FOOD DERIVED FROM GLYPHOSATE-TOLERANT SUGAR BEET LINE H7-1

A Safety Assessment

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SUMMARY

Food derived from genetically modified (GM) sugar beet line H7-1 has been assessed for its safety for human consumption. Sugar beet line H7-1, known commercially as Roundup Ready® sugar beet, has been genetically modified to be tolerant to applications of the herbicide glyphosate.

Criteria addressed in the assessment included: characterisation of the transferred genes, their origin, function and stability; changes at the DNA, protein and whole food levels; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed proteins to be allergenic or toxic to humans.

History of use

Sugar beet has a long history of food use, as a source of sugar; it accounts for approximately one-third of world sugar production. Sugar beet is processed to yield white sugar, molasses and pulp. The pulp may be used as food fibre, but is primarily used in animal feed, as is the molasses. By-products from sugar beet (tops, leaves and post-processing trash) are used as cattle feed.

Description of the genetic modification

Glyphosate-tolerant sugar beet line H7-1 was generated by the insertion of one new gene: the bacterial *cp4 epsps* gene. This gene encodes a 5-enolpyruvyl shikimate-3-phosphate synthase enzyme that is not sensitive to glyphosate, allowing the plants to function normally in the presence of the herbicide. The *cp4 epsps* gene is derived from the native soil microorganism, *Agrobacterium* sp. strain CP4.

Detailed molecular and genetic analyses of sugar beet line H7-1 indicate that a single *cp4 epsps* gene was transferred to the plant genome, resulting in the expression of one novel protein, the CP4 EPSPS enzyme. The genetic modification is stable and inherited in a predicted Mendelian fashion from one generation to the next.

Characterisation of novel protein

One novel protein is expressed in sugar beet line H7-1, namely the CP4 EPSPS enzyme. The mature CP4 EPSPS produced in line H7-1 is substantially similar to the EPSPS enzyme naturally present in all food crops, and in foods from fungal and microbial sources.

Protein expression analyses indicate that CP4 EPSPS is expressed at similar levels in the top (161 parts per million, ppm) and the root tissue (181 ppm) of H7-1 sugar beet plants.

Dietary exposure to CP4 EPSPS from consumption of food products derived from sugar beet line H7-1 is expected to be virtually zero, as plant proteins are not present in processed sugar. In addition, the potential toxicity and potential allergenicity of the CP4 EPSPS protein has been assessed previously by FSANZ in

relation to its use in other food crops such as corn. Additional biochemical studies relating to sugar beet line H7-1, together with detailed bioinformatic analyses on the protein, demonstrate that the CP4 EPSPS protein is not toxic and is not likely to be allergenic.

Comparative analyses

Compositional analyses were performed on key constituents of sugar beet with a particular focus on the root tissues used for processing into sugar for human consumption.

A comprehensive series of compositional analyses compared key constituents in sugar beet line H7-1 to those in the non-GM counterpart, and to a number of commercial sugar beet reference varieties. The constituents measured in beet top (leaf) and root (brei, the shredded roots used in the first step of sugar processing) tissues were: dry matter, crude protein, fibre, ash and fat, carbohydrates, 18 amino acids and the natural toxicant saponin.

In all parameters measured, the levels in sugar beet H7-1 were equivalent to the corresponding levels in tissues from the conventional counterpart, or to other commercial sugar beet varieties. Minor differences were noted in the levels of two amino acids in the root samples. However, these differences are within the natural range for conventional sugar beets and therefore are not significant with respect to food safety. Moreover, refined sugar contains no detectable plant proteins, including the novel protein introduced into line H7-1.

The detailed compositional studies therefore demonstrate that food derived from sugar beet line H7-1 is compositionally equivalent to food derived from non-GM sugar beet and other sugar beet lines.

Conclusion

No public health and safety concerns have been identified in the assessment of this glyphosate-tolerant sugar beet. Based on the available scientific evidence, food derived from sugar beet line H7-1 is equivalent to that derived from current commercial varieties of sugar beet in terms of its safety for human consumption. These conclusions are consistent with previous assessments of other glyphosate-tolerant food crops that use this genetic modification.

FOOD DERIVED FROM GLYPHOSATE-TOLERANT SUGAR BEET LINE H7-1

A SAFETY ASSESSMENT

BACKGROUND

A safety assessment has been conducted on food derived from sugar beet that has been genetically modified (GM) for tolerance to the herbicide glyphosate. The modified sugar beet is referred to as glyphosate-tolerant sugar beet line H7-1 but is know commercially as Roundup Ready® sugar beet.

Weed competition in commercial sugarbeet fields constitutes a significant crop production problem. Glyphosate is the active ingredient of the herbicide Roundup® which is used widely as a non-selective pre-emergent weed control agent in primary crops including sugarbeet. Glyphosate acts by specifically binding and blocking the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is essential for the biosynthesis of aromatic amino acids in all plants, bacteria and fungi (Steinrucken and Amrhein, 1980). Biochemical studies of the EPSPS enzyme have shown that natural variation in glyphosate-enzyme binding affinity exists across a variety of organisms, particularly across bacterial species (Schulz et al 1985). Tolerance to glyphosate in plants can therefore be achieved by introducing a bacterial version of the *epsps* gene that encodes for a version of the EPSPS protein with a reduced binding affinity for glyphosate, thus allowing plant aromatic amino acid synthesis to function normally in the presence of the herbicide.

Sugar beet line H7-1 contains the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4, which encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS). The CP4 EPSPS enzyme is structurally and functionally similar to native plant EPSPS enzymes, but has a lower affinity for the herbicide glyphosate (Padgette et al 1996). In non-GM plants, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby depriving plants of essential components (Steinrucken and Amrhein 1980). However, in glyphosate-tolerant sugar beet line H7-1, the CP4 EPSPS enzyme is not inactivated by glyphosate, so that growth and development of the plant can continue in the presence of the herbicide.

Currently sugar beet is the major sugar crop grown in temperate regions of the world. The most important food product derived from sugar beet is sucrose. Sugar beets are processed into white sugar, pulp and molasses. Each of these fractions have multiple uses for food, feed or industrial application, but sugar and to a much lesser degree molasses, are the principal food products derived from sugar beet.

In Australia, sugar is produced entirely from sugar cane. While refined sugar from sugar beet is not specifically imported into Australia or New Zealand, it may occur as an element within ingredients used in locally produced processed foods or as an ingredient within imported processed foods.

HISTORY OF USE

Sugar beet has been grown for sugar production since the late 18th century, when 'white Silesian beet' was identified as a source of sugar in Europe. Sugar beet currently accounts for approximately one-third of world sugar production, with some 35% being produced in the European Union, 20% in Russia and 10% in the US (Macrae et al 1993).

The root (i.e. the beet) of sugar beet is processed into two major food products — pure sucrose and molasses. Sugar beet pulp is a by-product of processing, which has occasionally been purified and sold as food fibre. Waste products from both pre-processing (leaves and tops) and post-processing (trash) are used as cattle feed.

Glyphosate-tolerant sugar beet line H7-1was derived from the KWS (seed company) proprietary multigerm line designated 3S0057, a cultivar of *Beta vulgaris* L. ssp. *vulgaris* (sugar beet). *Beta vulgaris* is normally biennial, developing a large succulent root during the first year and a seed stalk the second year. To induce the reproductive stage of sugar beet, a period of low temperature (vernalisation) is required.

Sugar beet is the major sugar crop grown in temperate regions of the world. Sugar beets are processed into white sugar, pulp and molasses. Each of these fractions has multiple uses for food, feed or industrial applications, but the principal food products derived from sugar beet are sugar and (to a much lesser degree) molasses.

DESCRIPTION OF THE GENETIC MODIFICATION

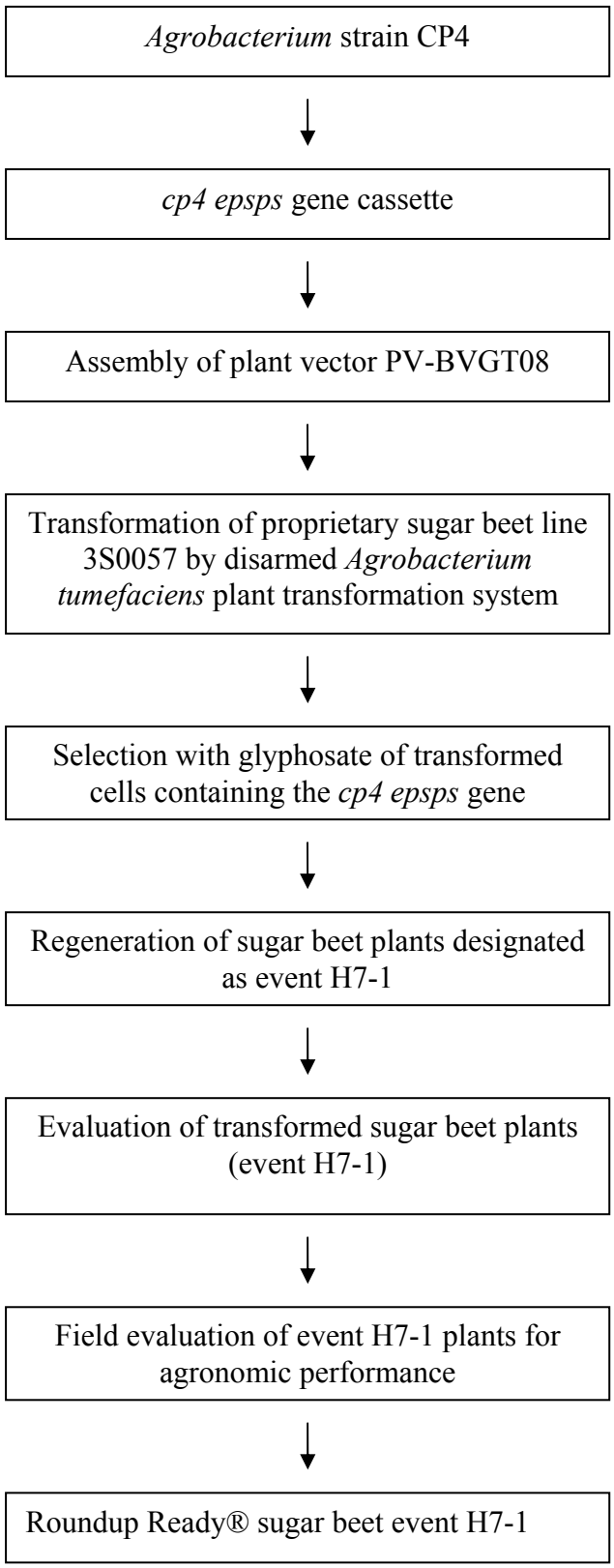
Method used in the genetic modification

Sugar beet genotype 3S0057 (the KWS proprietary line) was transformed by *Agrobacterium tumefaciens*-mediated transformation, using the binary vector PV-BVGT08.

The Agrobacterium-mediated DNA transformation system is the basis of natural plasmid-induced crown-gall formation in many plants and is well understood (Zambryski, 1992). The genes of interest were inserted into the plasmid between DNA sequences known as the Left and Right Borders (LB and RB). These border sequences were isolated from the Ti plasmid of Agrobacterium and normally delimit the DNA sequence (T-DNA) transferred into the plant.

For transformation, Agrobacteria were co-cultivated with sugar beet explants. After several days, the Agrobacteria were removed by treatment with antibiotics. Stably transformed sugar beet cells were selected for tolerance to glyphosate by using glyphosate as a selection agent in the cell culture media. The tolerant cells were regenerated to fertile plants and further analysed. The flow diagram shown in Figure 1 illustrates the steps used to develop line H7-1.

Figure 1: Development of event H7-1



Function and regulation of novel genes

The vector PV-BVGT08 used in the transformation contains a region of DNA (T-DNA) that is delineated by left and right border sequences, and contains a single *cp4 epsps* gene with essential regulatory elements necessary for expression in the chloroplasts of the sugarbeet plants. The organisation of the T-DNA, corresponding to approximately 3.4 kb is depicted in Figure 2. The function of each of the genetic elements present in the expression cassette is described in Table 1.

Figure 2: Diagram of DNA insert in sugar beet line H7-1

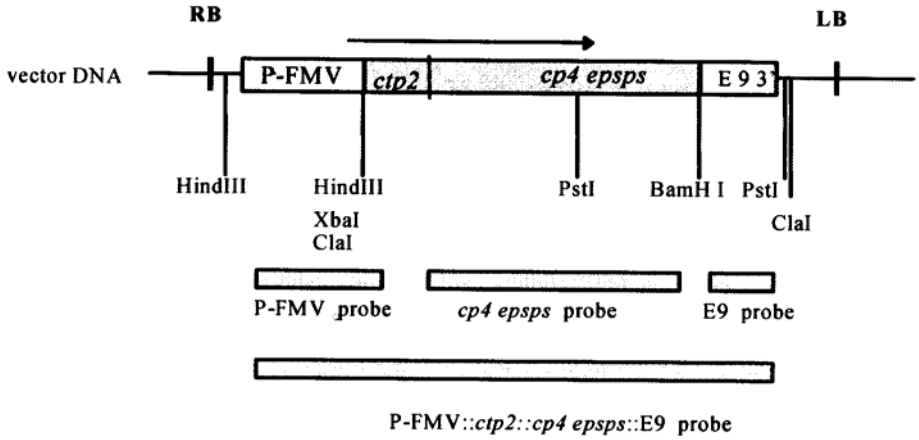


Table 1: Summary of genetic elements used in transformation

Genetic element	Description and reference	Function
Right border	A 21–25 bp nucleotide sequence, originally isolated from *A. tumefaciens* plasmid pTiT37 (Depicker et al 1982).	Acts as the initial point of DNA transfer into plant cells.
P-FMV	The 35S gene promoter from a modified figwort mosaic virus (FMV) (Sheperd et al 1987; Richins et al 1987; Gowda et al 1989; Sanger et al 1990).	Promoter of high level constitute gene expression in plant tissues.
ctp2	The N-terminal chloroplast transit peptide sequence from the *Arabidopsis thaliana epsps* coding region (Timko et al 1988).	Directs the EPSPS protein into the chloroplast, where it is active.
cp4 epsps	The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) coding region from *Agrobacterium sp*. strain CP4 (Padgette et al 1995).	Maintains aromatic amino acid synthesis through its insensitivity to glyphosate.
E9 3'	The 3' end of the *Pisum sativum rbc*S E9 gene, containing polyadenylation sites that direct mRNA processing and polyadenylation (Coruzzi et al 1984, Morelli et al 1985).	Contains signal sequences for termination of transcription.
Left border	A 21–25 bp nucleotide sequence, originally isolated from *A. tumefaciens* plasmid pTi 15955, a derivative of the octopine type plasmid, pTiA6 (Barker et al 1983).	Delimits the T-DNA transfer into plant cells.

The cp4 epsps gene

EPSPS is an essential enzyme involved in the biosynthesis of aromatic amino acids via the shikimate metabolic pathway. This metabolic pathway is present in all plants, bacteria and fungi (Haslam, 1993). Plant variants of the EPSPS enzyme are inhibited by the herbicide glyphosate, however, bacterial variants of the EPSPS enzyme are, in general, not inhibited due to reduced binding affinity to the herbicide (Schulz et al 1985). *Agrobacterium* sp. strain CP4 produces an EPSPS enzyme that is naturally tolerant to glyphosate (Padgette et al 1996). The *cp4 epsps* coding sequence has been shown to provide high levels of tolerance to glyphosate when introduced into plants (Padgette et al 1996).

The initiation of transcription of the *ctp2::cp4 epsps* coding region is controlled by the 35S gene promoter, derived from figwort mosaic virus (FMV). The 35S promoter is constitutively active in plants (Sheperd et al 1987; Richins et al 1987; Gowda et al 1989; Sanger et al 1990). The 3' end of the pea rbcS E9 gene (E9 3') terminates transcription and contains sequences that will direct the polyadenylation of the mRNA. The mature, active EPSPS enzyme is located in the chloroplasts. Therefore, in the construction of PV-BVGT08, a chloroplast transit peptide coding sequence (*ctp2*, which encodes 76 amino acids) from the *Arabidopsis thaliana epsps* coding region (Klee et al 1987) was joined to the *cp4 epsps* coding sequence, to provide a mechanism for transport to the sugar beet chloroplast.

Characterisation of the genes in the plant

Traditional molecular techniques were used to analyse the inserted DNA in sugar beet line H7-1. Southern blot analysis was used to determine the insert number; the copy number; the integrity of the promoters, coding regions and polyadenylation sequences; and the presence or absence of the transforming plasmid backbone sequence. Polymerase chain reaction (PCR) analyses were performed to verify the sequences at the 5' and 3' ends of the insert.

Insert and copy number

Southern hybridisation was used to determine the number and nature of DNA insertions in line H7-1. The genomic DNA was digested and probed with an internal sequence of the *cp4 epsps* coding region (covering basepairs 447–1555 of the plasmid). Using three different restriction enzymes, only a single hybridisation fragment was detected in each case, indicating that H7-1 represents a single integration event.

Theoretically, one integration site could contain more than one copy of the inserted DNA, but this is unlikely based on the size of the fragments detected in the Southern hybridisation experiments. Additional analyses were therefore done, to confirm that only one copy of the inserted DNA was present at a single genomic site in sugar beet line H7-1. The presence of a 1.2 kb and a 4.9 kb fragment confirmed that only one copy of the inserted DNA was present in line H7-1.

Integrity of gene cassette

The integrity of the gene cassette, with respect to the P-FMV promoter, *cp4 epsps* coding region and E9 3' polyadenylation signal region, was assessed by digestion with various restriction enzymes. Based on the size of the fragments generated by the digests, all the elements were found to be intact.

In addition, Southern blot analyses showed that bacterial genetic elements from outside of the T-DNA border sequences on the plasmid (i.e. *ori-V*, *ori-322* and *aad*) were not transferred to the sugar beet genome.

Study submitted:
Kraus J (2003). Sequencing of the T-DNA insert including plant genome flanking sequences in Roundup Ready® Sugar Beet event H7-1. Plant GmbH Technical Report, Study No. PLT6010-5MA, Einbeck, Germany.

PCR and sequence analysis

The DNA sequence of the insert in sugarbeet H7-1 was compared to the DNA sequence of the PV-BVGT08 plasmid. The aim was to determine whether the transformation process had altered the DNA sequence inserted into the plant.

Based on the alignment of the DNA sequences between the insert and the plasmid, the sequences were found to be almost identical, with the exception of four nucleotide differences. Three of the differences are located in non-coding regions of the introduced DNA, and therefore have no impact on the amino acid sequence of the CP4 EPSPS protein. The fourth difference is a single base change within the *cp4 epsps* coding region, and corresponds to a change from thymidine (T) to a cytosine (C), in the third position within the codon. This change does not affect the deduced protein sequence because the translated amino acid remains as threonine (ACT to ACC). Apart from these four nucleotides, the DNA inserted into the plant does not differ from the plasmid used in the transformation, and the differences noted have not changed the amino acid sequence of the CP4 EPSPS protein.

Flanking regions

Agrobacterium-mediated transformation involves the integration of DNA sequences between the left and right borders into the plant genome. The ends of the integrated plasmid DNA should be within or near the *A. tumefaciens* right and left border sequences. Inverse PCR showed that the nucleotide homology stopped exactly within the left border sequences with 21 nucleotides of the left border inserted. On the right border, the homology was interrupted 18 nucleotides in front of the border sequence, such that no right border sequences are contained in sugar beet line H7-1.

Conclusion

Detailed molecular characterisation has shown that a single copy of the introduced DNA was inserted into the genome of sugar beet line H7-1. This insert contains one

intact copy of the P-FMV*::ctp2::cp4 epsps::*E9 3' gene expression cassette. Backbone sequence from the plasmid used for the transformation was not detected in the plants. In particular, the bacterial origins of replication and the *aad* gene are not present in line H7-1. These results are summarised in Table 2.

Table 2: Summary of sugar beet line H7-1 insert analysis

Genetic element	Copy number
P-FMV*::ctp2::cp4 epsps::*E9 3' cassette	One copy
Bacterial origin of replication	Not present
aad (confers resistance to streptomycin)	Not present
Other plasmid backbone sequences outside the T-DNA	Not present

Stability of the genetic changes

Conventional breeding techniques were used to examine how glyphosate tolerance is inherited in sugar beet line H7-1. The characteristics studied were the stability of the inserted DNA and the phenotype (determined through segregation analysis).

Stability of inserted DNA

The genetic stability of the insert contained within H7-1 was analysed at the molecular level over three generations. The original transformation line H7-1 was compared to three progenies of this line resulting from self-pollination of the line or crosses with nontransgenic sugar beet lines. Nontransgenic plants were used as controls.

Southern hybridisations were performed on genomic DNA, and probed with a labelled *cp4 epsps* fragment, to detect the presence of the transgene. The results showed no differences in the banding patterns across multiple generations of line H7-1, demonstrating that the inserted DNA was stably integrated into the plant genome.

Segregation analysis

In other glyphosate-tolerant commercial crops, glyphosate tolerance is inherited as a dominant trait in a Mendelian manner. The inheritance of the introduced DNA in the progenies from these crosses or multiplications was monitored phenotypically at the whole-plant level by application of glyphosate at the two-leaf stage in greenhouse experiments.
The results from the analysis show that, of the 27 experiments, 24 resulted in segregation patterns as expected from Chi-square tests, at a probability level of $P = 0.05$. Different results in a few experiments were explained as being due to the small number of plants tested on those occasions.

Conclusion

The results of the segregation analysis are consistent with a single site of insertion for the *cp4 epsps* gene and confirm the results of the molecular characterisation.

Analysis of a total of three generations indicates that the single T-DNA insert in sugar beet line H7-1 is integrated in the plant nuclear genome in a stable manner through subsequent generations.

CHARACTERISATION OF NOVEL PROTEINS

A single novel protein is present in sugar beet line H7-1. The CP4 EPSPS protein is 47.6 kDa and consists of a single polypeptide of 455 amino acids.

Biochemical function and phenotypic effects

The EPSPS enzyme is essential in the biosynthesis of the aromatic amino acids, via the shikimate metabolic pathway present in all plants, bacteria and fungi. In plants, the EPSPS enzyme is inhibited by glyphosate (Steinrucken and Armhein 1980), but bacterial EPSPSs, such as the CP4 EPSPS, have a reduced affinity for glyphosate.

In plants, EPSPS is found in the chloroplast. In sugar beet line H7-1, the CP4 EPSPS gene was fused to the *Arabidopsis thaliana* EPSPS chloroplast transit peptide (CTP), which targets the protein to the chloroplast. *In vitro* chloroplast uptake assays have shown that the *A. thaliana* EPSPS CTP delivers mature CP4 EPSPS to the chloroplast, following cleavage from the preprotein (della Cioppa et al 1986). The chloroplast transit peptide is rapidly degraded after cleavage *in vivo* by cellular proteases.

Protein expression analysis

Expression levels in plants

CP4 EPSPS levels were measured in brei (root) and top (leaf) samples from sugar beet line H7-1 treated with glyphosate, grown in six areas of Europe. Protein levels in extracts were estimated using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA).

The test uses a mouse monoclonal anti-CP4 EPSPS antibody as the capture antibody and a goat polyclonal anti-CP4 EPSPS conjugated to horseradish peroxidase (HRP) as the detection antibody.

On average, levels of CP4 EPSPS protein were similar in the top and root samples. The results are summarised in Table 3. The range of mean levels of CP4 EPSPS protein was not significantly different across the six European sites used.

Table 3: Summary of CP4 EPSPS levels in tissues of sugar beet line H7-1

Tissue type	CP4 EPSPS protein (µg/g tissue fresh weight)
Top[1]	
mean[2]	161
range[3]	112–201
Brei[4]	
mean[2]	181
range[3]	145–202

[1] One leaf approximately 5–10 cm^2 was sampled from 30 sugar beet line H7-1 plants for each replicate. Three replicates were collected per site. Collected leaves were placed in conical tubes and transferred on dry ice to the testing facility.
[2] The mean was calculated from the analyses of three replicate plant samples from each of the field sites.
[3] Range of mean values from the analyses of samples at each site; in top (leaf), *n* = 6 sites and in root (brei), *n* = 6 sites.
[4] Brei was prepared by a French laboratory, AGREN, using a sawing machine. Samples were immediately frozen on dry ice and then stored at ⁻80°C until analysed.

Protein levels in sugar

Protein levels in the sugar derived from sugar beet line H7-1 are anticipated to be very low, due to extensive processing. Using Western blotting (which has a limit of detection at two parts per billion) no CP4 EPSPS protein was detected in the processed sugar from sugar beet line H7-1. Therefore, exposure to CP4 EPSPS from sugar derived from sugar beet line H7-1 is likely to be virtually zero.

Potential toxicity of novel proteins

Equivalence of novel plant proteins with bacterially produced novel proteins

To generate sufficient quantities of the CP4 EPSPS protein required for toxicity, and biochemical studies, it is necessary to produce the protein in bacterial expression systems. Prior to use, the bacterially produced protein is compared to the protein produced in the plant, to demonstrate their equivalence. The CP4 EPSPS used for further analyses was produced in the laboratory using recombinant *Escherichia coli*.

The molecular identity and biochemical characteristics of the protein expressed *in planta* and in the bacterial expression system were examined using a range of biochemical methods. These studies established that microbially-produced CP4 EPSPS protein was equivalent to the protein produced by sugar beet line H7-1.

The amino acid sequence of the mature CP4 EPSPS protein produced in sugar beet line H7-1 is virtually identical to the amino acid sequence of the bacterial CP4 EPSPS protein (homology more than 99%) and to that of the EPSPS protein produced in a number of other glyphosate tolerant food crops that have already been assessed. The CP4 EPSPS protein produced in sugar beet line H7-1 is identical to the CP4 EPSPS protein produced in sugar beet line 77, which was approved for use in Australia and New Zealand in 2001 (Application A378).

Potential toxicity of novel protein

The potential toxicity of the CP4 EPSPS protein has been assessed by FSANZ in a number of previous safety assessments of GM foods derived from glyphosate tolerant crops. The previous assessments concluded that the CP4 EPSPS protein is not toxic and is therefore safe for human consumption.

The mature CP4 EPSPS protein in sugar beet line H7-1 is substantially similar to the EPSPS proteins already consumed in a variety of food and feed sources. The CP4 EPSPS protein is homologous to plant EPSPS enzymes naturally present in food crops (eg soybean and corn), and in fungal and microbial food sources such as baker's yeast (*Saccharomyces cerevisiae*) and *Bacillus subtilis* (Mountain 1989), which have a history of safe consumption by humans (Padgette et al 1996; Harrison et al 1996).

Acute oral toxicity was tested in a supplementary evaluation (Harrison et al 1996). The mature CP4 EPSPS protein was administered to mice as a single high dose of 572 mg/kg (more than 1000 times the consumption level of food products potentially containing the protein). Despite this high dose, there was no mortality or morbidity, and there were no significant differences in terminal body weights of animals in the treated and control groups. Upon necropsy, body cavities were opened and organs examined *in situ* and removed. There were no pathological findings attributable to the treatment with the CP4 EPSPS protein.

Similarities with known protein toxins

The amino acid sequence of the CP4 EPSPS protein in sugar beet line H7-1 was compared to sequences in the public domain (ALLPEPTIDES) protein databases using the FASTA algorithm (Pearson and Lipman 1988), in order to assess sequence and structural homology to known proteins including toxins.

Overall, no structural similarities were observed between the CP4 EPSPS protein in sugar beet line H7-1 and pharmacologically active proteins that are known to cause adverse health effects in humans or other animals.

Potential allergenicity of novel proteins

Similarity to known allergens

The CP4 EPSPS protein was obtained from the naturally occurring soil-borne plant-pathogenic bacterium *Agrobacterium* sp. strain CP4. This source organism is not known to be allergenic to humans.

Bioinformatics analysis investigates whether there are sequence similarities between the introduced CP4 EPSPS protein and proteins that are known allergens.

Using updated versions of the allergen (ALLERGEN3) and public domain protein sequence databases, no sequence similarity between CP4 EPSPS and known protein allergens was found.

In vitro digestibility

The *in vitro* digestibility of CP4 EPSPS protein, isolated from large-scale fermentation of *E. coli,* was tested in simulated gastric fluid (SGF), containing pepsin, at pH 1.2. Digestibility was assessed by SDS polyacrylamide gel electrophoresis, Western blot analysis and an assay for EPSPS enzyme activity.

By all three detection methods, CP4 EPSPS protein was rapidly digested after incubation in SGF at 37^{o}C. At least 98% of the *E. coli*-produced CP4 EPSPS protein was digested within 15 seconds, as determined by colloidal blue staining. More than 95% of the protein was digested in SGF within 15 seconds as determined by Western blot analysis. EPSPS activity was reduced by > 90% within 15 seconds of incubation of the CP4 EPSPS protein in SGF.

In summary, the three detection methods all demonstrate that *E. coli*-produced CP4 EPSPS protein is rapidly degraded in SGF.

Conclusion

Sugar beet line H7-1 expresses one novel protein, CP4 EPSPS, which is targeted to the plant chloroplasts. It is expressed in the root and leaves at low levels, with the highest expression level being 202 µg/g tissue fresh weight, found in brei.

A combination of bioinformatics, toxicity studies and *in vitro* biochemical studies was used to evaluate the potential toxicity and allergenicity of the CP4 EPSPS protein. The results from these studies demonstrate that the CP4 EPSPS protein is unlikely to be toxic or allergenic to humans. This assessment agrees with the conclusions of previous safety assessments of the CP4 EPSPS protein.

COMPARATIVE ANALYSES

A comparative approach is considered the most appropriate strategy for assessing the safety and nutrition of GM foods (WHO 2000). This approach examines the similarities and differences between the GM food and its conventional counterpart, to identify potential safety and nutritional issues, based on the history of safe use of the traditional non-GM food. The critical components to be measured are determined by identifying the main nutrients, toxicants and antinutrients for the food source in question (FAO 1996); these may be major constituents (e.g. fats, proteins and carbohydrates) or quantitatively minor ones (e.g. minerals and vitamins). Important toxicants are any toxicologically significant compounds that are known to be inherently present in the plant, and may be significant to health (e.g. solanine in potatoes, which is toxic if present in high levels). The main components of sugar beet that have been considered in this comparison include proximates, carbohydrates, minerals, quality parameters, 18 amino acids and saponins (OECD 2002).

Compositional analysis

To determine whether the genetic modification resulted in changes to the nutrient composition of sugar beet line H7-1, a range of compositional parameters in line H7-1 were compared to the non-GM control lines with genetic backgrounds similar to H7-1, as well as to commercially available varieties grown in the same field trials. Components analysed in both top (leaf) and root (brei) samples were: proximate content (moisture, fat, protein, fibre and ash), carbohydrates, minerals (sodium and potassium), quality parameters (percentage sucrose, invert sugar and alpha-amino nitrogen), 18 amino acids and saponins.

Sugar beet line H7-1 and its non-GM control were grown in 1999, in sites in France, Germany, Italy, Spain and the United Kingdom. Sugar beet top and root tissues were collected from sugar beet line H7-1, the non-expressing segregant (control) and eight different commercial sugar beet varieties grown at the same field locations. Root tissue from all sugar beets was processed into brei before analysis.

Replicate samples from each plot were analysed individually, and the results combined to determine mean values across the five sites. Statistical analyses were conducted on the combined mean values, to identify statistically significant differences between sugar beet line H7-1 and its non-GM control at $P < 0.05$ (a P-value of < 5% would indicate that the effect of genotype was statistically significant at the 5% level). The results were also compared with values from the literature.

Proximate and carbohydrate analyses

Tables 4 and 5 summarise the results of the proximate analyses in top and root tissue, respectively. One minor statistically significant difference between sugar beet line H7-1 and the control line was observed in the mean level of dry matter in top tissue. However, the mean for the dry matter in top samples of H7-1 is within the range for controls, reference varieties and the literature, and the range is within that observed for reference sugar beet varieties. There were no significant differences observed in the results obtained for the root (brei) tissue samples.

Table 4: Summary of proximate analyses of top (leaf) tissue from sugar beet line H7-1

Analysis	Unit	Control sample[1]		Line H7-1[1]		Reference varieties[2]		Literature range[3]
		Mean	Range	Mean	Range	Mean	Range	
Dry matter[4]	%	16.37	13.69–19.83	17.98*	13.94–21.23	16.18	11.37–26.81	16–20
Crude protein[5]	% DM	16.02	14.46–19.80	15.27	11.16–18.31	16.65	12.75–24.47	8.4–23.2
Crude fibre[6]	% DM	11.43	9.23–16.82	11.50	8.56–15.99	11.62	7.84–19.71	5.9–15.9
Crude ash[7]	% DM	19.50	15.39–21.96	21.95	17.84–31.90	21.80	16.20–27.91	11.5–34.4
Crude fat[8]	% DM	0.87	0.74–1.15	0.95	0.85–1.09	1.02	0.53–1.46	0–4.7

Control sample = nontransgenic control with a genetic background similar to line H7-1.

[1] *n* = 5 sites, single analyses of replicate samples.

[2] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.

[3] See reference DLG 1991.

[4] Dry matter was determined using an oven method.

[S] Crude protein was determined using a Kjeldahl method.

[6] Crude fibre was determined using the Weende analysis.

[7] Crude ash was determined using an oven method.

[8] Crude fat was determined using a Soxhlet method.

***** indicates a significant difference at 5% level when compared with the corresponding nontransgenic control.

Table 5: Summary of proximate analyses of root (brei) tissue from line H7-1

Analysi	Unit	Control sample[1]		Line H7-1[1]		Reference varieties[2]		Literature range[3]
		Mean	Range	Mean	Range	Mean	Range	
Dry matter[4]	%	24.01	21.75–25.83	25.46	22.87–28.88	22.74	19.05–26.33	23
Crude protein[5]	% DM	5.62	4.13–6.98	5.51	4.50–6.57	5.06	3.72–6.93	1.2–12.4
Crude fibre[6]	% DM	4.84	4.57–5.04	4.54	3.73–5.20	4.75	3.65–5.69	3.4–7.4
Crude ash[7]	% DM	2.54	1.78–3.21	2.51	1.73–3.35	2.41	1.74–3.89	1.3–17.7
Crude fat[8]	% DM	0.20	0.06–0.38	0.13	0.08–0.18	0.16	0.05–0.25	0–1.8

Control sample = nontransgenic control with a genetic background similar to line H7-1.
[1] *n* = 5 sites, single analyses of replicate samples.
[2] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.
[3] See reference DLG 1991.
[4] Dry matter was determined using an oven method.
[5] Crude protein was determined using a Kjeldahl method.
[6] Crude fibre was determined using the Weende analysis.
[7] Crude ash was determined using an oven method.
[8] Crude fat was determined using a Soxhlet method.

Table 6 summarises the results of the analyses of levels of soluble carbohydrates in top and root tissue samples of sugar beet line H7-1, the control line, reference varieties and those reported in the literature. There were no statistically significant differences between mean levels of soluble carbohydrates in top and root samples of H7-1 when compared to the non-GM control samples.

Table 6: Summary of soluble carbohydrate determination from line H7-1

Analysi	Unit	Control sample[2]		Line H7-1[2]		Reference varieties[3]		Literature range[4]
		Mean	Range	Mea	Range	Mea	Range	
Top	% DM	52.18	45.50–58.03	50.35	44.60–54.68	48.92	43.30–56.52	38.3–64.5
Root	% DM	86.80	84.44–89.02	87.31	85.58–89.04	87.62	83.31–90.05	67.3–90.9

Control sample = nontransgenic control with a genetic background similar to line H7-1.
[1] Carbohydrate calculation = 100% – (crude protein + crude ash + crude fibre + crude fat).
[2] *n* = 5 sites, single analyses of replicate samples.
[3] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.
[4] Reference DLG 1991.

Mineral and quality analysis

The minerals potassium and sodium were analysed, together with sugar content (measured by polarisation), invert sugar (glucose + fructose) and alpha-amino nitrogen. Table 7 shows the results of these analyses. The mean levels of the five components measured in sugar beet root tissues from line H7-1 were not significantly different from the mean levels in the non-GM control samples.

Table 7: Summary of mineral and quality analyses of root (brei) tissue from line H7-1

Analysis	Unit	Control sample[1]		Line H7-1[1]		Reference varieties[2]		Literature range[3]
		Mea	Range	Mea	Range	Mea	Range	
Potassium[4]	nmol/100 kg FW	3.85	3.08–4.87	3.85	3.08–5.13	4.10	2.82–5.38	2.95–10.21
Sodium[5]	nmol/100 kg FW	0.65	0.26–1.83	0.57	0.16–2.04	0.61	0.19–2.39	0.13–5.48
Polarization[6]	g/100g FW	18.12	16.11–19.23	18.54	16.14–20.21	17.08	14.14–19.51	10.8–20.7
Invert sugar[7]	nmol/100 kg FW	0.83	0.24–2.94	0.78	0.24–2.61	0.67	0.17–2.94	0.3–2.7
Amino-N[8]	nmol/100 kg FW	1.29	0.79–1.71	1.29	0.86–1.93	1.21	0.63–2.50	0.80–5.62

Control sample = nontransgenic control with a genetic background similar to line H7-1.
[1] *n* = 5 sites, single analyses of replicate samples.
[2] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.
[3] See references Märländer et al 1996 and Smed et al 1996.
[4] Potassium was determined using a spectrophotometer.
[5] Sodium was determined using a spectrophotometer.
[6] Polarisation was determined using a polarimeter.
[7] Invert sugar was determined using the Institute of Berlin method.
[8] Amino-N was determined using a spectrophotometer.

Fatty acid analysis

The only food product obtained from sugarbeet is a highly refined sugar. This product does not contain fats and therefore fatty acid analysis is not relevant for this application.

Amino acid analysis

Tables 8 and 9 show the results of analyses of levels of amino acids in sugar beet top and root samples, respectively. For top samples, mean levels of 14 of the 18 amino acids measured were not significantly different between sugar beet H7-1 and non-GM control samples. Mean levels of four amino acids (alanine, histidine, phenylalanine and tyrosine) were statistically different when compared to the corresponding mean levels from the non-GM control samples. However, the ranges observed for these four amino acids from sugar beet line H7-1 either mainly overlapped or were completely within the ranges of values for the non-GM control samples and the commercial reference varieties.

Table 8: Summary of amino acid analyses of top (leaf) tissue from line H7-1

Analysis[1]	Unit	Control sample[2]		Line H7-1[2]		Reference varieties[3]	
		Mean	Range	Mea	Range	Mea	Range
Alanine	% total aa	6.44	6.29–6.61	6.67*	6.29–7.07	6.53	5.98–6.97
Arginine	% total aa	5.36	5.13–5.69	5.44	5.06–5.91	5.42	4.68–5.96
Aspartic acid	% total aa	10.29	10.16–10.36	10.53	9.96–11.37	10.55	9.55–11.40
Cystine	% total aa	1.73	1.18–2.24	1.90	0.87–3.20	1.77	1.07–3.05
Glutamic acid	% total aa	13.46	12.20–14.78	13.10	12.35–13.56	13.92	12.29–15.55
Glycine	% total aa	6.86	6.23–7.32	6.80	6.45–7.47	6.78	6.21–7.80
Histidine	% total aa	2.46	2.00–2.73	2.29*	1.73–2.54	2.26	1.75–2.68
Isoleucine	% total aa	4.49	4.26–4.86	4.39	4.12–4.67	4.44	4.11–4.97
Leucine	% total aa	8.20	7.58–9.19	8.13	7.66–9.06	8.01	7.16–9.01
Lysine	% total aa	5.36	4.82–5.81	5.25	3.75–5.81	5.35	4.60–5.85
Methionine	% total aa	1.83	1.37–2.45	2.20	1.13–4.05	1.74	1.06–3.18
Phenylalanine	% total aa	5.12	4.76–5.46	4.98*	4.65–5.30	4.99	4.47–5.40
Proline	% total aa	7.04	5.53–7.95	7.04	6.55–7.47	7.15	5.46–9.58
Serine	% total aa	5.80	5.48–6.24	5.94	5.47–6.33	5.78	5.34–6.21
Threonine	% total aa	4.82	4.42–5.05	4.71	4.09–5.07	4.47	3.39–5.23
Tryptophan	% total aa	1.60	1.30–1.86	1.69	1.17–2.13	1.81	1.37–2.45
Tyrosine	% total aa	3.65	3.28–3.87	3.46*	3.05–3.69	3.63	3.21–4.70
Valine	% total aa	5.51	5.29–6.17	5.49	5.26–5.99	5.37	4.97–6.17

[1] Amino acids were determined using a high-performance liquid chromatography (HPLC) method.
[2] *n* = 5 sites, single analyses of replicate samples.
[3] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties
[*] indicates a significant difference at 5% level when compared with the corresponding non-transgenic control.

For root samples, mean levels of 16 of the 18 amino acids measured were not significantly different between sugar beet H7-1 and the non-GM control samples. Mean levels of two amino acids (alanine and glutamic acid) were statistically different when compared to the corresponding mean levels from the non-GM control samples. However, the ranges observed for these two amino acids either mainly overlapped or

were completely within the range of values for the non-GM control samples and the commercial reference varieties.

Table 9: Summary of amino acid analyses of root (brei) tissue from line H7-1

Analysis[1]	Unit	Control sample[2]		Line H7-1[2]		Reference varieties[3]	
		Mea	Range	Mean	Range	Mea	Range
Alanine	% total aa	5.29	4.69–6.33	5.91*	5.04–6.73	6.27	4.73–9.43
Arginine	% total aa	4.91	4.50–5.18	5.30	4.94–5.63	4.80	4.02–5.74
Aspartic acid	% total aa	13.35	12.32–14.75	13.21	12.66–14.46	13.19	11.57–15.53
Cystine	% total aa	1.38	1.28–1.53	1.42	1.25–1.72	1.45	0.84–2.28
Glutamic acid	% total aa	18.58	15.66–21.85	16.87*	14.72–19.98	19.21	13.76–25.07
Glycine	% total aa	4.23	3.74–4.54	4.73	4.15–5.21	4.61	3.83–6.18
Histidine	% total aa	2.69	1.58–3.33	2.95	2.00–3.45	2.87	1.78–3.43
Isoleucine	% total aa	4.01	3.90–4.24	4.23	4.15–4.35	3.95	3.27–4.95
Leucine	% total aa	6.09	5.55–6.61	6.47	5.90–7.18	6.07	4.82–6.95
Lysine	% total aa	5.42	3.50–6.88	5.73	4.29–6.52	5.49	3.69–7.11
Methionine	% total aa	1.35	1.23–1.46	1.29	1.05–1.57	1.28	0.70–2.04
Phenylalanine	% total aa	3.36	2.98–3.69	3.45	3.29–3.66	3.26	2.75–3.75
Proline	% total aa	5.94	5.53–6.46	5.39	3.29–7.02	5.11	1.71–9.29
Serine	% total aa	7.34	6.61–8.49	7.55	6.86–8.45	7.58	6.63–8.72
Threonine	% total aa	4.76	4.11–5.30	4.98	4.51–5.28	4.75	3.96–5.51
Tryptophan	% total aa	2.30	1.11–4.26	1.82	1.06–2.44	1.93	1.02–5.40
Tyrosine	% total aa	3.53	3.27–3.86	3.55	3.09–4.23	3.34	2.51–4.23
Valine	% total aa	5.47	5.10–5.82	5.14	3.79–5.87	4.84	1.99–7.49

Control sample = nontransgenic control with a genetic background similar to line H7-1.
[1] Amino acids were determined using a high-performance liquid chromatography (HPLC) method.
[2] *n* = 5 sites, single analyses of replicate samples.
[3] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.
[*] indicates a significant difference at 5% level when compared with the corresponding nontransgenic control.

Naturally occurring toxicants

Sugar beet naturally contains low levels of toxic saponins. As their name implies, saponins are a group of compounds with properties resembling soap and detergents. They are a complex and chemically diverse group of compounds incorporating both triterpenes and steroids linked to one or more sugar groups. Saponins are found naturally, and in significant amounts, in commonly used food and forage plants such as clover, alfalfa, soybeans, chickpeas, eggplant, silver beet and spinach (Oakenfull and Sidhu 1989), and are characterised by having a bitter and astringent taste. The predominant sapogenic form in sugar beet is oleanolic acid. Due to their surface-active properties, saponins can cause problems with foaming and turbidity during production of sugar from sugar beet; therefore, efforts are made to reduce saponin levels through processing.

The wide range of chemical and physical properties of saponins is reflected in the extent and range of their physiological and pharmacological properties. For example, saponins have been shown to interact with biological membranes (due to their detergent qualities) and to both inhibit and stimulate enzymes and metabolic activity (Oakenfull and Sidhu 1989). There has been a tendency to treat saponins exclusively as antinutritional or toxic constituents; however, recent work has shown several beneficial dietary effects of saponins, including improved nutrient absorption during digestion and lower blood cholesterol levels (Oakenfull and Sidhu 1989).

Table 10 shows the results of analysis of saponin levels in the roots and tops of sugar beet line H7-1 and the non-GM control sugar beet plants. The results show that saponin levels for sugar beet line H7-1 are not significantly different from those of the non-GM control samples, or the values reported in the literature.

Table 10: Summary of saponin analyses of top and root tissues from line H7-1

Analysis[1]	Unit	Control sample[2]		Line H7-1[2]		Reference varieties[3]		Literature range[4]
		Mean	Range	Mean	Range	Mean	Range	
Top	mg/kg FW	65	47–100	58	34–93	76	27–200	50–600
Root	mg/kg FW	90	54–120	92	54–150	99	63–170	75–965

Control sample = nontransgenic control with a genetic background similar to line H7-1.
[1] Saponin was determined using a high-performance liquid chromatography (HPLC) method.
[2] *n* = 5 sites, single analyses of replicate samples.
[3] *n* = 5 sites, single analyses of replicate samples, eight commercial varieties.
[4] See reference Lüdecke et al 1958.

Conclusions

Detailed compositional analyses of sugar beet line H7-1, using the non-GM counterpart as a control, included proximate analyses, carbohydrates, minerals, quality components, 18 amino acids and saponin. All parameters were analysed in both top and root tissues, except for minerals and quality components, which were analysed in root tissue only.

A total of 55 statistical comparisons were made between sugar beet line H7-1 and the non-GM control line for both tissues. Seven of these comparisons were significantly different at $P < 0.05$, but in each case, the ranges overlapped with, or were completely within the range of, values observed for the non-GM control, the commercial reference varieties and available published values for commercial sugar beet varieties. These results indicate that the nutrient composition in top and root tissues of sugar beet line H7-1 is equivalent to that of commercial sugar beet varieties. The minor differences in composition that were noted do not raise any food safety concerns with respect to the sugar product. Minor differences in composition for other constituents (eg amino acids) will not impact on the final food.

NUTRITIONAL IMPACT

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further reassurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients, or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

In the case of glyphosate-tolerant sugar beet line H7-1, no significant compositional differences were evident. On the basis of these findings, feeding studies were not considered warranted in this case.

Furthermore, the principal human food derivative from sugar beet line H7-1 is highly refined sugar which is composed of 96–99% sucrose and 0.6–1.2% other sugars such as glucose and fructose. The extensive processing involved in the production of sugar effectively eliminates all plant proteins, including the novel protein, from the final food product. Refined sugar from any source has a long history of safe use as a human food.

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