A Review of the Food and Feed Safety of the Cry1Ac Protein

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INTRODUCTION

This document provides a comprehensive review of information and data relevant to the assessment of the protein Cry1Ac for food and feed safety. To date, five genetically engineered[1] (GE) crops (cotton, maize rice, soybean, and tomato) in which the Cry1Ac 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 17 countries or regions including the European Union (EU), representing 23 transformation events. In total, there are about 110 regulatory approvals in these countries[2].

All sources of information reviewed herein 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 consequence.

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 a protein (Cry1Ac) and not on GE crops containing the protein, not all the safety assessment elements in the Codex Guidance are relevant. The three sections covered in this monograph are "Origin and Function of Cry1Ac (including its mechanism of action on targeted species), "Expression of Cry1Ac in Insect-Resistant GE Plants" (including the expression levels of Cry1Ac in various parts of the crops), and "Food and Feed Safety of the Cry1Ac Protein" (including information on toxicology and allergenicity assessments).

Key words

Cry1Ac, *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 Cry1Ac events in GE crops for food and/or feed uses [9].

Species	Event Name	Argentina	Australia	Brazil	Burkina Faso	Canada	China	Columbia	EU	India	Japan	Korea	Mexico	Philippines	South Africa	Taiwan	Uruguay	USA
Gossypium hirsutum **(Cotton)**	3006-210-23					x					x		x					x
	281-24-236 x 3006-210-23, also called DAS-21Ø23-5 x DAS-24236-5 (MXB-13)		x	x					x		x	x	x					
	31807/31808					x					x							x
	281-24-236 x 3006-210-23x MON1445										x	x	x					
	281-24-236 x 3006-210-23x MON88913										x	x	x					
	Event-1									x								
	MON15985		x	x	x	x	x	x	x	x	x	x	x	x	x			x
	LLCotton25 x MON15985										x	x	x					
	GHB614×LLCotton25×15985										x							
	MON15985 x MON1445		x						x[2]		x	x	x	x				
	MON531 x MON1445	x	x	x				x	x		x	x	x	x	x			
	MON15985 x MON88913		x	x				x			x	x	x	x	x			
	MON88701 × 15985 × MON88913										x							
	COT102 × 15985 × MON88913										x							
	COT102 × 15985										x							
	281×3006×COT102×MON88913										x							
	MON531/757/1076	x	x	x		x	x	x	x	x	x	x	x	x	x			x
Zea mays **(Maize)**	DBT418	x	x			x					x	x	x	x		x		x
Glycine max **(Soybean)**	DAS-81419-2		x			x					x							x
	MON87701		x			x			x		x	x		x				x
	MON87701 x MON89788	x		x				x	x		x	x					x	
Solanum lycopersicum **(Tomato)**	5345					x												x
Oryza sativa **(Rice)**	gHvNAS1-1										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. 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 Cry1Ac 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.

ORIGIN AND FUNCTION OF CRY1AC

Bacillus thuringiensis and the Cry1Ac 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 organism which provided the genetic elements used in the development of the GE crop [10].

The donor organism of Cry1Ac, (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 [11]–[16]. The species has been studied extensively and used commercially for many years due to its ability to synthesize proteins that possess selective pesticidal properties [17]–[22]. Cry1Ac is one of the many pesticidal proteins synthesized by the bacteria. Preparations of natural isolates of Bt were first used as a commercial insecticide in France in 1938 [22], and Bt subspecies *kurstaki (Btk)* has been registered with the Environmental Protection Agency (EPA)

of the United States since 1961 [23]. Microbial preparations of Bt are currently approved for use around the world including in Australia, Canada, the EU, and the United States [19], [23]–[30].

Several hundred pesticidal substances have been isolated from Bt cultures [18], [31], [32], and these substances display tremendous variety in chemical structure, mode of action, and target specificity [17], [20], [19], [21], [33]–[35]. 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 [22], [36], [37]. 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 [17], [20], [19], [38]. These substances may interact with each other to influence the toxicity and activity spectrum of individual bacterial preparations [20], [19]. 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 [20]. 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 [18], [20], [31], [38]–[42]. 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 [17], [20], [19], [28]. A systematic nomenclature for identifying and differentiating Cry proteins was proposed in 1989 and widely adopted [17], [20]. 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 [20], [43]. This document uses the most recent nomenclature (Cry1Ac for the protein, *cry1Ac* for the gene) but the protein in question is synonymous with the older nomenclature CryIA(c).

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 [17], [43]. 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% [20], [28], [43]–[45].

Mechanism of Cry1Ac insecticidal activity

Although there is significant variability in amino acid sequence and target range, the general mechanism by which Cry proteins (including Cry1Ac) achieve insecticidal activity is believed to be common across the group [17], [20], [28], [43]–[45]. The Cry1 proteins are produced in the form of protoxins of 130–140 kDa in size containing 1100–1200 amino acid residues [20], [28], [44], [45]. 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 [20], [28], [42], [46], [47]. 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 [20], [28], [30], [44], [45], [47], [48]. 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 [20], [28], [44], [45]. It is believed that these pores form ion channels that disrupt the transmembrane potential, causing osmotic lysis [17], [20], [28], [44], [45], [47]. 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 [44], [47], [49]. 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 [30], [48]–[50]. 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 [20], [45], [51]. 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 [45].

EXPRESSION OF CRY1AC IN INSECT-RESISTANT GE PLANTS

It is important to know the concentration levels of Cry1Ac 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 effect of processing on levels of Cry1Ac 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 [52].

The level of expression of Cry1Ac 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 expression levels of Cry1Ac in GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents[53]–[88]. For example, the mean level of the Cry1Ac protein in the seed of MON 87701 soybean is 4.2 μg/g

fresh weight [89]. The dietary exposure 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 [90]. The highest levels of Cry1Ac in GE plants are summarized in Table 2. Tissue types and collection methods differed between studies but all used an enzyme-linked immunosorbent assay (ELISA) or Western blot to quantify the amount of Cry1Ac protein present in a given sample.

Table 2. Highest reported protein concentrations of Cry1Ac in GE plant tissues from representative approved events.[1]

Species	Event	Tissue	Expression Level	Reference
Gossypium hirsutum (Cotton)	MON-15985-7	Seed	3.35±0.63[2] µg/g	[91]
	DAS-21023-5	Young Leaf	0.46-3.5 µg/g DW[3]	[92]
	DAS-21023-5 X DAS-	Flower	1.83 µg/g DW[4]	[58]
	31807/31808	Seed[5]	2.5 µg/g FW	[86]
	MON-00531-6	Young Leaf	5.00 ± 1.84[2] µg/g	[91]
Zea mays (Maize)	DKB-89614-9	Harvest Leaf	626.8 ± 141.62[6] ng/g FW	[93]

Table 2 Notes:
[1] These values are not cross-comparable due to differences in sample collection and preparation methodology.
[2] Standard Deviation.
[3] DW = dry weight.
[4] FW = fresh weight.
[5] Only tissue reported.
[6] Standard Error.

Typically, one or more samples of plant tissue were taken at a field trial site and pooled for analysis. The determination of Cry1Ac level was normally on a dry weight basis and then a ratio was calculated to provide values relative to the total fresh weight of the sample (e.g., micrograms of Cry1Ac protein per gram of fresh weight). 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 cases, means are provided with the standard deviation or the standard error of means.

Variations in methodology for sample collection make it inappropriate to make direct statistical cross-comparisons of the data, but the weight of evidence from the above regulatory submissions suggests that Cry1Ac is expressed at very low levels (less than 5 µg/g) relative to the total protein synthesized by the plant.

Gene transfer from GE food to cells in the human digestive tract is extremely unlikely to occur [88], and unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods. In the case of the β-lactamase (bla) and other antibiotic resistance genes used in many GE crops, it was concluded that even should transfer occur, the health impacts would be negligible because this antibiotic resistance gene is already commonly carried by bacteria found in the environment as well as inhabiting the human digestive tract. Despite that some small differences were found in the levels of a few measurement endpoints, these differences were determined to be biologically insignificant, which further support the lack of unintended effects as a result of the genetic modifications [53]–[76], [86]–[89], [95]–[104], [108]–[116] . It is also considered extremely unlikely that Cry1Ac protein could affect the metabolic system of the recipient plant [77]–[81], [83], [84], [107], [112]–[114], [117]–[123]. Results from field trials did not show indications of unexpected changes in agronomic performance and phenotypic characteristics.

Modifications to the Cry1Ac gene and Cry1Ac protein in GE plants

There are two types of modifications to the *cry1Ac* gene from Bt that are relevant for its use in GE plants. In some Cry1Ac events (soybean line DAS-81419-2 and cotton line 281-24-236 x 3006-210-23 or called MXB-13), the gene expressing Cry1Ac protein is a synthetic chimera comprising sequences from three sources: the *cry1Ac1* gene expressing the core toxin of Cry1Ac which is originally isolated from Bt subsp. kurstaki strain HD73, the *cry1ACa3* gene expressing a non-toxin amino acid sequence which is originally isolated from Bt subsp. aizawai strain PS811 and the *cry1Ab1* gene expressing a non-toxin amino acid sequence which is originally isolated from Bt subsp. berliner 1715 [54][58].

Other Cry1Ac events (3006-210-23, MON531, MON757, MON1076, MON87701, and DBT418) contain a slightly modified Cry1Ac protein that is 99.4% similar to the natural bacteria-derived Cry1Ac protein. Despite the protein sequence differences, the insecticidal properties the modified Cry1Ac protein in the GE crops was confirmed and its equivalence to the natural bacterial Cry1Ac protein was established based on assessments of its biochemical, immunological, and toxicological properties [56], [57], [59], [61]–[63], [71]–[76], [89], [94].

FOOD AND FEED SAFETY OF THE CRY1AC 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 [124], [125]. 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 based on the literature on protein toxicology [126]. Therefore, EPA believes that no chronic exposure studies of laboratory animals of more than 90 days are necessary for evaluating the safety of proteins [126], [127]. Though long-term toxicological studies are not required, EPA does evaluate long-term studies if available [124].

EPA and FDA assess food safety of GE proteins and crops by focusing on toxicity and allergenicity [128]. 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 [129].

In Canada, Health Canada regulates foods and the Canadian Food Inspection Agency (CFIA) regulates livestock feed [130]. 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 [131]. 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 [132].

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 [133]. 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 CRY1AC 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 levels many times higher than intended levels of consumption (typically 1000 mg/kg or more), 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 [134].

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 Cry1Ac gene has been stably integrated into the genome of the GE plants and is stably inherited from one generation to the next [53]–[76], [79]–[85], [87], [89], [94]–[111]. 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 Cry1Ac does not share structural similarities with protein toxins to humans or livestock animals [54]–[68], [71]–[76], [78], [89], [95]–[104].

Acute Toxicity studies on the Cry1Ac protein and GE crops

Acute toxicity studies have been required by regulatory agencies for assessing food and feed safety of Cry1Ac derived from GE crops. The studies they reviewed include acute oral toxicity tests in rodents exposed to the protein at levels up to 5050 mg/kg body weight for up to 14 days and model digestion system studies. In all cases, the regulators have concluded that the Cry1Ac protein is toxic to lepidopteran insects but non-toxic to humans and livestock [54]–[66], [69], [71]–[76], [89], [95]–[100], [114].

Besides acute animal studies submitted to regulatory agencies, there are also some acute toxicity studies on Cry1Ac in the peer-reviewed literature. For example, there is an *in vitro* study on acute genotoxic effect on human lymphocytes, hemolytic effect on human and animal erythrocytes, and antimicrobial effects on some strains of bacteria and yeast cells. These experiments involved exposures to Cry1Ac at concentration up to 1000 g/ml for a time duration of 1 to 72 hours. No adverse effects were identified in these acute assays [135].

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 [112]–[114]. The authorities came to the conclusion that stacked events containing Cry1Ac 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 [71], [72], [74], [76], [79], [83], [84], [98], [107], [112]–[114], [117]–[121], [123].

Allergenicity of the Cry1Ac 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 Cry1Ac 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 [136].

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 [137], and immunological properties (via IgE binding assays) [136]. 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 [10]. Although proposed by some scientists [136], 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 [10].

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], [137]–[139]. 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 [140], it was proposed that these digestibility assays should be combined with immunological assays to provide greater certainty in allergenicity assessment [136], [137].Digestion conditions are known to influence the outcome of the digestibility assay, such that a standard set of conditions should be utilized [141]. 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 [136].

The physicochemical and structural properties of the Cry1Ac protein such as sequence and stability in digestive fluids have been determined to be different from those of known allergens. The *cry1Ac* gene originates from Btk, a soil microorganism that is not known to be allergenic. Amino acid sequence analysis of Cry1Ac did not identify any significant similarities to known allergens [77]–[85], [104], [107], [114], [121]–[123]. The resistance to degradation of the Cry1Ac 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. Cry1Ac was well digested within two to seven minutes in gastric fluid [53]. The stability of Cry1Ac in simulated gastric fluids and/or simulated intestine fluids were also assessed in other regulatory submissions or peer-reviewed studies and

found consistently that it was rapidly digested [54]–[69], [71]–[76], [95]–[104], [114], [142]–[144]. Some regulatory agencies (such as the Brazilian National Technical Biosafety Commission-CTNBio) also assess IgE binding of the introduced protein in a GE crop if the crop is traditionally an allergenic food. These assessments did not identify any allergenic hazards in Cry1Ac [97].

A study also showed that Cry proteins including Cry1Ab are not allergenic, as supported by three lines of evidence by sequence homology results for 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[145].

FEEDING STUDIES ON FOOD AND FEED DERIVED FROM GE CROPS EXPRESSING THE CRY1AC PROTEIN

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 [146]. 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 [147] 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 Cry1Ac 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. Several authorities also assessed feeding studies to ensure the nutritional equivalence of the GE crops with their conventional counterparts. The impacts of diets containing the GE events on performances of various animals (general health including growth, organ development, blood biochemical parameters, and histopathological changes) were analyzed in these studies and regulatory reviews indicate that no significant safety issues were identified [71]–[76], [97], [100]–[104], [108], [112]–[114].

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 Cry1Ac protein.

In a feeding study, lactating cows were fed Bt cottonseed containing Cry1Ac protein at 0.195 mg/g for 4 weeks. Body weight gain, nutrient intake and digestibility, milk yield and composition, body condition score, and blood parameters were measured and not found to vary significantly between the control and the treatment groups [148]. In a similar study [149], the dry matter intake (DMI), milk yield, milk composition, body weight, and body condition score did not differ from controls in Argentinean Holstein dairy cows fed Bt cottonseed derived from cotton containing the Cry1Ac protein. A 7-week feeding study on growing broiler chickens fed Bt cottonseed containing Cry1Ac did not identify any deleterious effect on growth performance, blood biochemistry, or various carcass characteristics [150].

A review article summarized the findings of feeding studies in which animals were fed with various types of GE feed including those containing Cry1Ac 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 [151]. According to another review article, numerous studies have consistently shown that the performance and health of animals fed with GE feed including feed containing Cry1Ac are comparable with those of animals fed with isogenic lines [152].

CONCLUSION

The Cry1Ac protein expressed in insect-resistant GE plants (tomato, rice, maize, cotton, and soybean) 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 Cry1Ac does not share sequence or structural characteristics with known human or livestock toxins. Toxicity studies on Cry1Ac submitted with regulatory dossiers did not identify any toxic effect in humans or livestock at any tested concentration, including concentrations far exceeding expected levels in food derived from Cry1Ac expressing GE plants. Bt is not a known source of allergens and Cry1Ac 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 Cry1Ac is not likely to be a food allergen.

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