INTRODUCTION

This document provides a comprehensive review of information and data relevant to the assessment of the EPSPS protein for food and feed safety. To date, nine genetically engineered (GE) crops (alfalfa, canola, cotton, creeping bentgrass, maize, potato, soybean, sugar beet and wheat) in which the EPSPS 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 more than twenty jurisdictions including the European Union (EU), representing 91 transformation events. In total, there are 364 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 a non-GE parent line or closely related variety [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 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 mainly on the safety of a protein (EPSPS) 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 EPSPS (including its mechanism of action on targeted species),” “Expression of EPSPS in Glyphosate-tolerant GE Plants” (including the expression levels of EPSPS in various parts of the crops), and “Food and Feed Safety of the EPSPS Protein” (including information on toxicology and allergenicity assessments).

Key words

EPSPS, Bacillus thuringiensis, insect resistance, genetically engineered, environmental risk assessment

1GE 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.

2Regulatory 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 EPSPS events in GE crops for food and/or feed uses [9].

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**ORIGIN AND FUNCTION OF EPSPS**

**EPSPS enzymes and their function**

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS: EC 2.5.1.19) family of enzymes is ubiquitous in plants and microorganisms. EPSPS has been isolated from both sources, and their properties have been extensively studied. The bacterial and plant enzymes are mono-functional with a molecular mass of 44-48 kD [10]. EPSPS proteins catalyze the transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate [11]. Because of the stringent specificity for substrates, EPSPS enzymes bind only to PEP and glyphosate. The only known metabolic product produced is shikimate-3-phosphate, the penultimate product of the shikimic acid pathway. Shikimate is a substrate for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as many secondary metabolites, such as tetrahydrofolate, ubiquinone, and vitamin K. Importantly, the shikimate pathway and, hence, EPSPS
proteins, are absent in mammals, fish, birds, reptiles and insects [11]. It has been estimated that aromatic molecules, all of which are derived from shikimic acid, represent 35% or more of the dry weight of a plant [12].

The _cp4 epsps_ gene, most commonly used in genetically engineered plants was isolated from _Agrobacterium_ sp. strain CP4, a common soil-borne bacterium. It has been sequenced and encodes a 47.6 kD EPSPS protein consisting of a single polypeptide of 455 amino acids. The _mepsps_ and _2mepsps_ genes, also used in genetically engineered plants, were isolated from maize (_Z. mays_ L.) and resulted from two amino acid substitutions [13][14]. The gene _epsps grg23ace5_ is a synthetic gene with 97.6% primary sequence identity to the _epsps grg23_ gene from the common soil bacterium _A. globiformis_ [15]. The CP4 EPSPS and the variant forms of the protein expressed in GE glyphosate tolerant plants are functionally equivalent to endogenous plant EPSPS enzymes with the exception that these proteins display reduced affinity for glyphosate [12] [13].

**Mechanism of glyphosate tolerance**

In plants that are not glyphosate tolerant, glyphosate binds to the endogenous plant EPSPS enzyme and blocks the biosynthesis of 5-enolpyruvyl-shikimate-3-phosphate, thereby starving plants of essential amino acids and secondary metabolites [16]. Inhibition of EPSPS enzyme activity has been shown to proceed through the formation of a ternary complex of EPSPS-S3P-glyphosate. Formation of the complex occurs in an ordered fashion with glyphosate binding occurring only after the formation of a binary EPSPS-S3P complex. Glyphosate binding effectively blocks the binding of PEP and prevents EPSPS catalysis of S3P and PEP. In CP4 EPSPS or other mutant EPSPS forms, however, affinity for PEP is much higher than affinity for glyphosate, so the CP4 EPSPS or a mutant form of EPSPS preferentially binds PEP even in the presence of glyphosate and catalysis proceeds just as in the absence of glyphosate [12]. This difference in the glyphosate binding affinity is the basis for glyphosate tolerance in plants with introduced CP4 EPSPS- or a mutant form of EPSPS. The CP4 or mutant EPSPS enzyme continues to function in the presence of glyphosate, producing the aromatic amino acids and other metabolites that are necessary for normal plant growth and development (Figure 1).

**EXPRESSION OF EPSPS IN GLYPHOSATE-TOLERANT GE PLANTS**

It is important to know the concentration levels of EPSPS 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 EPSPS 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 considered for humans. For example, cotton oil (which contains no plant proteins) is consumed by humans as the sixth largest category of vegetable oil while cotton hulls and cottonseed meal (which do contain plant proteins) are typically used as stock feed [18].

Data for the level of expression of EPSPS proteins in glyphosate tolerant GE plants that have obtained regulatory approvals are available in publicly accessible regulatory submissions and decision documents [14], [15], [19]–[123]. Tissue types and collection methods that measure the expression levels of EPSPS differed between studies, but all of them used an enzyme-linked immunosorbent assay (ELISA) to quantify the amount of CP4 EPSPS (or other EPSPS) present in samples.

Typically, one or more samples were taken at one or more field trial sites and pooled for analysis. Samples were usually collected from several tissue types and at multiple growth stages providing data from plants over time and from multiple locations. The amount of EPSPS was calculated in comparison to the total fresh weight of the sample and represented in a ratio (e.g., micrograms of EPSPS protein per gram of fresh weight). In most cases the data were presented as a mean and a range. The mean is normally an average of means since values were averaged within a field trial and across trials as well. The range is
Variations in methodology for sample collection makes direct statistical cross-comparisons of the data inappropriate, but the weight of evidence suggests that GE plants express EPSPS at very low levels. The highest reported level of expression was for soybean leaves (798 ug/g fresh weight), and typically values in other plants were much lower. (See Table 2 for summary data.)

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<th>Reference</th>
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<td>MON88017</td>
<td>Pollen</td>
<td>280</td>
<td>[102]</td>
</tr>
</tbody>
</table>

Table 2 Notes: 'These values are not cross-comparable due to differences in sample collection and preparation methodology.

FOOD AND FEED SAFETY OF THE EPSPS PROTEIN

General considerations in assessing food and feed safety of GE crops

In assessing food safety for GE crops, comparative assessment is a key step, though it is not a safety assessment by itself. This concept is used to identify similarities and differences between the new food and its conventional counterpart. It helps to identify potential safety and nutritional issues and therefore is widely accepted [6]. When statistical differences are identified between a GE crop and its traditional counterpart for the levels of some substances, the biological relevance needs to be assessed for these differences. A difference is typically considered to have no biological relevance when the level of the substance in the GE crop is within the natural variation observed in the population of conventional crop varieties with confirmed history of safe use.

Regulatory agencies around the world regulate GE crops for food and/or feed use based on safety assessments of the specific GE crop products. Although countries follow the same Codex Guidance, the data requirements for regulatory approvals are not 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 with additional consideration of the possibility of causing gluten-sensitive enteropathy, if the introduced genetic material is obtained from wheat, rye, barley, oats, or related cereal grains [6]. When the transformed crop has known allergenic properties (e.g., soybean, peanut, rice, etc.), then the level of endogenous allergenic proteins should not be increased in the GE crop.

In the United States, the Food and Drug Administration (FDA) is in charge of the food safety of whole GE plants that contain EPSPS. FDA assesses food safety of proteins introduced into the plants by focusing on toxicity and allergenicity. Before the proteins are introduced into the food or feed supply, they are tested for heat and digestive stability, as well as their structural similarity to known allergenic proteins [125].

In Canada, Health Canada regulates foods, and the Canadian Food Inspection Agency (CFIA) regulates livestock feed [126]. Health Canada regulates GE food through its authority over novel foods. Toxicology studies are not considered necessary if the substance of interest or a closely related substance has a safe consumption history at equivalent consumption levels or if the new substance is not present in the food or feed. Otherwise, conventional toxicology studies on the new substance are 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 agronomic and compositional analysis. Besides testing proteins, testing of the whole GE food is also considered, since unexpected changes to the genome, caused as a result of the genetic engineering process, could result in accumulation of toxic substances either of endogenous or exogenous origin [127]. 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 residues in livestock products, and impact on agricultural workers or people nearby [128].
In the EU, European Food Safety Authority (EFSA) is the authoritative agency performing food/feed safety assessment for GE crops, though it does not have regulatory power. EU 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 [129]. However, it is well known that it can be extremely difficult for whole food exposure studies to detect potential adverse effects and attribute these effects conclusively to an individual characteristic of the food [6].

In study design and data interpretation, it is important to consider the impact of food processing (especially heating) on the level of the transferred gene and the expressed protein. It was found in a study that the cp4 epsps gene was not detectable after processing but the CP4 EPSPS protein persists in some of the final food products prepared [130].

TOXICOLOGICAL STUDIES ON THE EPSPS PROTEIN AND GE CROPS

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 CP4 EPSPS gene has been stably integrated into the genome of the GE plants and is stably inherited from one generation to the next. In addition, comparative analyses with known toxins do not indicate any potential for the CP4 EPSPS protein to be toxic to humans [14], [15], [19]–[21], [23], [25]–[27], [29]–[35], [38]–[50], [52]–[61], [63]–[80], [82]–[84], [86], [104]–[123], [131]–[234]. The databases searched were generally latest versions of protein toxin databases at the time of search, such as TOXIN5 [111], Uniprot_Swissprot, Uniprot_TriEMBL, PDB, DAD and GenPept [106]. BLASTP search program was often used when comparing the structure of interest with structures in the databases [106][14].

Acute toxicity studies on the CP4 EPSPS protein and GE Crops

In many countries where GE crops have been approved as food or feed, acute animal studies are required for assessing toxicity of a newly expressed protein because proteins typically exert toxicity via an acute mechanism. In fact, oral exposure to proteins has not been shown to have carcinogenic, teratogenic, or mutagenic effects [235] [236]. In the acute toxicity studies, rodents are exposed orally to the protein at levels up to 5000 mg/kg for up to 14 days. According to numerous regulatory decision documents of various countries, the EPSPS protein was consistently found to be non-toxic in acute mouse gavage tests using purified EPSPS proteins at very high doses.

In the studies assessed in these approval documents, the protein used for testing was often produced in Escherichia coli because of the difficulty in extracting sufficient amounts of the protein from the GE plant which only contains a very low level of the protein. The equivalence between the two sources of EPSPS proteins were established to validate the use of bacterial EPSPS proteins [14], [15], [19]–[23], [25]–[27], [29]–[35], [38]–[50], [52]–[59], [61]–[84], [86], [104]–[123], [131]–[157], [237]–[247].

Safety assessment of stacked events

In some countries, GE plants with stacked traits (i.e., those with more than one event introduced typically by cross-breeding two or more GE plant varieties of the same species) containing EPSPS were also assessed for biosafety. Besides the safety data on their parent GE plants, 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 may be considered when assessing food and feed safety of stacked events. These include possible impact on genetic stability of the introduced traits and level of expression of the involved novel proteins. The authorities came to the conclusion that stacked events, with one event expressing the CP4 EPSPS protein, 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. In fact, it is very unlikely that stacked events expressing novel proteins that participate in different metabolic pathways will interact [25], [26], [57], [60], [62], [63], [65]–[76], [83], [132]–[141], [158], [160]–[168], [170]–[173], [175]–[179], [181]–[188], [190]–[195], [197]–[204], [206], [207], [209], [210], [212]–[214], [217], [219], [222], [225]–[230], [237], [245], [248]–[264].

Allergenicity of the EPSPS protein

One concern with the safety of GE crops is the risk of introducing new allergens through the introduction of new genes and gene products into the crops. Here the primary focus is on the allergenicity of the EPSPS protein, not that of the whole GE crops.

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 a series of biochemical and cellular changes that finally result 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 [265].

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 [266] and simulated intestinal fluid [6], and in some cases immunological properties (via IgE binding assays) [265]. Note that IgE binding studies are only appropriate 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 [267]. Although proposed by some scientists [265], studies on the eliciting or sensitizing capacity of proteins are not conducted often since the predictive value or practicality of these assays, especially animal models for sensitization, have not been proven [267]. In fact, there is no validated animal model that is satisfactorily predictive of protein allergenicity, mainly due to a lack of understanding of the detailed mechanism of food-induced allergy [268], [269]. The assessment of allergenicity for a protein follows a weight-of-evidence approach by taking into account all of the information obtained, since none of the commonly used methods, taken alone, can provide confirmative evidence on allergenicity [4], [129], [266], [270]. 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 [271], it was proposed that these digestibility assays should be combined with immunological assays for less uncertainty in allergenicity assessment [265], [266]. Notably, digestion conditions are known to influence the outcome of the digestibility assay, such that a standard set of conditions should be utilized in order to compare the digestibility of one protein to another [272]. 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 [265].

The CP4 EPSPS protein has been determined to not share the properties of known allergens. The cp4 epsps gene originates from Agrobacterium sp. strain CP4, a soil microorganism that is not known to be allergenic. Amino acid sequence analysis of CP4 EPSPS did not identify any significant similarities to known allergens using the latest AllergenOnline.org database [264]. The resistance to degradation of the CP4 EPSPS protein was measured in a pepsin solution [269]. The integrity of the protein was analyzed by gel electrophoresis followed by protein staining. No CP4 EPSPS protein was detected within a few minutes of incubation. The stability of CP4 EPSPS in simulated gastric fluids and/or simulated intestinal fluids were also studied and found that it was rapidly digested [269].

In a study to examine the allergenicity of the CP4 EPSPS protein, CP4 EPSPS proteins extracted from a GE soybean and Escherichia coli were not found to bind significant quantities of IgE from two geographically distinct sensitive populations (Korean children with atopic dermatitis secondarily diagnosed with soybean and other food allergies and European adult individuals with clinically documented soy allergy) [273].

Various regulatory approval documents had the same conclusion that the CP4 EPSPS protein does not have characteristics that are typical of known food allergens, and there is no history that this family of EPSPS proteins are allergenic. There is also no evidence, from tests with human sera, that glyphosate–tolerant crops have increased allergenicity in comparison to conventional crops [14], [15], [19]–[23], [25]–[27], [29]–[35], [38]–[50], [52]–[84], [86], [104]–[123], [131]–[157], [159], [174]–[210], [212]–[232], [237]–[264].

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

Feeding studies are generally not designed specifically as toxicity tests but as nutritional studies to evaluate unknown factors that may present in GE crops that affect animal growth and well-being. Such feeding studies are typically of short duration because of the difficulties with interpreting the results of long-term whole food animal feeding studies [274].

By using farmed animals such as rats, chicken, fish, and dairy cattle to carry out feeding studies, regulatory authorities in various countries/regions came to the conclusion that processed or unprocessed meals derived from GE crops containing EPSPS protein do not cause significant health effects in the animals studied [14], [19]–[23], [25]–[27], [29]–[31], [33]–[35], [39], [42], [54], [56]–[59], [61]–[84], [86], [122], [123], [131], [132], [237], [238].

Besides feeding studies included in regulatory submissions, there are also feeding studies published in the peer-reviewed literature. In a study where one CP4 EPSPS alfalfa variety and three commercial conventional varieties of alfalfa grown in southeastern Washington State of the United States was fed to dairy cows as hay for 28 days, no difference was found for milk yield, milk fat, milk true protein, milk lactose, and solids-not-fat between the GE variety and the three conventional varieties [275].

In two 13-week feeding studies conducted by Monsanto, maize grains from Roundup Ready maize expressing CP4 EPSPS (NK 603 and MON 88017), were fed to Sprague–Dawley rats. The GE crops were grown in field test sites and sprayed with a glyphosate formulation during the growing season at commercial application rates. No differences were identified in overall health, body weight, food consumption, clinical pathology parameters (hematology, blood chemistry, and urinalysis), organ weights, and gross and microscopic appearance of tissues between the treatment and the control groups [276], [277].

A 90-day study on GE soybean (3Ø5423 x 40-3-2) expressing CP4 EPSPS examined similar endpoints and did not identify any biologically relevant changes [278]. Two Polish studies on calves and chicken fed soybean meal (Roundup Ready; MON 40-3-2) for 90 days and 28 days respectively did not identify any significant effects
after evaluating feed efficiency, immunological indictors, nutritional value and a series of organ, cellular, and biochemical changes in tissues as well as transfer of transgenic DNA (tDNA) to tissues [279], [280].

A 42-day feeding study addressing nutritional concerns in chicken fed with glyphosate-tolerant soybeans (MON 89788) did not find any differences in feed intake, weight gain, adjusted feed conversion, or any measured carcass and meat quality parameters between the treatment and control groups [281].

Tufarelli and colleagues [282] reviewed a large number of poultry nutrition studies that evaluated the wholesomeness of transgenic crops including those containing CP4 EPSPS by examining performances of animals during growth or egg laying. They 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 engineered feeds are substantially equivalent, and they are as safe as existing conventional feeds.

CONCLUSION

The EPSPS protein expressed in glyphosate-resistant GE plants is encoded by a gene which is either from a soil bacterium, maize with genetic modifications, or synthesized. Genetic analyses showed that the introduction of the protein into crops did not impact the genetic stability of the receiving crops. Compositional analyses consistently showed that it does not induce any unintended effects with biological significance. Bioinformatic analyses, in vitro studies of the stability of EPSPS under heating or with presence of gastrointestinal fluids did not identify any property in the protein that is typical of a protein toxin or protein allergen. Various acute oral toxicological studies and feeding studies with either toxicological or nutritional considerations did not identify any changes of biological significance. Regulatory agencies across the world evaluated the data in regulatory submissions for more than 90 transformation events containing EPSPS and they consistently concluded that the presence of this protein in the GE crops does not pose any significant risk in addition to what has already been accepted for conventional crops.

REFERENCES


