A Review of the Food and Feed Safety of the Cry1Ab Protein

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December 31, 2016

INTRODUCTION

This document provides a comprehensive review of information and data relevant to the assessment of the protein Cry1Ab for food and feed safety. To date, three genetically engineered (GE)[1] crops (cotton, maize and rice) in which the Cry1Ab protein is expressed have been approved in at least one country (Table 1). To date, regulatory approvals for the food and/or feed use of these crops have been issued in 18 countries and the European Union (EU), representing 42 transformation events. In total, there are 162 regulatory approvals in these countries[2].

All sources of information cited in this document are publicly available and include: dossiers presented to regulatory authorities; decision summaries prepared by regulatory authorities; peer reviewed literature; and product summaries prepared by product developers. The safety assessments in these documents typically involve comparisons to an untransformed parent line or closely related isoline [1]–[8]. The point of these comparisons is to identify risks to the food supply that the GE plant might present beyond what is already accepted for non-GE varieties of the plant. Any identified risks can then be assessed for their potential consequences.

The Codex Alimentarius Guidance CAC/GL 45-2003 (Codex Guidance) covers safety assessment of foods derived from GE plants [6], and provides a framework for conducting food safety assessment on GE plants. Safety assessments related to the use of GE plants in food and feed are conducted on a case-by-case basis, taking into account the following factors:

- The biology of the unmodified plant;
- The traditional uses of the unmodified plant in food and feed;
- The intended uses of the GE plant in food and feed;
- The nature of the transgene, the donor organism, and the protein it produces;
- The phenotype conferred by the transgene;
- Compositional analyses of key components including metabolites;
- The presence of known toxins, allergens, and anti-nutritional substances;
- Toxicologic and allergenic properties of the expressed protein;
- Feeding studies for GE plant that is intended to confer nutritional improvement;
- The potential impact of food and feed processing on safety.

Since this monograph is on the safety of the Cry1Ab protein and not on GE crops containing the protein, not all the safety assessment elements in the Codex Guidance are relevant. The three topics covered in this monograph are "Origin and Function of Cry1Ab (including its mechanism of action on targeted species), "Expression of Cry1Ab in Insect-Resistant GE Plants" (including the expression levels of Cry1Ab in various parts of the crops), and "Food and Feed Safety of the Cry1Ab Protein" (including information on toxicology and allergenicity assessments).

Key words

Cry1Ab, *Bacillus thuringiensis*, insect resistance, genetically engineered, environmental risk assessment

[1]GE crops are crops that have been modified using techniques of modern biotechnology to impart one or more desirable traits such as protection from insects, resistance to herbicides, and improved nutrient profiles.

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

Table 1. Global regulatory approvals of Cry1Ab events in GE crops for food and/or feed uses [9].

Species	Event Name	Argentina	Australia	Brazil	Canada	China	Columbia	El Salvador	EU	Japan	Korea	Mexico	Netherlands	Philippines	Russia	South Africa	Switzerland	Taiwan	UK	Uruguay	USA
Gossypium hirsutum **(Cotton)**	COT67B (IR67B)		x		x					x											x
	T304-40		x	x	x	x			x	x											x
	GHB614 x T304-40 x GHB119			x						x	x										
	T304-40xGHB119			x	x						x										x
Zea mays **(Maize)**	Bt11	x	x	x	x	x	x		x	x	x	x		x		x	x		x	x	x
	Bt176	x	x		x	x			x	x	x		x	x		x	x				x
	MON801																				x
	MON802				x																x
	MON805																				x
	MON809				x																x
	MON810	x	x	x	x	x			x	x	x	x		x	x	x	x			x	x
	MON830																				x
	MON831																				x
	MON832																				x
	3272 × Bt11 × MIR604 × GA21									x	x	x		x							
	Bt11 x DAS-59122 x MIR604 x TC1507 x GA21									x	x	x		x				x			
	BT11 x GA21	x		x					x	x	x	x		x		x				x	
	BT11 x MIR162									x											
	BT11 x GA21 x MIR162	x		x						x	x			x		x				x	
	BT11 x MIR162 x 1507 x GA21									x				x							
	BT11 x MIR162 x MIR604 x GA21	x								x	x			x		x					
	BT11 x MIR604								x	x	x	x		x		x					
	BT11 x MIR604 x GA21								x	x		x		x		x					
	GA21 x MON810								x	x				x		x					
	MON810 x LY038									x				x							
	MON810 x MON88017								x	x		x		x		x					
	MON863 x MON810								x	x		x		x		x					
	MON863 x MON810 x NK603								x	x		x		x		x					
	NK603 x MON810	x		x				x	x	x		x		x		x				x	
	T25 x MON810									x											
	TC1507 x MON810			x																	
	TC1507 x MON810 x MIR162									x	x	x		x				x			
	TC1507 x MON810 x MIR162 x NK603									x											
	TC1507 x MON810 x NK603			x						x											
	TC1507 x MON810 x MIR604 x NK603									x											
	1507 × 59122 × MON810 × NK603									x											
	1507 × 59122 × MON810 × NK603 × MIR604									x											
	Bt11×MIR604× 1507× 5307×GA21									x											
	Bt11×MIR162×MIR604× 1507×5307×GA21									x											
	X4334CBR and X4734CBR				x																
Oryza sativa **(Rice)**	Huahui-1 (TT51-1)					x															
	Shanyou63					x															

Table 1 Notes:

1. An "X" means an approval. This table presents information on regulatory authorizations that have been granted for food and feed use of the indicated GE plants to date. It does not consider the timeframe for any authorizations, and should not be used to determine if a particular plant is currently on the market in any particular jurisdiction.
2. Existing stacked event authorizations are included in this table because they rely on safety data relevant for assessing the safety of Cry1Ab protein. Some countries (such as the United States) do not require regulatory approval for "stacked events" that are generated through conventional breeding of two or more approved GE plants.
3. Though some countries (namely Netherlands and UK) are EU members, they have separate regulatory approval documents. Accordingly, these cases are separately listed in the table.

ORIGIN AND FUNCTION OF CRY1AB

Bacillus thuringiensis and the Cry1Ab insecticidal protein

As pointed out in Article 18 of the Codex Guidance [6], an important step in assessing the safety of a GE crop is to characterize the donor organisms which provided the genetic elements used in the development of the GE crop. The donor organism of Cry1Ab, *Bacillus thuringiensis* (Bt), 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 [10]–[12]. The species has been studied extensively and used commercially for many years due to its ability to synthesize proteins that possess selective pesticidal properties [13]–[18]. Preparations of natural isolates of Bt were first used as a commercial insecticide in France in 1938 [16], and *t*Bt subspecies *kurstaki* (*Btk*) has been registered with the Environmental Protection Agency (EPA) of the United States since 1961 [19]. Microbial preparations of Bt are currently approved for use around the world including in Australia, Canada, the EU, and the United States [17], [19]–[26].

Several hundred pesticidal substances have been isolated from Bt cultures [13], [27], [28], and these substances display tremendous variety in chemical structure, mode of action, and target specificity [14], [15], [17], [18], [29]–[31]. Insecticidal preparations derived from cultured cells of Bt bacteria may contain a complex mixture of the pesticidal substances produced by the particular Bt strain used [16], [32], [33]. They include antifungal compounds, vegetative insecticidal proteins (Vip), the cytolytic (Cyt) proteins, ß-exotoxin, and the δ-endotoxins, a group that includes the insecticidal crystalline (Cry) proteins [14], [15], [17], [34]. These substances may interact with each other to influence the toxicity and activity spectrum of individual bacterial preparations [15], [17]. Therefore, the activity spectrum of sprays made from Bt bacterial cultures may be much broader when compared to the activity spectrum of individual Bt proteins produced by a GE plant [15]. The Cry proteins have been studied extensively and used widely in agriculture as environmentally safe pesticides that control a broad range of economically significant insect pests [13], [15], [27], [34]–[38].

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 Bt [14], [15], [17], [24]. A systematic nomenclature for identifying and differentiating Cry proteins was proposed in 1989 and widely adopted [14], [15]. Under this nomenclature, the Cry proteins were grouped into four initial classes I, II, III, and IV based on their toxicity to particular orders of insects. 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. This system has been subsequently updated to account for additional Cry proteins and expanding knowledge of their molecular structure, function and relatedness, leading to some minor discrepancies in naming relative to earlier literature [15], [39]. This document uses the most recent nomenclature (Cry1Ab for the protein, *cry1Ab* for the gene) but the protein in question is synonymous with the older nomenclature CryIA(b).

The Cry1 proteins are classified based on amino acid sequence and the proteins designated as Cry1A (including Cry1Aa, Cry1Ab and Cry1Ac) are more than 85% identical in amino acid sequence [14], [39]. 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% [15], [24], [39], [40][41].

Mechanism of Cry1Ab insecticidal activity

Although there is significant variability in amino acid sequence and target range, the general mechanism by which Cry proteins (including Cry1Ab) achieve insecticidal activity is believed to be common across the group [14], [15], [24], [39]–[41]. The Cry1 proteins are produced in the form of protoxins of 130–140 kDa in size containing 1100–1200 amino acid residues [15], [24], [40], [41]. For Cry1A these protoxins are cleaved by proteases in the gut of sensitive organisms to generate active toxins consisting of 60–70 kDa fragments from the N terminal portion of the protein [15], [24], [38], [42], [43]. There are multiple theories about how these active toxins cause cell death, however there is general agreement that the first step is binding of specific receptors on the plasma membrane of midgut epithelium cells in susceptible insects [15], [24], [26], [40], [41], [43], [44]. The most popular theory holds that, once bound to receptors, the toxin is able to insert into the plasma membrane through the formation of oligomeric transmembrane pores [15], [24], [40], [41]. It is believed that these pores form ion channels that disrupt the transmembrane potential, causing osmotic lysis [14], [15], [24], [40], [41], [43]. A recent study found that two distinct functional pre-pores of Cry1Ab are formed after binding to the cadherin receptor, but before membrane insertion. Both pre-pores actively induce pore formation, although with different characteristics, and contribute to the insecticidal activity [45]. The biochemical process of membrane insertion is not completely

understood, but it is thought to involve the binding of additional cell surface receptors which facilitate oligomerization [40], [43], [46]. A competing theory, based on work in cell culture, suggests that binding to specific cell surface receptors is followed by exocytosis and the induction of a G-protein mediated signaling cascade which leads to oncotic cell death without oligomerization of Cry proteins or pore formation [26], [44], [46], [47]. 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 [15], [41], [48]. There is also some evidence based partly on experiments using sublethal concentrations, that there may be other relevant interactions between Cry proteins and their insect targets [41].

EXPRESSION OF CRY1AB IN INSECT-RESISTANT GE PLANTS

It is important to know the concentration levels of Cry1Ab in various parts of the GE plants because these levels, together with consumption information, can be used to estimate the human exposure for food safety assessment and animal exposure for feed safety assessment. Note that an exposure assessment also needs to consider the effects of processing on levels of Cry1Ab and the amount of GE crop consumed as a percentage of the diet. For feeding exposure assessment, the parts and proportions of GE crops consumed by the animals of interest are often different from those by humans. For example, cottonseed oil (which contains no plant proteins) is consumed by humans as the 6th largest category of vegetable oil while cottonseed hulls and cottonseed meal (which do contain plant proteins) are typically used as stock feed [49].

The level of expression of Cry1Ab in GE plants is determined by several factors related to the types of promoter, terminating sequences, and the gene insert site(s). Each transformation event therefore results in a different expression profile. Data for the level of expression of Cry1Ab in GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents [50]–[81]. For example, the level of the Cry1Ab protein in maize kernels of Bt-176 maize is less than 5 ng/g fresh weight. The dietary exposure from Bt-176 maize is expected to be lower than that experienced through eating products sprayed with Bt-based insecticides such as broccoli according to a study on dietary intake of Bt pesticides [50]. The level of the Cry1Ab protein was fairly low compared to levels of inherent dietary proteins in the kernel of Bt-11 maize, with a maximum level of 3.17 µg/g fresh weight. Once processed (canned) the level in the kernels was found to be less than 5 ng/g fresh weight, due to inactivation of the Bt protein by heating, based on enzyme-linked immunosorbent assay (ELISA) method which only detects active proteins [52]. The highest levels of Cry1Ab protein in GE plants is summarized in Table 2. Tissue types (leaf or whole plant) and collection methods differed between studies but all used an ELISA or Western blot to quantify the amount of Cry1Ab protein present in a given sample.

Table 2. Highest reported protein concentrations of Cry1Ab in GE plant tissues from representative approved events.[1]

Species	Event	Expression Level (ng/g fresh weight)	Tissue	Reference
Zea mays (Maize)	BT11	5300	Leaf	[52]
Zea mays (Maize)	BT176	3029	Leaf	[82]
Zea mays (Maize)	MON801	1770	Whole Plant	[83]
Zea mays (Maize)	MON802	9550	Leaf	[61], [66]
Zea mays (Maize)	MON809	1630	Leaf	[60], [63]
Zea mays (Maize)	MON810	10340[2]	Leaf	[51], [60], [64]

Table 2 Notes:
[1] Values are reported as mean unless otherwise noted. These values are not cross-comparable due to differences in sample collection and preparation methodology.
[2] Value represents highest observed value from a sample of 6 where the mean was 9350 ng/g fresh weight.

Typically, one or more samples of plant tissue were taken at a field trial site and pooled for analysis. The amount of Cry1Ab was normally determined on a dry weight basis then calculated to provide values relative to the total fresh weight of the sample and represented in a ratio (e.g., micrograms of Cry1Ab protein per gram of fresh weight) [50]–[81]. Samples were usually collected from several tissue types and at multiple growth stages providing data from plants over time and from multiple locations. In most cases the data were presented as a mean value (normally a mean of means as values were averaged within a field trial and across trials as well) and a range (normally also a range of means representing the average expression at a trial site, although this also varied depending on the individual example). In other data sets, means are provided with the standard deviation or the standard error of means [50]–[81].

Variations in methodology for sample collection make direct statistical cross-comparisons of the data inappropriate, but the weight of evidence from the above regulatory submissions suggests that Cry1Ab is expressed at very low levels relative to the total protein synthesized by the plant.

It is considered extremely unlikely that Cry1Ab protein could affect the metabolic system of the recipient plant [76], [84]–[86]. Results from field trials did not show indications of unexpected changes in agronomic performance and phenotypic characteristics.

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

There are two types of modifications to the *cry1Ab* gene from Bt that are relevant for its use in GE plants. The first type involves modifications to the nucleotide sequence which do not alter the amino acid sequence of the protein [53]–[61]. These modifications are primarily used to increase the translation of the gene either by modifying codon usage to align with plant preferred codons, or

through the insertion of plant introns to improve the efficiency of translation [53]–[61], [87].

The second type of modification involves changes to the nucleotide sequence which ultimately affect the amino acid sequence of the resulting protein. In all but one of GE plants expressing Cry1Ab protein, only truncations of the protein have been submitted for regulatory approvals [51]–[55], [60], [62], [65], [67], [76]–[78]; while in event COT67B in cotton, full-length Cry1Ab is used [88]. This means that the protein expressed in plants contains a subset of the amino acids in the native, full length protein from Bt. These truncated proteins mimic the "activated" form of the Cry1Ab protein following protease digestion in the insect midgut. They still require binding to a specific receptor or receptors in the insect midgut and they retain the species specificity found in the full length protein [51]–[55], [60], [62], [65], [67], [76]–[78]. There is adequate information to show that Cry1Ab from Bt bacteria is biochemically and functionally similar to that extracted from the GE crops [89]. Besides the truncation, the only other change to the amino acid sequence is an addition of a motif in some events (such as COT67B in cotton) called "Geiser motif" which was added to enhance the production efficiency of the Cry1Ab protein at the time of incubation of Bt [84].

FOOD AND FEED SAFETY OF THE CRY1AB PROTEIN

General considerations in assessing food and feed safety of GE crops

In assessing food safety for GE crops, comparative assessment is a key concept, although it is not a safety assessment in and of itself. This concept is used to identify relevant differences between the new food and its conventional counterpart. It helps to identify potential safety and nutritional issues and therefore is widely accepted as the most appropriate strategy for safety assessment of GE foods [6].

Regulatory agencies around the world regulate GE crops for food and/or feed use based on safety assessment of the specific GE crop products. Although countries follow the same Codex Guidance, the data requirements for regulatory approvals may not be the same in all countries/regions.

According to the Codex Guidance [6], when assessing potential toxicity of an expressed protein in GE crops, the following aspects should be considered: primary sequence similarity between the protein and known protein toxins and anti-nutrients, stability to heat or processing and to enzymatic degradation, and oral toxicity studies in cases where the protein present in the food is not similar to proteins that have previously been consumed safely in food. In addition, allergenicity of the protein should be assessed. The possibility of causing gluten-sensitive enteropathy, if the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains should also be considered.

In the United States, both FDA and EPA are in charge of the food/feed safety of the food and feed derived from GE crops containing biopesticides. EPA regulates pesticide proteins (referred to as Plant Incorporated Protectants, or PIPs) but it does not consider genetic materials in GE crops to be pesticidal nor does it consider GE crops themselves [90], [91]. Acute exposure studies in laboratory animals of up to 14 days should suffice given that the toxicity of a protein can usually be identified in acute toxicity studies [92][93]. Therefore, EPA believes that no chronic exposure studies in laboratory animals, of more than 90 days, are necessary for evaluating Cry1Ab protein safety [89], [93], [94]. Though long-term toxicological studies are not required by default, EPA does evaluate long-term studies if available [90].

EPA and FDA assess food safety of GE proteins and crops by focusing on toxicity and allergenicity [95]. Besides toxicity testing, non-toxicological safety evaluation methods are also applied, which include the heat and digestive stability of these proteins, as well as their structural similarity to known allergenic proteins which can be examined by comparing the protein structures with protein structures in a database of known protein allergens [96].

In Canada, Health Canada regulates foods and the Canadian Food Inspection Agency (CFIA) regulates livestock feed [97]. Health Canada regulates GE food as a type of novel food. Toxicology studies are not considered necessary if the substance of interest or a closely related substance has a safe consumption history at equivalent consumption level or if the new substance is not present in the food. Otherwise, conventional toxicology studies on the new substance will be required. The toxicity assessment of proteins covers structural homology, stability to heat, processing, and enzymatic degradation. If the expected exposure is oral only, it is generally not necessary to study long-term toxicological effects (direct-acting carcinogens, mutagens, teratogens or reproductive toxicants). Acute oral toxicity studies on the novel proteins are appropriate for assessing their potential toxicity. The detection of unintended changes relies on compositional analysis. Besides testing proteins, testing of the whole GE food is also considered since potentially unexpected changes to the genome could result in accumulation of toxic substances either of endogenous or exogenous origin [98]. When assessing feed derived from GE crops, CFIA considers nutritional data, toxicological data, allergenicity data, feeding trials, and environmental safety. Toxicological considerations include toxicity to livestock through feed intake, health effects to humans through ingestion of livestock-derived food products, and impact on bystanders or people through occupational exposure [99].

In the EU, European Food Safety Authority (EFSA) is the authoritative agency performing safety assessment for GE crops. In contrast to the United States and Canada, EFSA requires the newly expressed proteins to be tested in a repeated dose 28-day oral toxicity study in rodents that should be performed according to OECD guideline 407. Depending on specific profiles, the whole food and feed derived from the GE crop should be tested and the testing program should

include a 90-day toxicity study in rodents. Post market monitoring (PMM) might also be required on a case-by-case basis [100]. In whole food exposure studies, it can be extremely difficult to detect potential adverse effects and attribute these effects conclusively to an individual characteristic of the food [6].

TOXICOLOGICAL STUDIES ON THE CRY1AB PROTEIN AND GE CROPS

Safety studies on Bt proteins used as biopesticides

Information on prior safe use in food can be informative for food safety assessment of GE plants. A review on the safety of Btk summarized laboratory studies involving human oral exposure at high levels many times higher than intended levels of consumption, epidemiological studies involving human occupational exposure via inhalation, skin, and eyes, reported human infection cases, human dietary exposure through food consumption, human cell culture studies, and testing on large mammals. The review concluded that no human health effects have been conclusively attributed to Bt products appropriately applied on crops used for human consumption [101].

Toxicity prediction based on genetic stability and bioinformatics

Though not a part of safety studies, data on genetic stability is often included as part of a regulatory submission. The Cry1Ab gene has been stably integrated into the genome of the GE plants and is stably inherited from one generation to the next. To assess the safety of GE crops, one important consideration is possible protein structural similarities of the introduced proteins to known protein toxins in TOXIN6, GenBank, RefSeq, Uniprot Swissprot, PIR (Protein Information Resource), PRF (Protein Research Foundation) and PDB (Protein Data Bank) or other protein toxin databases. Various regulatory authorities have assessed the bioinformatic analyses related to this concern and came to the conclusion that Cry1Ab does not share structural similarities with protein toxins to humans or livestock animals [50]–[53], [55], [56], [58], [61]–[66], [81], [102]–[114].

Acute toxicity studies on the Cry1Ab protein and GE crops

Acute toxicity studies have been required by regulatory agencies for assessing food and feed safety of Cry1Ab derived from GE crops. The studies they reviewed include acute oral toxicity tests in rodents exposed to the protein for up to 14 days at levels up to 5050 mg/kg body weight for up to 14 days and model digestion system studies. In all cases, regulators have concluded that the Cry1Ab protein is toxic to lepidopteran insects but non-toxic to humans and livestock [50]–[53], [55], [56], [58], [61]–[66], [81], [102]–[114].

Safety assessment of stacked events

In some countries, GE plants with stacked events (i.e., those with more than one gene introduced typically by cross-breeding two or more GE plant varieties of the same species) were also assessed for biosafety. Besides the safety data on their parent GE plants, data on possible changes and potential adverse effects (such as gene silencing, metabolic changes, compositional changes, agronomical changes, toxicity, and allergenicity) as a result of interactions between the introduced genetic modifications are taken into account when assessing food and feed safety of stacked events [115]–[118]. The authorities came to the conclusion that stacked events, expressing Cry1Ab and other Bt proteins, did not add extra food or feed risk via interactions between the expressed gene products since the expressed proteins are non-toxic to humans and animals and the expression levels are too low to trigger synergistic, antagonistic, or other combined effects [116]–[160].

Allergenicity of the Cry1Ab protein

Another consideration for the safety of GE crops is the risk of introducing new allergens through the introduction of new genes and gene products. Here the primary focus is on the allergenicity of the Cry1Ab protein, not that of the whole plant.

Immunoglobulin E (IgE) mediated food allergy (type I food allergy) has two phases: a sensitization and an elicitation phase. Sensitization usually occurs by a primary exposure to the given dietary protein in susceptible individuals. In elicitation phase, re-exposure to the same protein leads to degranulation of mast cells which results in allergic symptoms. Since many food allergens are thought to sensitize through the gastrointestinal (GI) tract, resistance to proteolysis in the GI tract has been proposed to be a prerequisite for sensitization [161].

The following aspects are commonly considered when assessing allergenicity hazard of a protein: structural similarity to known allergens, whether it is glycosylated or not, stability to heat, processing, and enzymatic degradation in simulated gastric fluid [162], and immunological properties (via IgE binding assays) [161]. Note that IgE binding studies may be necessary when the gene donor is a known source of allergens or if structural similarity is found between the protein and known allergens. Since risk depends on exposure, the level of expression in the food for consumption should also be estimated [163]. Although proposed by some scientists [161], studies on the eliciting or sensitizing capacity of proteins are not conducted often since the predictive values or practicality of these assays especially animal models for sensitization have not been proven [163].

The assessment of allergenicity for a protein usually follows a weight-of-evidence approach by taking into account all of the information obtained, since none of the commonly used experimental methods can provide confirmative evidence on allergenicity [4], [162], [164], [165]. Though allergens are typically water-soluble glycoproteins

and are stable to treatment with heat, acid or proteases, many food allergens do not share such characteristics and some non-allergenic proteins can have these characteristics. Considering that digestibility assays are not as reliable as previously hypothesized [166], it was proposed that these digestibility assays should be combined with immunological assays to provide greater certainty in allergenicity assessment [161], [162]. Digestion conditions are known to influence the outcome of the digestibility assay, such that a standard set of conditions should be utilized [167]. In addition, besides the intact proteins, peptide fragments generated during the digestion process, especially those larger than 3.5 kDa, should be assessed for stability and allergenicity [161].

The physicochemical and structural properties of the Cry1Ab protein such as sequence and stability in digestive fluids have been determined to be different from those of known allergens. The *cry1Ab* gene originates from Btk, a soil microorganism that is not known to be allergenic. Amino acid sequence analysis of Cry1Ab did not identify any significant similarities to known allergens. The resistance to degradation of the Cry1Ab protein was measured in a pepsin solution at a pH of 1.2. The integrity of the protein was analyzed by gel electrophoresis followed by protein staining. No Cry1Ab protein was detected within two minutes of incubation [107]. The stability of Cry1Ab in simulated gastric fluids and/or simulated intestine fluids were also studied and found that it was rapidly digested [50]–[53], [55], [56], [58], [61]–[66], [72], [76], [81], [84], [85], [89], [102]–[112], [119], [168]–[171].

A study also showed that Cry proteins including Cry1Ab are not allergenic [172] as supported by three lines of evidence: by sequence homology results of several Cry proteins against two allergen databases - Allergen Online of Food Allergy Research and Resource Program (FARRP) and Structural Database of Allergenic Proteins (SDAP), levels of specific IgE in food sensitized patients sera to maize extracts, and IgE binding using immunoblot,.

FEEDING STUDIES ON FOOD AND FEED DERIVED FROM GE CROPS EXPRESSING THE CRY1AB PROTEIN

The role of feeding studies in food and feed safety assessment of GE crops

Feeding studies that aim to evaluate potential adverse effects of a whole food are difficult to design and the subsequent data interpretation is also difficult [173]. The challenges in designing whole food feeding studies are associated with the difficulty in choosing dose range. A good dose range should show a dose-response curve in case of a positive finding. However, unlike chemicals, some foods are major components of human or animal diets, making it virtually impossible to considerably increase the amount consumed to a sufficiently high level (such as a five to ten fold increase) that may be required to induce a toxic effect. Data interpretation is often challenging because in case of a negative finding, it is difficult to determine whether it is due to insufficient dose of a certain toxic ingredient (if any) in the food, or lack of toxicity of the food, or insufficient sensitivity of animal species to the toxic ingredients (if any) in the food.

It is worth noting that according to a review [174] on feeding studies using rats, many feeding studies either lack methodological details, methodological consistency , or defined criteria for outcomes that would be considered toxicologically or pathologically significant, making generalization difficult. However, such studies are periodically associated with food and feed safety review for GE plants, so studies related to Cry1Ab are reviewed here.

In association with some EU regulatory approvals, the EFSA GMO Panel also evaluated toxicity data prepared by the applicants from the peer-reviewed literature. This included whole food animal feeding studies which were reviewed for animal feed safety. The impacts of diets containing the GM events on performances of various animals were analyzed in these studies (including general health indicators such as growth, organ development, blood biochemical parameters and histopathological changes) and regulatory reviews indicate that no significant safety issues were identified [104]–[106], [169], [170].

Though 90-day feeding studies are generally not required for regulatory approval, there are peer-reviewed studies investigating subchronic effects of feeding GE crop derived food that contains Cry1Ab protein. In a 90-day study in Wistar rats fed with GE rice expressing Cry1Ab protein at a rate of 0.54 mg Bt toxin per kg body weight, no biologically relevant effects were found, though the authors added that the study design would be improved by adding an additional group of animals fed with a diet spiked with pure recombinant protein Cry1Ab [175]. No adverse effects were found in a 28-day study where Cry1Ab protein from Btk HD-1 was fed to F344 male rats that had gastrointestinal impairment [176]. A project funded by the European Commission found that in two 90-day feeding trials with two different GM maize MON810 varieties, the MON810 maize at a level of up to 33 % in the diet did not induce adverse effects in Wistar Han RCC rats [177]. Whole embryo culture (WEC) was used to study the embryotoxicity of GE food TT51, a transgenic rice with a synthetic Cry1Ab/CryAc gene. The embryos were cultured with serum obtained from rats exposed orally to the TT51 rice. No embryotoxicity to rats was found with TT51 rice diet exposure although Bt toxin had side effect on embryos at a concentration equal to the daily intake of Bt protein in TT51 diet [178]. A 90-day feeding study on calf fed with diet containing MON810 maize did not find any performance issue among the animals, in terms of growth rate and meat quality, or transfer of transgenic DNA to calf tissue [179]. The development of frogs were studied following dietary exposure to 30% GE rice expressing a Cry1Ab/1Ac fusion protein for 90 days and these frogs were not adversely affected [180].

In a two-generation reproduction study, 60% GE rice that contains Cry1Ab and Cry1Ac proteins, were fed to two generations of

rats, finding no biologically significant differences between the studied groups based on both clinical performance variables and histopathological responses [181].

A review article summarized the findings of feeding studies in which animals were fed with various types of GE feed including those containing Cry1Ab protein. A wide variety of endpoints were studied including general health status, blood parameters, immunological characteristics, histopathology and organ weight, microbial population of gastrointestinal tract, production performance, fate of transgenic DNA in the animals, and digestibility of nutrients, and quality of animal origin products of food producing animals. It was concluded that no biologically relevant effects were identified in these studies [182]. According to another review article, numerous studies have consistently showed that the performance and health of animals fed with GE feed are comparable with those of animals fed with isogenic lines [183]. More than 95% of the food-producing animals in the United States consume GE feed. Field data sets collected from these animals did not reveal unfavorable trends in livestock health and productivity [183]. No safety issues were identified in a study of transgenic maize containing the Bt gene (MON 810) fed to cows to examine the impact on the performance parameters, milk composition, blood serum metabolite profiles and transfer of tDNA into milk of cows [184].

Another review article [185] reviewed a large number of poultry nutrition studies that evaluated the wholesomeness of transgenic crops containing Cry1Ab or one of the other expressed proteins by examining performances of animals during growth or egg laying. It also reviewed studies examining the detectability of foreign DNA and proteins in meat, egg, and tissue samples from broiler chickens and laying hens fed diets containing transgenic feeds. This review concluded that genetically modified feeds are substantially equivalent and they are as safe as existing conventional feeds.

In a four-generation study [186] in which hens were fed with Bt maize containing Cry1Ab, no significant findings were identified after studying feed intake, growth, laying or breeding performance.

A 99-day feeding trial studied responses in Atlantic salmon juveniles fed diets containing Bt-maize MON810, finding that the Cry1Ab protein or other compositional differences in the GE feed caused minor alterations in intestinal responses (minor but significantly decreased digestive enzyme activities of leucine aminopeptidase and maltase) in juvenile salmon but without effects on overall survival, growth performance, development or health [187].

It is worth noting that according to a review [174] on rat feeding studies, many feeding studies are either lacking methodological details, inconsistent in methodology, or lacking defined criteria for outcomes that would be considered toxicologically or pathologically significant, making generalization difficult.

OTHER SAFETY FACTORS UNDER CONSIDERATION BY SOME REGULATORY AGENCIES

US EPA also considered the need for an additional margin of safety for infants and children when assessing Cry1Ab safety. It concluded that there is no such need considering that these sub-populations consume minimal residues of Cry1Ab. Cumulative effect of Cry1Ab exposure and exposures to other substances sharing a common mechanism of toxicity is also dismissed due to lack of mammalian toxicity of Cry1Ab.

CONCLUSION

The Cry1Ab protein expressed in insect-resistant GE plants (maize, common, and rice) is derived from the common soil bacterium Bt and is specifically toxic to Lepidoptera. Bioinformatic analyses in publically available regulatory submissions and peer reviewed literature demonstrate that Cry1Ab does not share sequence or structural characteristics with known human or livestock toxins. Toxicity studies submitted with regulatory dossiers did not identify any toxic effect in humans or livestock at any tested concentration of Cry1Ab, including concentrations far exceeding expected levels in food derived from Cry1Ab expressing GE plants. Bt is not a known source of allergens and Cry1Ab protein does not share sequence homology with known allergens. It is rapidly degraded by simulated gastric fluid, and regulators have consistently concluded based on a weight of evidence approach that Cry1Ab is not likely to be a food allergen.

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