tier I studies were conducted with the other two Cry proteins for which there was limited biosafety data (Avisar *et al.*, 2024). For Cry1Ab, the NOEL/C for the test organisms, calculated as the highest soluble recombinant Cry1Ab protein incorporated into the diet (or liquid habitat for D. magna), are shown in Table 6.

The highest levels of the three Cry proteins expressed based on anticipated routes of exposure to Cry1Ab in the relevant eucalyptus plant tissues (see Table 2.5 in Annex 2 for Cry1Ab expression levels in eucalyptus tissues) were used to calculate the EED or EEC (Avisar *et al.*, 2024). For honeybees, the highest pollen concentration measured in the field of 5.08 ug/g was converted with established pollen intake rates for honeybee larvae and adults to an EED of 0.0104 ug/g per larva and 0.0218 ug/g per adult worker bee (Table 6). Estimates for soil-dwelling earthworm and springtail were based on the maximum concentration in 1521K059 leaf tissue of 53.76 ug/g (Table 6). For D. magna, the maximum leaf concentration was converted to an EEC of 0.68 mg/L in the test water, using an estimate of the eucalyptus biomass according to the pond model based on maize (Table 6) (Avisar *et al.*, 2024). These EED/EECs were then compared to the NOEL/C to determine the margin of exposure (MOE) (Table 6). The margin of exposure for Cry1Ab ranged from 44 to 1694 times, indicating that the EED/EEC values were tens to hundreds of times lower than the respective NOEL/C (Avisar *et al.*, 2024).

This methodology is the globally accepted approach for Tier 1 testing in non-target organism assessment for Bt crops and the methodologies for its application have been well defined through the years of experience gained from GE crop risk assessment and approvals (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008; Romeis *et al.*, 2011; EFSA, 2010b; US EPA, 2025). Based on NTO assessment following this approach, regulatory authorities in countries where cultivation of Cry1Ab-expressing maize, cotton, cowpea, eucalyptus and sugarcane events are authorized have concluded that toxicity of Cry1Ab to non-lepidopteran NTOs is highly unlikely even at high doses, and at levels well beyond the exposure level expected in the environment (See Annex 1).

Table 6: Calculated values for the no-observed effect level/concentration (NOEL/C), estimated environmental concentration/dose (EED/EEC), and the margin of exposure of the Cry1Ab protein in eucalyptus event 1521K059 (extracted from Avisar *et al.*, 2024).

Species	NOEL/C	EED/EEC	MoE (times)
Honeybee *Apis mellifera* larvae	4 ug/larvae	0.0104 ug/larvae	386
Honeybee *Apis mellifera* adults	37 ug/day	0.0218 ug/day	1694
Earthworm *Eisenia fetida*	2600 ug/g	53.76 ug/g diet	48
Springtail *Folsomia candida*	2600 ug/g	53.76 ug/g diet	48
Daphnia magna	30 mg/L	0.68 mg/L	44

Protein Interaction Effects in NTO Assessment

The purpose for stacking multiple insecticidal substances such as the Cry proteins in crops is often to delay the development of resistance in the target organism by using different modes of action or to increase the efficiency of the product by targeting a wider spectrum of pests. However, as the introduction of GM crops with combinations of insecticidal proteins increased, concerns were raised over the potential for these combinations to have interactive effects (synergism, antagonism, or additive) that present an increased hazard to NTOs (Liu *et al.*, 2024; US EPA, 2025). Some regulatory authorities have requested data, including for 'breeding stacks' derived from approved single transformation events, to evaluate the potential for synergistic effects (i.e., greater than additive) as part of the NTO assessment (EFSA, 2010; US EPA, 2009, 2025; Sul *et al.*, 2021). Data requirements for stacked pesticidal proteins have included concentration-response studies, challenging sensitive insects (often the susceptible target organism) with individual and combinations of two or more test substances, to test for synergistic effects (EFSA, 2010; US EPA, 2025; Liu *et al.*, 2024).

Bt crops expressing the Cry1Ab protein in combination with other Cry proteins have been the subject of concentration-response studies (Koch *et al.*, 2015; Graser *et al.*, 2017; Walters *et al.*, 2018; Liu *et al.*, 2024). In general, the literature strongly supports only rare occurrence of synergistic interactions. In a review, Walters *et al.* (2018) considered 179 interaction experimental results and concluded that synergistic interactions are rarely found between Cry1, Cry2, Cry 3, Cry 9, and Vip3A insecticidal proteins, and elevated levels of synergism are even more unlikely. For combinations of these Cry proteins in GM crops, it should be possible to apply scientific rationale in the place of laboratory studies to determine that a hypothesis for harm to NTOs due to high levels of synergism is not plausible (US EPA, 2025).

NTO Assessment on Non-Target Lepidoptera

GE crops expressing the Cry1Ab protein are intended to control lepidopteran pest species of those crops. Lepidopterans directly exposed to the Cry1Ab protein through feeding on those crop plants are generally considered pests. Non-pest, non-target Lepidoptera that might also be susceptible to the Cry1Ab protein have been assessed considering incidental or indirect routes of exposure. If exposure at levels that are harmful to non-target lepidoptera is not likely, Tier 1 testing should not be necessary. However, 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). This study lacked proper experimentation methods and interpretation and was strongly criticized by the scientific community (Shelton & 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 (Hansen Jesse & 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 (Hellmich *et al.*, 2001; Stanley-Horn *et al.*, 2001) indicated that Bt176 (which has a pollen-specific promoter driving expression) maize pollen caused lethality at low concentrations but Bt11 and MON810 pollen showed negligible effects at concentrations up to and exceeding 1000 pollen grains/cm2. 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/cm2 (Hellmich *et al.*, 2001; Pleasants *et al.*, 2001; OECD, 2007). 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. This confirmed earlier risk assessments which predicted negligible impacts due to the low exposure of non-target Lepidoptera to pollen or other plant tissue containing Cry1Ab (See Annex 1).

Field Studies on Bt Crops and NTOs

Following the NTO tiered testing approach, higher-tier field tests are only performed when data generated in lower tier tests, such as laboratory tests, identify potential risks to a

particular group of NTO. For Bt crops if no adverse effects on NTOs are likely, given the specificity of the protein, the pattern of exposure and the results of Tier I testing, NTO field testing is not necessary. However, in the early years, when much less information was available on Bt crops, some regulatory authorities requested generic NTO field testing, comparing arthropod population numbers in Bt and non-Bt events. A number of independent research studies have also been conducted. These studies, primarily on Bt-maize (including Cry1Ab-expressing maize), have been the subject of a number of meta-analyses that have synthesized aggregate data from dozens to hundreds of independent studies (Marvier *et al.*, 2007; Duan *et al.*, 2008, 2010; Wolfenbarger *et al.*, 2008; Naranjo, 2009; Comas *et al.*, 2014; Pellegrino *et al.*, 2018; Krogh *et al.*, 2020; Meissle *et al.*, 2022). The consensus among these meta-analyses is that the cultivation of Bt crops does not result in adverse effects (e.g., reduction in overall abundance, species richness, or function) on non-target arthropods when compared to untreated, conventional non-Bt control crops (Marvier *et al.*, 2007; Wolfenbarger *et al.*, 2008; Comas *et al.*, 2014; Meissle *et al.*, 2022). The meta-analysis on the effects of Bt-maize on non-target invertebrates by Meissle *et al.* (2022) used more data, more recent studies, and a more systematic-review framework to largely confirm the conclusions of earlier reviews and meta-analyses that effects of Bt-maize on non-target invertebrates are generally small and mostly neutral. In this analysis, it was noted that the Cry proteins most studied were the lepidoptera-active Cry proteins, led by Cry1Ab and including Cry1A.105, Cry1Ac, Cry1F, Cry1Ie, Cry2Ab, alone or in breeding stacks (Meissle *et al.*, 2022).

One noted exception to the neutral effects on population levels of NTOs in field studies is that populations of specialist parasitoids and certain predators can be lower in Bt fields (Wolfenbarger *et al.*, 2008; Naranjo, 2009; Meissle *et al.*, 2022). These declines, however, are thought to represent a predictable food-web effect and are not caused by the toxicity of the Bt Cry proteins; because Bt crops effectively eliminate the target pest, the specialists that rely entirely on those pests for hosts or prey experience localized population declines (Romeis *et al.*, 2006, 2014; Meissle *et al.*, 2022). Meta-analyses that specifically compared hazard results from Tier 1 laboratory testing (both direct bi-trophic exposure and indirect tri-trophic exposure) against actual population abundance data gathered in the field also concluded that negative effects on natural enemies, occasionally reported in tri-trophic laboratory studies involving feeding on nutritionally compromised, Bt-intoxicated prey, are rarely realized in the field (Naranjo, 2009; Duan *et al.* 2010). In contrast, when Bt-maize was compared with pyrethroid-treated non-Bt-maize, predator populations were often higher in Bt fields. A conclusion that Bt-maize has less impact on non-target invertebrates than conventional broad-spectrum insecticide programs has been reinforced from other analyses of field studies (Marvier *et al.*, 2007; Wolfenbarger *et al.*, 2008; Naranjo, 2009; Meissle *et al.*, 2022). Overall, data from field studies support the conclusion in regulatory environmental risk assessments that adverse effects on NTO diversity, abundance, or function in crops expressing the Cry1Ab protein are unlikely (See Annex 1).

Cry1Ab Protein NTO Assessment Summary

Regulatory authorities have determined that adverse effects on NTOs are unlikely for several reasons (See Annex 1). First, Cry1Ab has a narrow spectrum of pesticidal activity. Second, Tier I studies have demonstrated that Cry1Ab has no observable effect on representative vertebrate species. Third, Tier I laboratory assays, employing a range of invertebrate species present in maize, cotton and soybean agricultural ecosystems, or surrogates for those species, have shown that Cry1Ab causes no significant observable effects in these species. Fourth, the levels of Cry1Ab used in these Tier I assays were much higher than those measured in the GE maize, cotton, and soybean tissues growing in the field. Fifth, adverse effects on Cry1Ab sensitive non-target Lepidoptera insects are considered unlikely due to low exposure in the environment of these non-pest insects to Cry1Ab at expression levels in crop tissues. Sixth, synergistic interactions between Cry1Ab and other insecticidal substances combined in GE crops that could lead to adverse effects on NTOs are considered unlikely. Seventh, meta-analyses of numerous field studies of Bt crops (primarily maize, including Cry1Ab maize) varieties suggest that adverse effects on NTO diversity is unlikely, and traditional insect control using chemical pesticides significantly alters species diversity and harms non-target species compared to insect control via Cry proteins, including Cry1Ab. Together, these findings indicate that Cry1Ab is unlikely to have adverse effects on natural populations of organisms, except for the target lepidopteran crop pests it is meant to control.

ESTABLISHMENT AND PERSISTENCE OF CRY1AB-EXPRESSING PLANTS

Biology of the Plant Species

The biology of the non-GE plant species in the receiving environment is typically the starting point for environmental risk assessments of GE plants (OECD, 2007, 2020, 2023; OGTR, 2013). Information about the biology of the non-GE plant can be used to identify species-specific characteristics that may be affected by the Cry protein that may contribute to increased weediness in agricultural fields, invasiveness in natural habitats, or 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, whether the plant species is an annual or perennial species or is self-pollinated or wind pollinated may be relevant to assess the likelihood of the GE plant establishing and persisting outside

of cultivation. The Organization for Economic Cooperation and Development (OECD) has produced crop biology documents for maize (OECD, 2003), cotton (OECD, 2008a), sugarcane (OECD, 2013), eucalyptus (2014), cowpea (OECD, 2015), and rice (OECD, 2021) that have been used for the environmental risk assessment of GE crops expressing Cry1Ab.

Phenotypic Data

Information about the phenotypic characteristics of GE plants expressing Cry1Ab compared to the non-GE plant 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 (Craig *et al.*, 2008; EFSA, 2010a; EFSA, 2015; Jose *et al.*, 2020). Phenotypic data in regulatory submissions and peer reviewed publications have focused on characteristics of the plant that might contribute to its establishment or persistence (i.e., potential weediness), or those that may negatively affect agricultural performance (e.g., disease susceptibility and yield data). Some authorities have also considered data from comparisons of compositional characteristics of crops to identify unintended changes in phenotype that might impact environmental safety (EFSA, 2010a), although this data is more typically generated for food safety assessment of GE crops (Codex, 2003b; EFSA, 2011).

The phenotypic observations take into account the desired phenotype resulting from the transgenic trait, in this case insect resistance mediated by Cry1Ab. Some of the collected data are quantitative (e.g., plant height or percent seed germination) while other data are qualitative and observational (e.g., symptoms of disease susceptibility). Where differences between GE plants and controls are detected, an assessment will consider the biological significance of the difference and whether the phenotype of the GE crop is within the reported range for the non-GE crop. Collectively, the phenotypic data considered in risk assessments for cultivation approvals do not support the hypothesis that the expression of Cry1Ab protein has any unintended impact on the agronomic or phenotypic characteristics of the GE plant (See Annex 1).

Weediness and Invasiveness of the GE Crop

In environmental risk assessments of GE crops, the potential for adverse effects due to increased weediness of the crop plant in agricultural environments or increased invasiveness of the crop plant in non-agricultural environments are key considerations (EFSA, 2010a; OGTR, 2013; OECD, 2023). An understanding of the biology of the crop is central to these assessments (Bachman *et al.*, 2021). To determine potential weediness in GM crops, regardless of the introduced trait, risk assessments consider whether there is inherent weediness in the crop plant, or if it has "weedy" characteristics, and the ability of the crop plant to volunteer or to establish in agricultural fields without human intervention (Raybould, 2010). Similarly, risk assessments consider whether the crop plant is known to be invasive outside of agriculture or if it has invasive characteristics. This information is included in the OECD consensus documents on biology of the approved crops that express Cry1Ab (OECD, 2003, 2008, 2013, 2014, 2015, 2021). Although weedy or invasive characteristics in these six crops (maize, cotton, rice, cowpea, eucalyptus and sugarcane) vary considerably, none are considered weeds or invasive in the regions where they are grown as crops and therefore are less likely to become so from the addition of a new trait.

Concurrent to consideration of the weediness or invasiveness of the non-GE crop, a risk assessment considers the likelihood that the introduced trait, in this case expression of Cry1Ab protein for resistance to certain lepidopteran pest insects, might increase the 'fitness' (i.e., survival or persistence) of the crops in or outside of agricultural fields, thereby increasing the weediness or invasiveness of the GE crop compared to the non-GE crop (Raybould & Cooper, 2005; OECD, 2023). In the case of the approved crops expressing Cry1Ab protein, considering the biology of the crop including potential interactions with Cry1Ab sensitive organisms, regulatory decisions have determined that resistance to certain lepidopteran insects (target or non-target) is unlikely to increase establishment or persistence of the crop in agricultural fields or outside of agricultural fields to a level of environmental concern (See Annex 1).

Adverse Effects Due to Gene Flow from the GE Crop

Pollen-mediated movement of a gene from a GE plant to compatible relatives, or gene flow, is possible if a crop is growing in proximity to sexually compatible wild or 'free-living' populations of a sexually compatible plant (Raybould & Cooper, 2005; Ellstrand *et al.*, 2013). The rate of pollen flow and the production of reproductively viable hybrids depend on the biology of the crop species and the physical and temporal proximity of the GE plants to sexually compatible species (Raybould & Wilkinson, 2005). The relevant reproductive characteristics and the location of compatible relatives are described for the six crops (maize, cotton, rice, cowpea, eucalyptus and sugarcane) in the OECD Consensus Documents (OECD, 2003, 2008, 2013, 2014, 2015, 2021). Insect resistance due to expression of the Cry1Ab protein is not expected to increase the frequency of gene flow compared to that naturally occurring between the non-GE crop and compatible relatives where they are grown.

Should there be gene flow, risk assessments consider whether the introduced trait, in this case expression of the Cry1Ab protein for insect resistance, is likely to increase the establishment or persistence of the wild relative or hybrid populations such that these plants become weeds in agriculture or invasive outside of agriculture. The assessment considers whether any lepidopteran insect pests with susceptibility to the Cry1Ab protein may limit the establishment or persistence of the wild relative or hybrids

between wild relatives and the non-GE crop in comparison to other limiting factors, and whether there could be release from a natural constraint imposed by such an insect (Raybould & Cooper, 2005; EFSA, 2010a; OECD, 2023). In addition, assessments consider whether there might be adverse effects (e.g. reduction of abundance or loss of function) to NTOs following gene flow to compatible wild relatives, and this may depend on interaction of Cry1Ab susceptible insect species with the wild relative of the crop.

Regulatory decisions have concluded that it is unlikely that there will be adverse effects related to gene flow from the approved crops expressing the Cry1Ab protein (See Annex 1). For most of these approved crops, a lack of proximity to wild relatives reduces the likelihood of adverse effects related to gene flow. In the case of the approved Cry1Ab insect resistant cowpea in Ghana and Nigeria, the domesticated cowpea (*Vigna unguiculata* ssp. *unguiculata* var. *unguiculata*) is grown in proximity to a compatible wild progenitor variant of the species, *Vigna unguiculata* ssp. *unguiculata* var. *spontanea*, which persists in fields and field margins, and gene flow is considered likely in this 'crop-weed complex' (Huesing *et al.*, 2011; OECD, 2015). However, based on what is known about the weedy or invasive characteristics of cowpea and this progenitor variant and about factors limiting the hybrid populations and the interaction of lepidopteran insects with wild cowpea, an adverse effect from the expression of Cry1Ab in wild cowpea or hybrid populations is not expected (Huesing *et al.*, 2011; CSIR-SARI, 2020).

CONCLUSION

The Cry1Ab protein expressed in insect resistant GE crops is derived from the common soil bacterium *B. thuringiensis* and is toxic to certain lepidopteran insect species. Six GE crops expressing Cry1Ab protein have been approved for cultivation and subjected to environmental risk assessment in as many as 20 countries. The spectrum of activity of the Cry1Ab protein for organisms in the environment other than the target pest is well characterized. Toxicity testing with a range of representative non-target organisms (NTOs) produced NOEL/C values at concentrations many times higher than the expected environmental exposure to Cry1Ab in the approved crops. Meta-analyses of field studies confirm that the Cry1Ab protein expressed in crops is unlikely to have adverse effects on non-target arthropods. Cry1Ab in plants can be toxic to non-target Lepidoptera, but regulatory risk assessments for approved products based on laboratory and field studies have concluded that the low likelihood of exposure results in negligible additional risk compared to other agricultural practices. Analyses of phenotypic data demonstrate that Cry1Ab expression in approved GE crop events does not alter the agronomic or phenotypic characteristics of the plant other than the expression of Cry1Ab for lepidopteran insect resistance, and the expression of Cry1Ab protein in these crops is not likely to increase weediness or invasiveness in the crops compared to their conventional counterparts. If gene flow were to occur from these crops to other compatible plants, the Cry1Ab protein is unlikely to increase the weediness or invasiveness of those other plants or adversely affect non-target organisms exposed to the Cry1Ab protein in those other plants. To date, regulators have consistently concluded based on a weight-of-evidence approach that Cry1Ab in GE crops does not pose an environmental safety concern.

ANNEX 1: REGULATORY DOCUMENTS OF SOME CROP EVENTS EXPRESSING THE CRY1AB PROTEIN APPROVED FOR CULTIVATION

Crop/ Event	Title	Reference
Maize Bt176	Petition for Determination of Nonregulated Status of CIBA Seeds Corn Genetically Engineered to Express the Cry1A(b) protein from *Bacillus thuringiensis* var. kurstaki	USDA APHIS 1994
	Plant Pesticide Bacillus Thuringiensis CryIA(b) Delta-Endotoxin and the Genetic Material Necessary for its Production (Plasmid Vector pCIB4431) in Corn	US EPA 1995
	Decision Document DD96-09: Determination of Environmental Safety of Event 176 Bt Corn (*Zea mays* L.) Developed by Ciba Seeds and Mycogen Corporation	CFIA 1996a
	Approved Type 1 Use Regulation. Maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Modified cry1Ab, bar, *Zea mays* subsp. mays (L.) Iltis) (Event176, OECD UI:SYN-EV176-9)	J-BCH 2007a
Maize Bt11	Petition for Determination of Nonregulated Status for: Insect Protected Corn (*Zea mays* L.) Expressing the Cry1A(b) Gene from *Bacillus thuringiensis* var. kurstaki	USDA APHIS 1995
	Decision Document DD96-12: Determination of Environmental Safety of Northrup King Seeds' European Corn Borer (ECB) Resistant Corn (*Zea mays* L.)	CFIA 1996b
	Opinion of the Scientific Panel on Genetically Modified Organisms on a request from the Commission related to the notification (Reference C/F/96/05.10) for the placing on the market of insect resistant genetically modified maize Bt11, for cultivation, feed and industrial processing, under Part C of Directive 2001/18/EC from Syngenta Seeds	EFSA 2005
	Approved Type 1 Use Regulation. Maize resistant to Lepidoptera and tolerant to glufosinate herbicide (Modified cry1Ab, pat, *Zea mays* subsp. mays (L.) Iltis) (Bt11, OECD UI:SYN-BTØ11-1)	J-BCH 2007b
	Technical Opinion No 1255-2008. Commercial release of genetically modified insect-resistant corn event Bt11	CTNBio 2008
	Cry1Ab and Cry1F *Bacillus thuringiensis* (Bt) Corn Plant-Incorporated Protectants	US EPA 2010
Maize MON810	DD1997-19: Determination of the Safety of Monsanto Canada Inc.'s YieldgardTM Insect Resistant Corn (*Zea mays* L.) Line MON810	CFIA 1997
	Approved Type 1 Use Regulation. Lepidoptera resistant maize (cry1Ab, *Zea mays* L.) (MON810, OECD UI:MON-ØØ81Ø-6)	J-BCH 2004
	Technical Opinion No 1100-2007. Commercial release of genetically modified insect-resistant corn event MON810	CTNBio 2007
	Cry1Ab and Cry1F *Bacillus thuringiensis* (Bt) Corn Plant-Incorporated Protectants	US EPA 2010
	Scientific Opinion updating the risk assessment conclusions and risk management recommendations on the genetically modified insect resistant maize MON 810	EFSA 2012
Cotton COT67B	Petition for determination of nonregulated status event COT67B.	USDA APHIS 2007
	BRAD *Bacillus thuringiensis* modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton	US EPA 2008
	Approved Type 1 Use Regulation. Cotton resistant to Lepidoptera (Modified cry1Ab, *Gossypium hirsutum* L.) (COT67B, OECD UI: SYN-IR67B-1)	J-BCH 2012
	Decision Document DD2011-83 Determination of the Safety of Syngenta Seeds Canada Inc.'s Cotton (*Gossypium hirsutum*) Event COT67B	CFIA 2017
Cotton T304-40	Petition for determination of nonregulated status for Insect resistant and glufosinate ammonium-tolerant cotton (T304-40)	USDA APHIS 2008
	Decision Document DD2011-87: Determination of the Safety of Bayer CropScience Inc.'s TwinLink (T304-40 X GHB119) cotton (*Gossypium hirsutum*) and Cotton Events T304-40 and GHB119	CFIA 2011
	Final Cry1Ab-Cry2Ae-TwinLink BRAD 01-26-12	US EPA 2012
	Approved Type 1 Use Regulation. Cotton tolerant to glufosinate herbicide and resistant to Lepidoptera (modified bar, modified cry1Ab, *Gossypium hirsutum* L.) (T304-40, OECD UI: BCS-GHØØ4-7)	J-BCH 2013
Cowpea AAT709A	Decision Document for a permit for the Commercial release of Pod Borer - Resistant Cowpea (PBR – Cowpea)-event AAT709A, genetically modified for lepidopteran insect pest (Maruca vitrata) resistance, issued to Institute for Agricultural Research (IAR), Zaria	NBMA-Nigeria 2019
	Council for Science and Industrial Research (CSIR) - Savanna Agricultural Research Institute. 2020. Insect Resistant Cowpea Event 709A. Regulatory Dossier Number AAT/DPS-20VUIR-GH.	CSIR-SARI 2020
	Review of Risk Assessment on an Application from the Council for Scientific and Industrial Research (CSIR), Savanna Agricultural Research Institute (SARI) for Commercial use of Cowpea (*Vigna unguiculata* L. Walp.) Genetically Modified for Resistance to Maruca pod borer (Maruca vitrata) Event AAT-709AA-4 (hereafter referred to as 709A cowpea) in Ghana	NBA-Ghana 2022
Eucalyptus 1521K059	Technical Opinion No.8393/2023. Commercial release of genetically modified eucalyptus 1521K059 and its derivatives	CTNBio 2023
Sugarcane CTC175-A	Technical Opinion No 5483/2017. Commercial release of genetically modified sugarcane for insect resistance	CTNBio 2017

ANNEX 2: SUMMARY OF CRY1AB PROTEIN EXPRESSION DATA

The tables that follow present summary data from regulatory submissions and decision documents. 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 2.1a: Specific concentration of Cry1Ab protein in Bt11 transgenic corn tissues during the life cycle (From USDA, 1995)

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 Pith							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)

* 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.

** Stalk includes: stalk, ear shank, tassel, cob, roots, and silk.

Table 2.1b: Cry protein tissue expression in Bt11 (From US EPA, 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)

**Values indicate fresh tissue weight unless otherwise noted.*

Table 2.2: Protein expression levels in the insect-protected corn lines as determined by ELISA analysis (MON810 - From 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 2.3: Mean Cry1Ab protein expression levels in Event T304-40 Cotton (From US EPA, 2012)

Tissue Type	A.1. Cry1Ab Protein (µg/g dry weight ± SD)*
Roots	3.8 ± 0.9 – 8.6 ± 2.6
Stems	0.88 ± 0.37 – 6.2 ± 1.1
Leaves	0.45 ± 0.08 – 4.6 ± 0.6
Squares	2.90 ± 1.0
Apex	1.3 ± 0.3
Bolls	0.38 ± 0.2
Whole plants	2.2 ± 1.1
Pollen	NA[a]
Nectar	NA[a]
Flowers	10.6 ± 1.9
Cottonseed	4.10 ± 1.6

**Range reflects means at different growth stages*
[a]Not applicable due to the insufficient availability of material.

Table 2.4: Concentrations of Cry1Ab and NPTII in tissue samples derived from 709A cowpea (From CSIR-SARI, 2020)

Tissue Samples	Cry1Ab (µg/g FWT)[a]	NPTII (µg/g FWT)
Leaf	5.1 (1.9–8.1)	0.33 (0.21–0.45)
Flower	4.5 (3.2–5.2)	0.21 (0.21–0.21)
Pods	3.7 (2.9–4.0)	0.35 (0.31–0.41)
Green Cotyledon	2.2 (1.95–3.0)	0.16 (0.15–0.16)
Dry Seed	2.5 (2.3–2.8)	1.5 (1.3–1.6)
Pollen	0.12 (0.06–0.18)	0.22 (0.21–0.23)
Anther Wall	1.1 (0.98–1.2)	0.30 (0.29–0.32)
Roots	0.25 (0.18–0.33)	0.18 (0.06–0.30)

[a] Values represent the mean of replicate samples with the lowest and highest individual values shown in parentheses. Concentrations are uncorrected for extraction efficiency and expressed in µg protein per gram fresh weight tissue (FWT).

Table 2.5: Maximum measured protein expression levels across farms and tree age (µg/g). The BOLD highlighted results were used for EED/D estimations. (Eucalyptus Event 1521K059 - From Avisar *et al.*, 2024):

Tissue	Age	Cry1Ab	Cry1Bb	Cry2Aa
Young leaves	6	51.75	5.8	9.73
	12	40.21	4.71	7.27
	24	28.96	3.22	9.38
Mature leaves	6	53.76	8.33	9.24
	12	30.15	3.07	6.44
	24	18.7	3.26	6.65
Stem	6	21.66	0	1.05
	12	13.72	0	0.51
	24	8.01	0	0.33
Roots	6	7.61	0	0.77
	12	4.59	0	0
	24	2.04	0	0
Pollen	During flowering	5.08	0	1.53

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