FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Safety assessment - **Application A1280**

Food derived from herbicide-tolerant and insect-protected corn line DAS1131

Executive summary

Application A1280 seeks approval for the sale and use of food derived from corn line DAS1131 that has been genetically modified (GM) for tolerance to the herbicide glyphosate and protection from lepidopteran insect pests.

Protection against lepidopteran insect pests is conferred by the expression of the insecticidal *cry1Da2* gene encoding the Cry1Da2 protein. The *cry1Da2* gene is composed of two sequences from *Bacillus thuringiensis*: (1) the insecticidal core toxin sequence of the *cry1Da2* gene; and (2) a segment of the *cry1Ab* gene. Tolerance to the herbicide glyphosate is achieved by the expression of the *dgt-28 epsps* gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (DGT-28 EPSPS) from *Streptomyces sviceus*. The Cry1Da2 and DGT-28 EPSPS proteins have not previously been assessed by FSANZ.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of use

Corn has a long history of safe use in the food supply. Corn-derived products are routinely used in a large number and diverse range of foods e.g. cornflour, starch products, breakfast cereals and high fructose corn syrup.

Molecular characterisation

The genes encoding Cry1Da2 (*cry1Da2*) and DGT-28 EPSPS (*dgt-28 epsps*) were introduced into corn line DAS1131 via *Agrobacterium*-mediated transformation. Detailed molecular analyses of corn line DAS1131 indicate that a single copy of each of the linked *cry1Da2* and *dgt-28 epsps* cassettes is present at a single insertion site in the genome. There are no extraneous plasmid sequences, selectable marker cassettes or antibiotic resistance marker genes, present in this line.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be present within a single locus and stably inherited across multiple generations.

Characterisation and safety assessment of new substances

Newly expressed proteins

Cry1Da2 and DGT-28 EPSPS are newly expressed proteins present in DAS1131. Cry1aD2 is expressed at a low level in grain and at a high level in pollen, while DGT-28 EPSPS is expressed at a low level in roots and at a high level in leaf tissue. Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity to known protein toxins or allergens. Laboratory studies demonstrated the Cry1Da2 and DGT-28 EPSPS proteins are susceptible to the action of digestive enzymes and would be thoroughly degraded before being absorbed during passage through the gastrointestinal tract. The proteins are also susceptible to heat denaturation and degradation at the high temperatures typically used in food processing. Taken together, the evidence supports the conclusion that Cry1Da2 and DGT-28 EPSPS are not toxic or allergenic to humans.

Herbicide metabolites

For glyphosate, the metabolic profiles resulting from the protein/herbicide interactions have been established through a significant history of use. There are no concerns that the spraying of corn line DAS1131 with glyphosate would result in the production of metabolites that are not also produced in the non-GM crops sprayed with the same herbicide and already used in the food supply.

Compositional analyses

Detailed compositional analyses were performed on DAS1131. Statistically significant differences were found between grain from DAS1131 and the control for 7 of the 70 analytes evaluated, however these differences were small and all within the range established for existing commercial non-GM corn cultivars. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in grain from DAS1131 compared to non-GM corn cultivars available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant and insect-protected corn line DAS1131. On the basis of the data provided in the present application and other available information, food derived from DAS1131 is considered to be as safe for human consumption as food derived from conventional non-GM corn cultivars.

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List of Abbreviations

Abbreviation	Description
ADF	acid detergent fibre
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
CI	confidence interval
COMPARE	COMprehensive Protein Allergen REsource
DNA	deoxyribonucleic acid
dw	dry weight
ELISA	enzyme-linked immunosorbent assay
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FASTA	fast alignment search tool – all
FSANZ	Food Standards Australia New Zealand
g	gram
GM	genetically modified
HFCS	high fructose corn syrup
kDa	kilodalton
LLOQ	lower limit of quantitation
mg	milligram
Min	Minutes
MT	million tons
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
ng	nanogram
NGS	next generation sequencing
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PCR	polymerase chain reaction
PEP	Phosphoenolpyruvate
RF	reading frame
SbS	Southern-by-sequencing
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
S3P	Shikimate -3-phosphate
TDF	total dietary fibre
µg	microgram
USDA	United States Department of Agriculture

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Corteva Agriscience Australia Proprietary Limited to vary Schedule 26 in the Australia New Zealand Food Standards Code (the Code). The variation is to include food from a new genetically modified (GM) corn line DAS1131, with the OECD Unique Identifier DAS-Ø1131-3. This corn line is tolerant to the herbicide glyphosate and protected against lepidopteran insect pests.

Protection against lepidopteran insect pests is conferred by the expression of the insecticidal *cry1Da2* gene encoding the Cry1Da2 protein. The *cry1Da2* gene is composed of two sequences from *Bacillus thuringiensis*: (1) the insecticidal core toxin sequence of the *cry1Da2* gene; and (2) a segment of the *cry1Ab* gene.

Tolerance to the herbicide glyphosate is achieved by the expression of the *dgt-28 epsps* gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (DGT-28 EPSPS) from *Streptomyces sviceus*. The DGT-28 EPSPS protein is fused to TraP8, a chimeric chloroplast transit peptide derived from *Brassica napus* and *Brassica rapa,* enabling targeting to the chloroplast.

Many different Cry and EPSPS proteins are found in a variety of different GM crops grown around the world (FAO 2023; ISAAA 2023). FSANZ has assessed and approved 29 applications where Cry proteins have been introduced into crops for pest protection and 25 applications where EPSPS proteins have been used in crops for herbicide-tolerance. Crop species with Cry and EPSPS proteins that FSANZ has assessed and approved include corn, cotton, potato and soybean. A1280 is the first application to assess the specific Cry and EPSPS proteins, Cry1Da2 and DGT-28 EPSPS.

If approved, food derived from DAS1131 corn line may enter the Australian and New Zealand food supply as imported food products.

2 History of use

2.1 Host organism

The host organism is corn (*Zea mays*) which is also referred to as maize. The inbred corn line B104 was used as the parental variety for the genetic modification described in this application.

Corn was one of the first plants to be cultivated by humans (Ranum et al. 2014) and is now the world's dominant cereal crop, with global production of 1,151 MT[1] in 2022/23, ahead of wheat (788 MT) and rice (513 MT) (USDA 2023). Due to its economic importance, corn has been the subject of extensive study[2].

The United States is the world's largest producer of corn, producing 349 MT in 2022/23 (USDA 2023). Canada produced 14.5 MT in 2022/23 (USDA 2023). Of the corn grown in the

[1] million tons

[2] Refer to detailed reports published by the OECD (OECD 2002), the Grains Research and Development Corporation (GRDC 2017) and the Office of the Gene Technology Regulator (OGTR 2008).

United States and Canada, an estimated 92% and ~90%, respectively, is GM[3,4,5].

Corn is not a major crop in Australia or New Zealand. In 2021 these amounted to 0.306 and 0.209 MT respectively (FAOSTAT 2023). No GM corn is currently grown commercially in Australia or New Zealand.

To supplement their limited local production of corn, Australia and New Zealand import both corn grain and processed corn products. For example, in 2021 the imported quantities of corn flour into Australia and New Zealand were 11,626 and 1,284 tonnes respectively, while imports of corn oil totalled 1,106 and 122 tonnes respectively (FAOSTAT 2023).

Corn has a long history of safe consumption as food by humans[6]. Food products derived from processing of corn kernels include corn flour, meal, oil, starch and sweeteners such as high fructose corn syrup (HFCS). In Australia and New Zealand, corn starch is used in dessert mixes and canned foods, and HFCS is used in breakfast cereals, baking products, corn chips and extruded confectionary.

2.2 Donor organisms

2.2.1 Bacillus thuringiensis

The gene encoding the Cry1Da2 protein is derived from *Bacillus thuringiensis*, a facultative anaerobic, gram-positive spore-forming bacterium found in soil (Palma et al. 2014). *B. thuringiensis* expresses a number of insecticidal proteins, including the well-characterised Cry proteins. These toxins are highly specific to their target insects, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al. 2007). *B. thuringiensis* strains have a long history of use for the control of agricultural insect pests[7] (Nester et al. 2002; CERA 2011). Currently, there are approximately 44 biopesticide products based on *B. thuringiensis* registered in Australia (APVMA 2023) and 10 in New Zealand (ACVM 2023).

B. thuringiensis has been linked to human diarrheal illness due to its close relationship with the species *Bacillus cereus*. Some *B. cereus* strains contain enterotoxin genes and are pathogenic to humans (Ehling-Schulz et al. 2019; Biggel et al. 2022), However, it is unlikely *B. thuringiensis* strains and its subspecies or strains are causal agents of food-induced diarrhoea (Raymond and Federici 2017; Biggel et al. 2022).

2.2.2 Streptomyces sviceus

The source of the *dgt-28 epsps* gene is bacterium *Streptomyces sviceus*, a gram-positive, aerobic bacterium commonly found in soil. The *Streptomyces* genus has long been used in the pharmaceutical and biotechnology industries as a source of antibiotics and industrial enzymes (Watve et al. 2001). As soil dwelling bacteria, *Streptomyces* produces and secretes a large amount of extracellular enzymes to break down chemically complex materials produced by plants to obtain nutrients (Chater et al. 2010). Recently, the tannase enzyme from *S. sviceus* has been researched for use in food, feed, beverage and pharmaceutical

[3] For more information please see USDA Economic Research Service: http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx
[4] USDA Grain Report, CA14062, 2014: https://apps.fas.usda.gov/newgainapi/api/Report/DownloadReportByFileName?fileName=Agricultural%20Biotechnology%20Annual_Ottawa_Canada_7-14-2014
[5] Statistics Canada, 2023: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210004201
[6] A large proportion of corn produced is also used as animal feed.
[7] Since 1938 in France and 1961 in the United States

application (Wu et al. 2015).

2.2.3 Brassica napus and Brassica rapa

The chimeric chloroplast transit peptide, TraP8, is sourced from *B. napus* (rapeseed) and *B. rapa* (field mustard) from the Brassicaceae family, also commonly referred to as the mustard or cabbage family. A number of vegetable species belong to this family, including broccoli, cabbage, turnip and radish. The species *B. napus* and *B. rapa* are generally known as oilseed crops, i.e. are sources of cooking oils, and have a long history of safe consumption as food by humans.

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Details of the breeding history of DAS1131 were provided in the application as Confidential Commercial Information (CCI). While the full details of CCI cannot be provided in this public report, FSANZ has had regard to this information in its assessment.

3.1 Transformation method

To create the DAS1131 corn line, the inbred B104 corn line was transformed using the plasmid PHP88492 (Figure 1). The methodology is outlined in the flowchart in Appendix 1 and summarised below.

Transformation of the B104 line was achieved by co-culturing immature embryos excised from a post-pollinated corn ear with *Agrobacterium tumefaciens* containing the PHP88492 plasmid. Immature embryos were then placed on selective media containing glyphosate and carbenicillin. Glyphosate inhibits the growth of untransformed plant cells, while carbenicillin suppresses the growth of excess *Agrobacterium*.

After the transformed embryos reached the callus stage, the calli were placed on media to encourage shoot and root development. Rooted plants were screened using polymerase chain reaction (PCR) to identify T0 plants carrying the transfer DNA (T-DNA) (Figure 1). Following the evaluation of insert integrity, gene expression, phenotypic characteristics and agronomic performance, corn line DAS1131 was selected.

3.2 Detailed description of inserted DNA

Corn line DAS1131 contains T-DNA from plasmid PHP88492 (Figure 1) and includes the *cry1Da2* and *dgt-28 epsps* cassettes. The T-DNA contains *att*B recombination sites, engineered landing pad (ELP) regions and zinc finger nuclease target recognition sites (ZFN). The presence of these sites does not result in recombination in the corn genome since these sites require recombinase enzymes not found naturally in plants to function (Cody et al. 2020).

Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. Additional detail, including the plasmid backbone and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 2.

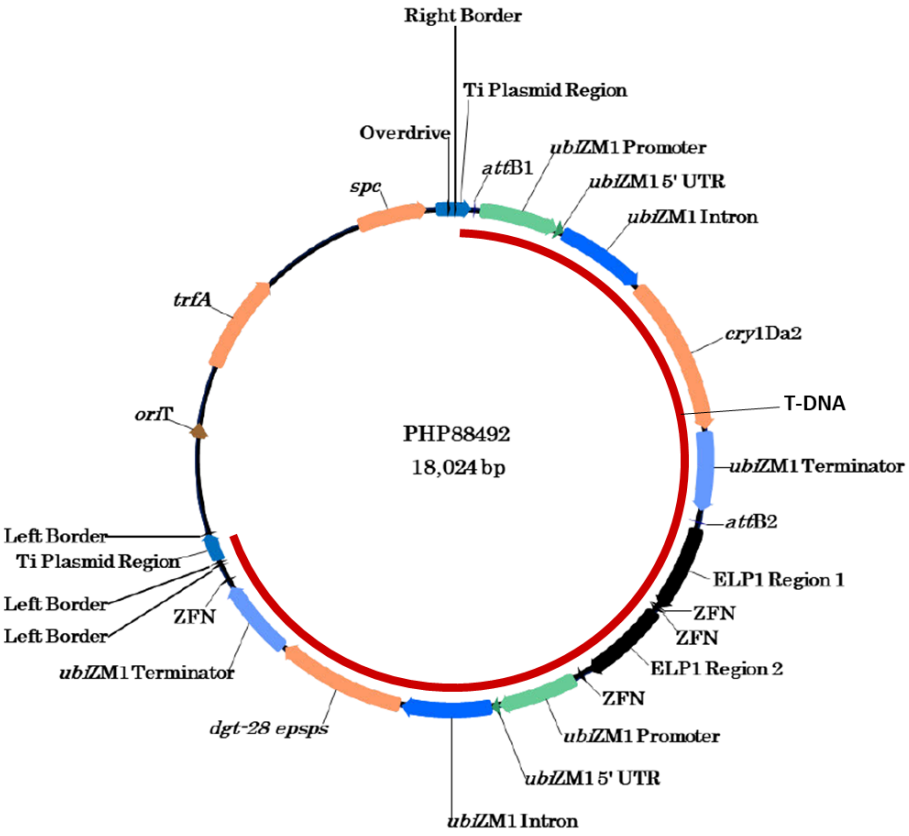

***Figure 1**: Plasmid map of PHP88492. The T-DNA region comprising the cry1Da2 and dgt-28 epsps expression cassettes is highlighted using the red line.*

Table 1: Expression cassettes contained in the T-DNA of PHP88492

Expression cassette	Promoter (Drives expression)	Intron-containing 5'UTR (Expression enhancer)	Coding sequence	Terminator (Polyadenylation and termination of transcription)
cry1Da2 expression cassette	Promoter region from *Z. mays ubiquitin gene 1*	Intron region *Z. mays ubiquitin gene 1*	Chimeric gene comprised of *cry1Da2* and *cry1Ab* gene from *B. thuringiensis*	Terminator region from *Z. mays ubiquitin gene 1*
dgt-28 epsps expression cassette	Promoter region from *Z. mays ubiquitin gene 1*	Intron region *Z. mays ubiquitin gene 1*	*epsps* gene from *Streptomyces sviceus* fused to a chimeric chloroplast transit peptide, TraP8 from *B. napus* and *B. rapa*	Terminator region from *Z. mays ubiquitin gene 1*

3.3 Development of the corn line from the original transformant

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of DAS1131

- ensuring that the DAS1131 event is incorporated into elite lines for commercialisation.

Table 2: DAS1131 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	section 3.4.1	T1	B104
Absence of backbone and other sequences	section 3.4.2	T1	B104
Insert integrity and site of integration	section 3.4.3	T1, T2, T3, T4 and T6	B104
Genetic stability	section 3.4.4.1	T1, T2, T3, T4 and T6	B104
Mendelian inheritance	section 3.4.4.2	BC1F1 [B104/PH184C[8]], BC1F1 [B104/PH1V5T], T2, T4, and T6)	N/A
Expression of phenotype over several generations	section 3.4.4.2	BC1F1 [B104/PH184C], BC1F1 [B104/PH1V5T], T2, T4, and T6)	B104
Compositional analysis	section 5	F1 (B104/PH4257)	B104/PH4257

3.4 Characterisation of the inserted DNA and site(s) of insertion

A range of analyses were undertaken to characterise the genetic modification in DAS1131. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

To characterise the number of integration sites, insert integrity and absence of extraneous sequences, the applicant made use of Southern-by-Sequencing (SbS) technology (Zastrow-Hayes et al. 2015; Brink et al. 2019).

3.4.1 Number of integration site(s)

Leaf-derived genomic DNA from ten plants from the T1 generation of DAS1131, along with DNA from a plant from the non-GM B104 corn as a control, was analysed by SbS. The ten DAS1131 plants analysed by SbS consisted of 6 transgenic and 4 null segregant plants. Additionally, positive control samples were generated using B104 DNA spiked with the PHP88492 plasmid used for the development of DAS1131.

Next generation sequencing (NGS) libraries were prepared on sheared genomic DNA that consisted of an average fragment size of 400 bp. The probe set was designed to collectively target all sequences within all plasmids. The DNA was enriched twice by hybridisation and was sequenced using an Illumina platform. Sufficient sequence fragments were obtained to cover the genomes being analysed, with a 100x depth of coverage.

The sequencing reads obtained by SbS were compared to the T-DNA sequence from plasmid PHP88492 (Figure 1), and to the endogenous corn genome to identify unique junctions attributable to inserted DNA. SbS analysis of each of the 6 transgenic plants yielded sequencing reads that aligned to the intended insertion, and identified two unique genome-insertion junctions. This result indicated that a single copy of the intended insertion, with the intended organisation, was integrated into the genome of DAS1131.

The sequencing coverage in the control and 4 null segregants was limited to regions derived

[8] PH184C, PH1V5T, PH4257 are Pioneer Hi-Bred inbred lines

from corn endogenous sequences present in the inserted DNA. No junctions between plasmid DNA and genomic DNA were identified in the control or in the 4 null segregant plants, confirming that the reads were only identifying endogenous sequences.

3.4.2 Absence of backbone and other sequences

The SbS analysis used a set of hybridisation probes covering the backbone sequence of the PHP88492 plasmid (Figure 1) used to create DAS1131. Alignment of NGS reads from the controls or DAS1131 to the plasmid sequence confirmed there was no integration of backbone sequences into DAS1131, including any antibiotic resistance genes.

3.4.3 Insert integrity and site of integration

The SbS analysis indicated that DAS1131 contains a single copy of the T-DNA from PHP88492, with the expected organisation, and no unintended sequences or rearrangements.

PCR and sequencing analysis of the insert and flanking corn genomic regions was consistent with the SbS data and confirmed that the organisation of the insert in DAS1131 is as expected. The identified corn sequences flanking the insertion site were further subjected to homology searches against the reference genome sequence of the conventional control[9] (Altschul et al. 1990). These searches located the T-DNA insert at a specific locus in the corn genome. The insertion did not disrupt any genes or any other known annotated feature in the corn genome.

The inserted T-DNA had a 27 base pair (bp) deletion at the right border (RB) and a 390 bp deletion at the left border (LB). Such changes are common during *Agrobacterium*-mediated plant transformation due to double-strand break repair mechanisms (Gheysen et al. 1991; Mayerhofer et al. 1991; Gelvin 2021). In addition, there was a single nucleotide A to G change at bp 1954 identified in the *ubi*ZM1 promoter. These changes would not affect the expression of the *cry1Da2* and *dgt-28 epsps* genes.

3.4.4 Stability of the genetic changes in corn line DAS1131

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation events) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.4.1 Genetic stability

Southern blot analysis was used to show the genetic stability of the inserted *cry1Da2* and *dgt-28 epsps* gene cassettes in DAS1131. Leaf-derived genomic DNA from five generations of DAS1131 (T1, T2, T3, T4 and T6) was extracted, digested with a restriction enzyme that possesses a single recognition site within the DAS1131 insertion, and hybridised with labelled probes specific for the *cry1Da2* and *dgt-28 epsps* gene cassettes. Genomic DNA from the non-GM parental line B104 served as a negative control, and B104 DNA spiked with the plasmid (Figure 1) served as a positive control.

Hybridisation of each probe to the digested genomic DNA from DAS1131 showed equivalent bands of the expected sizes across all five generations. The consistency of these results confirmed the inserted DNA is stably maintained in corn line DAS1131.

[9] Maize (B73) Public Genome Assembly (https://www.ncbi.nlm.nih.gov/assembly/GCF_902167145.1/)

3.4.4.2 Pattern of inheritance

Expressed phenotype over several generations

The inheritance pattern was assessed in five generations of DAS1131 (BC1F1 [B104/PH184C], BC1F1 [B104/PH1V5T], T2, T4, and T6), using 100 plants per generation. Plants from each generation were evaluated by both quantitative and qualitative PCR assays, using primers targeting the *cry1Da2* and *dgt-28 epsps* genes.

Plants were also examined phenotypically by observing plant survival after exposure to glyphosate. Each plant was assessed visually for glyphosate-tolerance four to five days after application of glyphosate spray. The absence of injury corresponded to a herbicide-tolerant (positive) phenotype.

Mendelian inheritance

A Chi-square (χ^2) analysis was undertaken over several generations to confirm the segregation and stability of the insert in DAS1131. Since the inserted DNA resides at a single locus within the DAS1131 genome, the inserted DNA would be expected to be inherited according to Mendelian principles. The expected segregation ratios for the BC1F1 (B104/PH184C and B104/PH1V5T) generations, based on Mendelian inheritance principles, were 1:1, 3:1 for the T2 generation, and homozygous positive for the T4 and T6 generations. The results demonstrated the expected segregation ratio for each generation (Table 3). These results were compared to the results from the phenotypic analysis and the co-segregation of genotype and phenotype was confirmed.

These data support the conclusion that the inserted DNA is present at a single locus in DAS1131 and is inherited predictably according to Mendelian principles in subsequent generations.

Table 3: Segregation results in five generations of DAS1131

Generation	Expected segregation ratio (positive:negative)	Observed number of plants			Statistical analysis	
		Positive	Negative	Total	χ^2	p-value
BC1F1 (B104/PH184C)	1:1	52	48	100	0.16	0.6892
BC1F1 (B104/PH1V5T)	1:1	53	47	100	0.36	0.5485
T2	3:1	70	30	100	1.33	0.2482
T4	Homozygous positive	100	0	100	-	-
T6	Homozygous positive	100	0	100	-	-

3.4.5 Reading frame analysis

A bioinformatic analysis of the DAS1131 insert, as well as the flanking DNA regions, was undertaken to identify whether any reading frames (RFs) had been created in DAS1131 as a result of the DNA insertion, and whether any putative peptides or polypeptides encoded by the identified RFs have the potential for allergenicity or toxicity.

All RFs of ≥ 8 amino acids (aa) in length spanning the 5' and 3' insert-flank junctions of DAS1131, or contained within the insert itself, were translated *in silico* from stop codon to

stop codon (TGA, TAG, TAA) in all six reading frames[10]. A total of 723 stop-codon bracketed RFs were identified that correspond to putative peptides of eight amino acids or greater in length. Among the identified RFs, are open RFs encoding the Cry1Da2 and DGT-28 EPSPS proteins. These analyses are theoretical only, as it is highly unlikely that any of the identified RFs or putative peptides would be expressed *in planta*.

3.4.5.1 Bioinformatic analysis for potential allergenicity

The putative peptides identified above were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE[11]) database, from the Health and Environmental Science Institute. At the date of the search (January 2021), there were 2,348 sequences in the allergen database.

A FASTA search algorithm (v35.4.4) (Pearson and Lipman 1988) was used to identify alignments between the query sequences and the COMPARE database, using a BLOSUM50 scoring matrix and an E-value threshold of 100 (Song et al. 2015). Only matches with a linear identity of greater than 35% over 80 amino acids were considered. In addition, a search for ≥ 8 contiguous aa matches to the allergens from the COMPARE database was performed using EMBOSS FUZZPRO.

Comparison of the 723 putative peptides against the COMPARE allergen database identified six that had ≥35% identity with allergens over an 80 aa sliding window. These putative peptides were identified using a highly conservative approach and further analysis confirmed that none of the identified putative peptide produced an eight contiguous amino acid match to an allergen. Therefore, risk of allergenic proteins with relevance to human safety being produced by these RFs or putative peptides is negligible.

3.4.5.2 Bioinformatic analysis for potential toxicity

The putative peptides encoded by the junction and insert sequences were compared *in silico* to an in-house toxin database (January 2021). This database is a subset of sequences derived from the UniProtKB/Swiss-Prot protein databases, filtered using keywords relating to potential toxicity or adverse health effects. A BLASTP algorithm with a BLOSUM62 scoring matrix and an E-value of $\leq 10^{-4}$ was used.

No alignments were found between the 723 putative peptides and any known protein toxins. The novel RFs in DAS1131 therefore do not present a toxicity concern.

3.4.6 Conclusion

Corn line DAS1131 contains a single copy of the intended DNA insertion, integrated at a specific locus in the corn genome. SbS and sequencing results confirmed that the *cry1Da2 and dgt-28 epsps* gene cassettes were inserted with the expected organisation. No backbone sequences from the plasmids used in the transformation are present, including any antibiotic resistance genes.

The inserted DNA is stably inherited and the glyphosate-tolerant phenotype is expressed across several breeding generations of DAS1131. No new RFs that raise potential allergenicity or toxicity concerns were created by the insertion.

4 Characterisation and safety assessment of novel

[10] Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.
[11] http://comparedatabase.org/database/

substances

Only a small number of dietary proteins have the potential to impair health, because they have anti-nutrient properties or they can cause allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutrient or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, detailed understanding of the biochemical function and phenotypic effects and concentration levels in the edible part of the plant is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

Two novel substances are expressed in DAS1131: the Cry1Da2 insecticidal protein, which provides protection against lepidopteran pests and the DGT-28 EPSPS protein, which provides tolerance to the herbicide glyphosate and acts as a selectable marker during the transformation process. In considering the safety of newly expressed substances it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects.

4.1 Cry1Da2

Cry proteins are contact pesticides, requiring ingestion by the target pest[12] and passage into the digestive system in order to function (Jurat-Fuentes and Crickmore 2017). Alkaline conditions and proteases in the insect midgut causes the proteolytic cleavage of the Cry protein's protoxin domain and activation of the insecticidal toxin. The activated protein functions by binding to a highly specific glycoprotein receptor on the surface of midgut epithelial cells, aggregating and forming pores in the cell membrane (Schnepf et al. 1998). This leads to loss of cell integrity in the midgut, leading to developmental delays (growth inhibition) and insect death.

One distinguishing feature of Cry proteins is their high specificity for particular target insects. Phylogenetic analyses has established that the diversity of the Cry family of proteins evolved by the independent evolution of three structural domains, and by swapping of domains between toxins (de Maagd et al. 2003). Similarities in the structural domains make it possible to engineer novel chimeric proteins in the laboratory through the exchange of homologous DNA domains between different *cry* genes (Deist et al. 2014).

Cry1Da2 is a chimeric protein composed of the insecticidal core toxin of *cry1Da2* gene and a segment of *cry1Ab* gene derived from *Bacillus thuringiensis*. The Cry1Da2 protein is designed with the goal to achieve high levels of activity against lepidopteran insect pests such as corn earworm and fall armyworm. These insects are serious corn pests and can cause significant economic losses.

The *cry1Da2* gene prepared by the applicant encodes a protein of 603 amino acids, with an apparent molecular weight of ~ 68 kDa.

4.1.1 Characterisation of Cry1Da2 expressed in DAS1131 and equivalence to a bacterially-produced form

The equivalence of the DAS1131- and bacterially-derived Cry1Da2 protein must be established before the safety data generated using bacterially-derived Cry1Da2 can be

[12] Lepidoptera, Coleoptera, Diptera and other invertebrates such as nematodes.

applied to DAS1131-derived Cry1Da2.

The plant-derived Cry1Da2 was purified from DAS1131 V9 leaf tissues. The methodology involved affinity chromatography which isolated any fraction bound to a Cry1Da2 antibody. The purified fractions containing Cry1Da2 were identified by SDS-PAGE and Western blot, and subsequently concentrated.

Bacterially-derived Cry1Da2 protein was generated from the fermentation of bacteria containing *a* plasmid that expresses Cry1Da2. Compared to DAS1131-derived Cry1Da2, the bacterially-derived Cry1Da2 protein sequence contains two amino acid modifications (K19Q and R27Q) intended to avoid truncation and maintain the N-terminus during bacterial production.

In order to confirm the identity and equivalence of the DAS1131- and bacterially-derived Cry1Da2, a series of analytical techniques were employed. The results are summarised below.

Molecular weight. Samples of purified DAS1131- and bacterially-derived Cry1Da2 were run on SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. The bacterially-derived Cry1Da2 was shown to migrate as a single band with an apparent molecular weight of 68 kDa consistent with the expected molecular weight. The DAS1131-derived Cry1Da protein migrated as two bands. The upper band had an apparent molecular weight of 68 kDa consistent with the expected molecular weight. The lower band migrated at approximately 66 kDa. The differences in banding pattern observed for DAS1131- and bacterially-derived Cry1Da2 protein are examined further below using N-terminal sequencing. The two DAS1131- and bacterially-derived Cry1Da2 protein bands were excised and used for the peptide mapping analysis.

Immunoreactivity. Western blot analysis with a Cry1Da2-specific antibody showed that the DAS1131-derived and bacterially-derived Cry1Da2 was in fact Cry1Da2 and they have equivalent immunoreactivity.

Peptide mapping. The two DAS1131-derived Cry1Da2 protein excised bands in the SDS-PAGE were digested with trypsin or chymotrypsin and analysed via mass spectrometry. When peptides were mapped, the combined sequence coverage for the upper band (~ 68 kDa) was 95.9% of the expected Cry1Da2 sequence[13] (578/603 amino acids), and the combined sequence coverage for the lower band (~ 66 kDa) was 91% (549/603) of the expected Cry1Da sequence.

The bacterially-derived Cry1Da2 was similarly digested with trypsin or chymotrypsin and analysed via mass spectrometry. Matched peptides for bacterially-derived Cry1Da2 accounted for 93% of the expected Cry1Da2 sequence (560/603 amino acids).

N-terminal sequencing. In the lower SDS-PAGE band for plant-derived Cry1Da2 the N-terminal peptide (amino acids 1-19) was not detected. This result indicates that there was likely a N-terminal truncation of the DAS1131-derived Cry1Da2, resulting in two bands in the SDS-PAGE. The truncation is likely due to proteolysis by trypsin-like proteases *in planta* or during extraction and purification.

The N-terminal peptide of DAS1131-derived Cry1Da2 in the SDS-PAGE upper band (68 kDa) and bacterially-derived Cry1Da2 were sequenced and were as expected. The two amino acid changes (K19Q and R27Q) made to the bacterially-derived Cry1Da2 were also

[13] The expected sequence refers to the translated sequence for the gene derived from the expression vector and used for production of the bacterially-derived Cry1Da2 protein.

confirmed by the sequencing analysis.

Glycosylation analysis. SDS-PAGE combined with a colourimetric glycoprotein detection procedure showed that both DAS1131- and bacterially-derived Cry1Da2 was equivalent and that neither is glycosylated. The positive control protein (horseradish peroxidase) showed a band indicative of glycosylation.

Functional activity. The biological activity of bacterially-derived Cry1Da2 was evaluated in a 7-day insect bioassay. In this assay, *Spodoptera frugiperda* larvae were fed either a diet containing 50 ng Cry1Da2 protein per mg diet wet weight or a control diet containing ultrapure water. Larvae fed a diet containing Cry1Da2 showed a mortality of 95%, compared to 0% in the control diet. This result demonstrates that bacterially-derived Cry1Da2 protein is functionally active against *S. frugiperda*.

The results outlined in this section demonstrated that bacterially-derived Cry1Da2 is structurally and biochemically equivalent to DAS1131-derived Cry1Da2. The biological activity of bacterially-derived Cry1Da2 was demonstrated in an insect bioassay and based on the structural and biochemical equivalence to DAS1131-derived Cry1Da2, the two proteins are expected to be functionally equivalent.

It can be concluded that bacterially-derived Cry1Da2 is a suitable surrogate for use in the safety assessment experiments described in section 4.1.3.

4.1.2 Expression of Cry1Da2 in DAS1131 tissue

For analysis of the expression levels of Cry1Da2 protein in DAS1131, tissues were collected from six field-trial sites in representative corn-producing regions of the United States and Canada during the 2020 growing season. Tissues were collected at varying stages of growth (see Figure 2 for a summary of corn growth stages). Cry1Da2 was extracted from tissues using standard methods and expression levels were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA). For each tissue analysed, four samples were processed from each of the six field-trial sites.

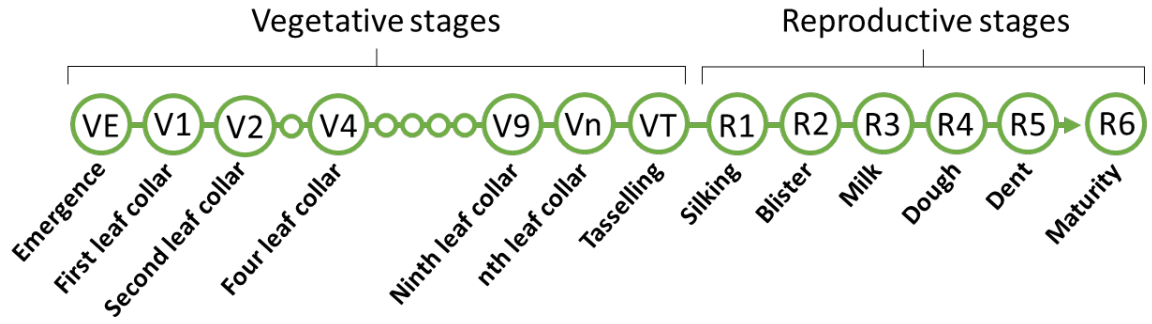

Figure 2: *Stages of corn growth. Grain is harvested at maturity (R6).*

Results from the ELISA show that the highest Cry1Da2 expression in DAS1131 was in pollen at the R1 stage (Figure 3). The lowest level of expression was in grain at the R6 stage.

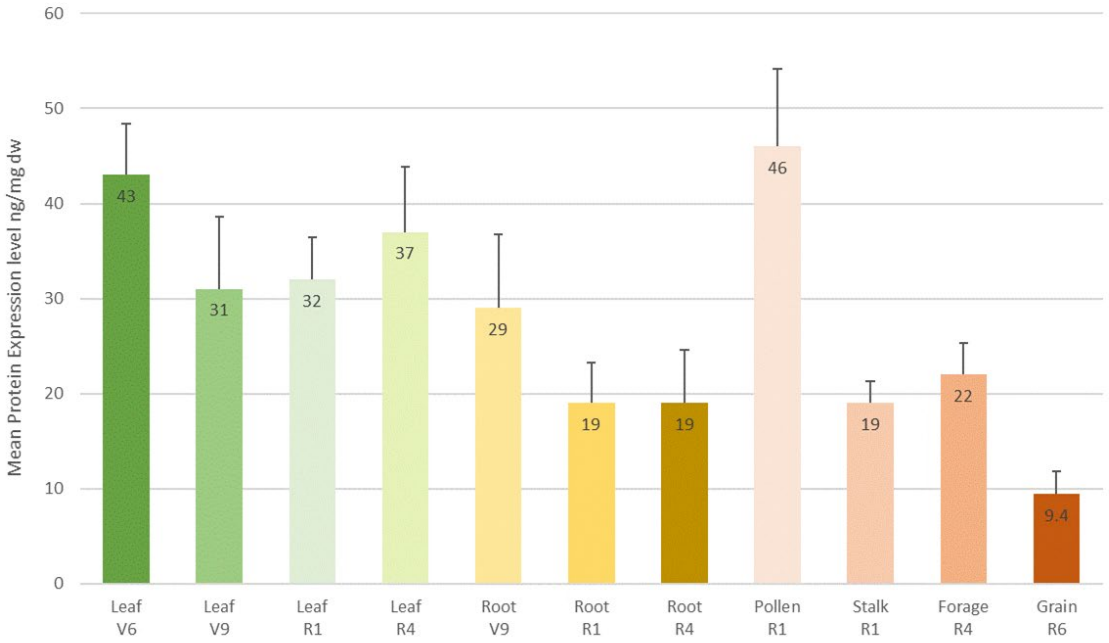

Figure 3: *Mean expression levels of Cry1Da2 protein (ng/mg dw) in DAS1131 tissues*

4.1.3 Safety of the introduced Cry1Da2

Bioinformatic analyses, as described in section 3.4.5.1, were performed to compare the Cry1Da2 amino acid sequence to known allergenic proteins in the COMPARE Allergen database (AD_2021). The search did not identify any known allergens with homology to Cry1Da2. No alignments had an E-value of $\leq 10^{-4}$ or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the Cry1Da2 sequence and proteins in the allergen database.

The Cry1Da2 amino acid sequence was compared with sequences in the Toxin database, as outlined in section 3.4.5.2. No alignments had an E-score of $\leq 10^{-4}$ indicating that the search did not identify any known toxins with homology to Cry1Da2.

4.1.3.1 Susceptibility of Cry1Da2 to digestion with pepsin and pancreatin

Bacterially-produced Cry1Da2 (test protein) was incubated at 37.4°C in simulated gastric fluid (SGF) system containing pepsin (10U enzyme/ug protein) at an acidic pH of ~1.2 for 0 - 60 min (Thomas et al. 2004). Controls included a no test protein control (pepsin only) and no pepsin control (test protein only) incubated for 0 and 60 min. Bovine serum albumin (BSA) and β-lactoglobulin were used as positive and negative controls respectively. The extent of digestion was visualised by SDS-PAGE with Coomassie Blue staining and Western blotting.

Visual inspection of the pepsin digestion showed that by 0.5 min, there was no intact Cry1Da2 remaining in the reaction mix. A band ~15 kDa was observed but was digested within 5 min. Some low molecular weight (~2 - 5 kDa) bands were evident in the Cry1Da2 samples throughout the 60 min time course. The BSA control was rapidly digested by 1 min, while the β-lactoglobulin remained present over the course of the reaction. These data indicate that Cry1Da2 is rapidly digested by pepsin.

Bacterially-produced Cry1Da2 was also incubated with pancreatin[14] [0.5% (w/v) enzyme/0.25

[14] Pancreatin is a mixture of proteolytic enzymes.

mg/ml of protein] at 37.7°C for 0 - 60 min, in a simulated intestinal fluid (SIF) system at a neutral pH range. Controls for this experiment included a no test protein control (pancreatin only) and no pancreatin control (test protein only) incubated for 0 and 60 min. β-lactoglobulin and BSA were also included as positive and negative controls, respectively. The extent of digestion was visualised by SDS-PAGE with Coomassie Blue staining and Western blotting.

Visual inspection of the pancreatin digestion showed that by 0.5 min, there was no intact Cry1Da2 remaining in the reaction mix. There were bands of ~20 kDa present throughout the experiment corresponding to fragments of Cry1Da2. The β-lactoglobulin control was digested within 60 min, while the BSA remained present over the course of the reaction.

In the human digestive system, pepsin digestion occurs before pancreatin digestion. Therefore a sequential digestion was performed on Cry1Da2. Cry1Da2 was first incubated with pepsin for 1 min, then with pancreatin over 0 – 30 min time course. The low molecular weight (~2 - 5 kDa) bands that were evident after 1 min of pepsin digestion were rapidly digested within 0.5 min of sequential pancreatin digestion. Taken together, these results indicate that Cry1Da2 would be fully degraded by gastric and intestinal enzymes in the human digestive system.

4.1.3.2 Bioactivity of Cry1Da2 after exposure to heat

The thermal stability of Cry1Da2 was evaluated by assessing the functional activity of the heat-treated Cry1Da2 protein in a 7-day insect bioassay. Bacterially-derived Cry1Da2 protein was incubated for 30-35 min at 25°C, 50°C, 75°C, or 95°C before incorporation into an artificial diet for *S. frugiperda* larvae. Control diets contained either ultrapure water, or unheated Cry1Da2. The test diets and the unheated control diet contained a target concentration of 50 ng Cry1Da2 protein per mg diet wet weight. Each diet was provided to 20 individual *S. frugiperda* larvae (except for the unheated control diet which was provided to 24 larvae) for a total of 7 days, with refeeding occurring on day 4. Mortality and the weight of surviving larvae were assessed after day 7. ELISA analysis confirmed the dose and homogeneity of the Cry1Da2 protein during the assay.

Table 4: Bioactivity of heat-treated Cry1Da2 in a diet fed to insect larvae

Treatment description	Incubation condition	Number of observations[1]	Total number of surviving organisms	Mortality (%)	p-value[2]	Weight of surviving organisms (mg)
Water diet	-	20	20	0	-	41.1 ± 9.83
Unheated control diet	-	20	3	85	-	24.4 ± 19.0
Test diet	25°C	20	0	100	1.0000	-
	50°C	20	2	90	0.8292	5.25 ± 7.42
	75°C	20	20	0	<0.0001	44.2 ± 7.83
	95°C	20	20	0	<0.0001	42.4 ± 9.45

The results demonstrated that when heated to temperatures of 75-95°C, the ability of the Cry1Da2 protein to cause *S. frugiperda* mortality was effectively abolished, with mortality rates of 0% for the larvae fed diets containing Cry1Da2 heat-treated at 75°C or 95°C (Table 4). Fisher's exact test was used to determine whether the mortality rate of *S. frugiperda* that had been fed diets containing the heated Cry1Da2 was smaller than that of those fed the unheated control diet. For Cry1Da2 heated to 75°C or 95°C, the decrease in activity against *S. frugiperda* larvae was statistically significant (p-value <0.05) compared to the unheated control. The Cry1Da2 protein that was heat-treated at 25°C or 50°C did not have a statistically significant decrease in activity compared to the unheated control (Table 4). These data indicate that Cry1Da2 is heat labile at temperatures ≥ 75°C.

4.1.3.3 14-day acute oral toxicity study

The potential acute toxicity of Cry1Da2 protein (>95% purity) was assessed in mice following a single oral exposure. Cry1Da2 protein was administered orally via gavage at doses of 0 or 5000 mg/kg body weight to mice (6/sex/group). The vehicle/negative control was water and another equivalent group of mice received bovine serum albumin protein (5000 mg/kg body weight), as a comparative control. The dose volume was 20 ml/kg body weight for all formulations.

The vehicle control, bovine serum protein and Cry1Da2 protein formulations were administered on day 1 in three split doses, separated by 4 hours. The mice were fasted prior to and throughout dosing. Mice were housed individually (males) or in pairs (females) under standard laboratory conditions. A 2-week observation period followed dosing. Mice were observed twice daily for animal health and daily for clinical condition. Additional clinical observations were made on day 1 (prior to administration of each formulation dose; and 30 minutes and 1-2 hours after each dose administration). Body weights were recorded on days 1, 2, 3, 5, 8 and 15. All animals were euthanised on day 15 and subject to a gross pathological examination of an extensive range of organs and tissues. The gastrointestinal tracts were prepared for histopathological examination but were later discarded as no microscopic evaluations were required.

All animals survived to study termination. There were no treatment related effects on body weight, clinical or gross observations. It was concluded that the oral LD_{50} of Cry1Da2 protein was greater than 5000 mg/kg body weight.

4.2 DGT-28 EPSPS

EPSPS proteins occur ubiquitously in plants and microorganisms, and have been extensively studied over a period of forty years. EPSPS catalyses a step in the shikimate pathway that is responsible for the biosynthesis of the aromatic amino acids phenylalanine, tryptophan and

tyrosine. Inhibition of this pathway in is lethal, as it deprives the organism of essential amino acids (aa).

In plants, the EPSPS enzyme is inhibited by glyphosate (Steinrücken and Amrhein 1980), but bacterial EPSPS enzymes, such as the CP4 EPSPS derived from *Agrobacterium tumefaciens* strain CP4, have a reduced affinity for glyphosate (Comai et al. 1983; Sost and Amrhein 1990; Padgette et al. 1991; Barry et al. 2001). Reduced affinity allows for the continued action of CP4 EPSPS in the presence of glyphosate. A single residue in the active site (Ala-100) renders CP4 EPSPS insensitive to glyphosate, whereas a highly conserved Gly residue is found at this position in known natural plant enzymes (Funke et al. 2006).

The DGT-28 EPSPS protein present in corn line DAS1131 is derived from *Streptomyces sviceus* and is functionally the same as the CP4 EPSPS protein (Figure 4B) (Griffin et al. 2021). The DGT-28 EPSPS expressed in DAS1131 is fused to a chimeric chloroplast transit peptide (CTP) TraP8, from *B. napus* and *B. rapa*, enabling expression in the chloroplast. Plants expressing DGT-28 EPSPS therefore are able to continue to function in the presence of the herbicide. The parental B104 corn line used for the genetic modification, as described in this application, contains the native EPSPS protein and would be inhibited by glyphosate (Figure 4A).

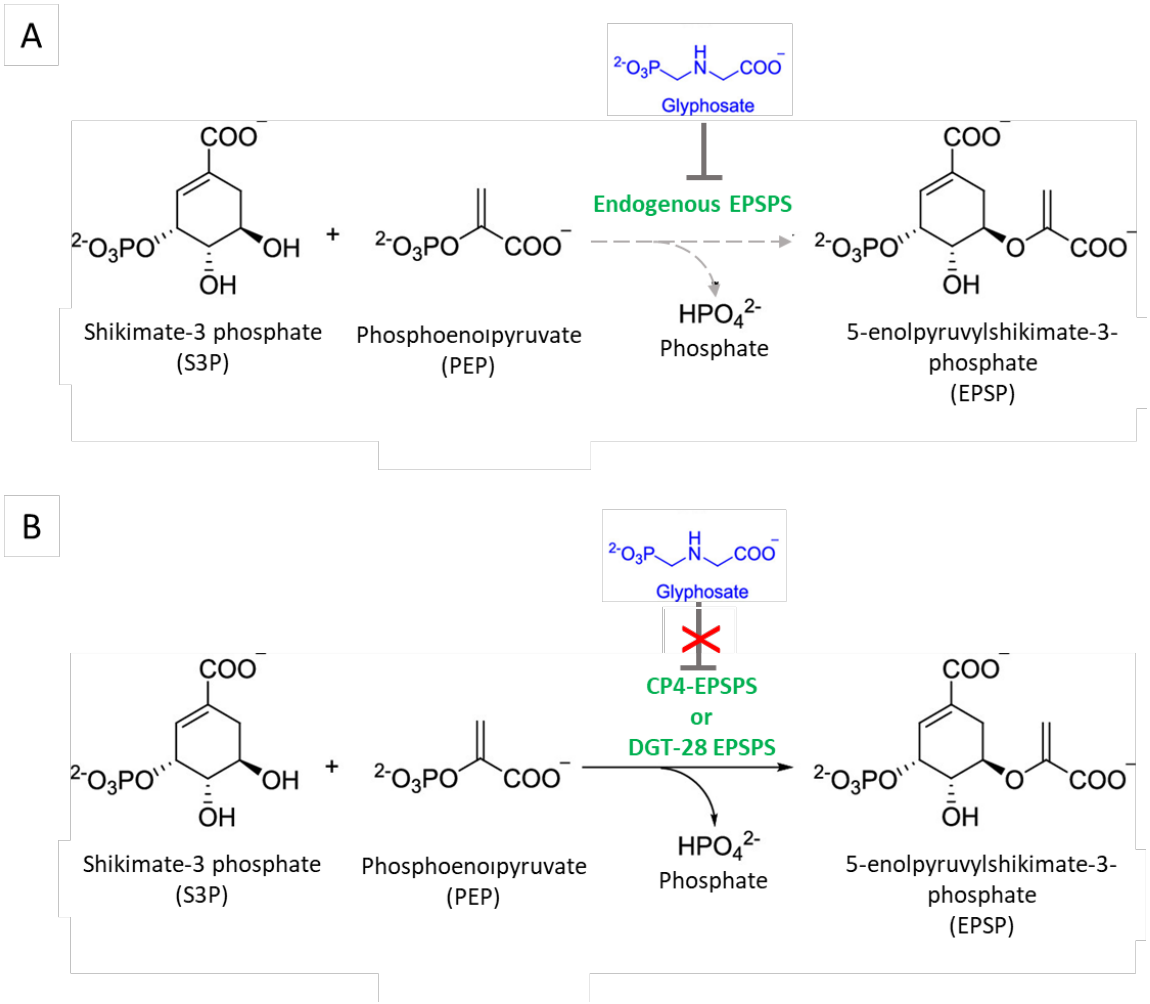

Figure 4: ***A****: Reaction catalysed by endogenous EPSPS.* ***B****: Reaction catalysed by CP4-EPSPS or DGT-28 EPSPS (adapted from Griffin et al. 2021).*

The *dgt-28 epsps* gene prepared by the applicant encodes a protein of 481 aa, which

includes the 65 aa CTP, 2 aa linker and 415 aa DGT-28 EPSPS protein. After the cleavage of the CTP, the mature protein will have an expected molecular weight of 45 kDa.

4.2.1 Characterisation of DGT-28 EPSPS expressed in DAS1131 and equivalence to a bacterially-produced form

The equivalence of the DAS1131- and *E.coli*-derived DGT-28 EPSPS proteins must be established before the safety data and conclusions generated using *E. coli*-derived DGT-28 EPSPS can be applied to DAS1131-derived DGT-28 EPSPS.

Plant-derived DGT-28 EPSPS protein was purified from DAS1131 leaf tissue using immunoaffinity chromatography. Purified DGT-28 EPSPS fractions were combined and subsequently concentrated. To obtain sufficient quantities of DGT-28 EPSPS for use in safety studies, DGT-28 EPSPS was also expressed in *E. coli*, fused to an N-terminal His-tag. The *E. coli*-derived DGT-28 EPSPS protein was purified using nickel affinity chromatography.

In order to confirm the identity and equivalence of the DAS1131- and *E. coli* derived DGT-28 EPSPS, a series of analytical tests were done, the results of which are summarised below.

Molecular weight. Samples of purified DAS1131- and *E. coli*-derived DGT-28 EPSPS were subjected to SDS-PAGE then visualised with Brilliant Blue G-Colloidal stain. Both proteins migrated with an apparent molecular weight of ~45 kDa, as expected.

Immunoreactivity. Western blot analysis with an DGT-28 EPSPS-specific antibody showed that the protein being expressed in DAS1131 and *E. coli* was indeed DGT-28 EPSPS, and that they have equivalent immunoreactivity.

Peptide mapping. DAS1131-derived and *E. coli*-derived DGT-28 EPSPS were digested with trypsin or chymotrypsin and analysed by LC-MS. For DAS1131-derived DGT-28 EPSPS, the combined tryptic and chymotryptic peptides covered 86% of the expected protein sequence. For *E. coli*-derived DGT-28 EPSPS, the peptide coverage was 81% of the expected protein sequence[15].

N-terminal sequencing. Edman sequencing of the DAS1131- and *E. coli*-derived DGT-28 EPSPS N-terminus showed the sequences were as expected. Two N-terminal sequences, corresponding to the first 10 residues, were observed for the DAS1131-derived DGT-28 EPSPS and is due to the alternate cleavage of CTP at either of two adjacent positions (A66 or A67). This is consistent with the observation that CTP cleavage can occur at multiple positions for some nucleus-encoded chloroplast proteins (Rowland et al. 2015).

Glycosylation analysis. SDS-PAGE combined with a colourimetric glycoprotein detection procedure showed that the DGT-28 EPSPS proteins from both DAS1131 and *E. coli* were equivalent and that neither is glycosylated. The positive control protein (horseradish peroxidase) showed a band indicative of glycosylation.

Functional analysis. A colourimetric assay that measures the amount of inorganic phosphate, the product formed from the EPSPS-catalysed reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP), showed that DAS1131- and *E. coli*-produced DGT-28 EPSPS proteins had equivalent functional activity.

The results outlined in this section demonstrated that *E.coli*-derived DGT-28 EPSPS is structurally, biochemically and functionally equivalent to DAS1131-derived DGT-28 EPSPS. It can be concluded that *E. coli*-derived DGT-28 EPSPS is a suitable surrogate for use in the

[15] The expected sequence refers to the translated sequence for the gene derived from the expression vector and used for production of the *E. coli*-derived DGT-28 EPSPS protein.

safety studies described in section 4.2.3.

4.2.2 Expression of DGT-28 EPSPS in DAS1131 tissue

The levels of DGT-28 EPSPS expression in DAS1131 tissues was determined in the same ELISA study described in section 4.1.2, using *E. coli*-produced DGT-28 EPSPS as an analytical reference for DAS1131-derived DGT-28 EPSPS.

Results from the ELISA show that the highest DGT-28 EPSPS expression in DAS1131 was in leaf at the R4 stage (Figure 5). The lowest level of expression was in root at the R4 stage.

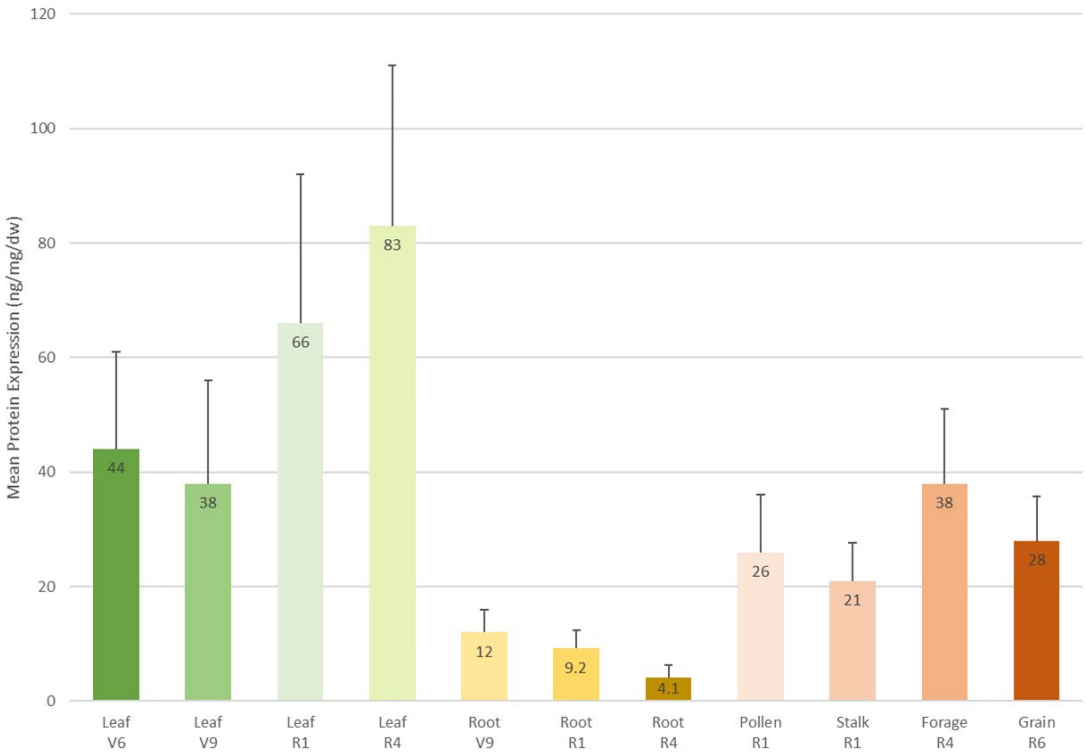

Figure 5: *Mean expression levels of DGT-28 EPSPS protein (ng/mg/dw) in DAS1131 tissues*

4.2.3 Safety of the introduced DGT-28 EPSPS

Bioinformatic analyses, as described in section 3.4.5.1, were performed to compare the DGT-28 EPSPS amino acid sequence to known allergenic proteins in the COMPARE Allergen database (AD_2021). The search did not identify any known allergens with homology to DGT-28 EPSPS. No alignments had an E-score of $\leq 1\times10^{-4}$ or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the DGT-28 EPSPS sequence and proteins in the allergen database.

The DGT-28 EPSPS amino acid sequence was compared with sequences in the Toxin database, as outlined in section 3.4.5.2. No alignments had an E-score of $\leq 1\times10^{-4}$ indicating that the search did not identify any known toxins with homology to DGT-28 EPSPS.

4.2.3.1 Susceptibility of DGT-28 EPSPS to digestion with pepsin and pancreatin

E. coli-derived DGT-28 EPSPS was subjected to digestibility assays (SGF, SIF and sequential digestion), as described in section 4.1.3.1.

Visual inspection of the pepsin digestion showed that by 0.5 min, there was no intact DGT-28 EPSPS remaining in the reaction mix. Some low molecular weight bands (~2 and ~5 kDa) were evident in the samples throughout the 60 min time course. The BSA control was rapidly digested by 1 min, while the β-lactoglobulin remained present over the course of the reaction. These data indicate that DGT-28 EPSPS is rapidly digested by pepsin.

The results from the pancreatin digestion showed that by 0.5 min, there was no intact DGT-28 EPSPS remaining in the reaction mix. Some low molecular weight bands remain present throughout the experiment. The β-lactoglobulin control was digested within 60 min, while the BSA remained present over the course of the reaction.

DGT-28 EPSPS was also subjected to a sequential digestion with pepsin followed by pancreatin. DGT-28 EPSPS was first incubated with pepsin for 2 min, then with pancreatin over a 0-30 min time course. The low molecular weight bands (~2 and ~5 kDa) that were evident after pepsin digestion were rapidly digested within 0.5 min of sequential pancreatin digestion. Taken together, these results indicate that DGT-28 EPSPS would be fully degraded by gastric and intestinal enzymes in the human digestive system.

4.2.3.2 Thermal stability of DGT-28 EPSPS after exposure to heat

E.coli-derived DGT-28 EPSPS was heated at 25°C, 37°C, 50°C or 75°C for 30-35 min. A control sample was kept on ice (~ 2 - 4ºC). In the Shikimate pathway, DGT-28 EPSPS enzyme catalyses the reaction of S3P with PEP to produce EPSP and inorganic phosphate (Figure 4). Enzyme activity of *E.coli*-derived DGT-28 EPSPS was determined based upon the presence or absence of inorganic phosphate as measured by the malachite green dye assay (see section 4.2.1).

The results demonstrated that the DGT-28 EPSPS protein was completely inactivated when heated to temperatures 50 – 75°C for 30-35 min. Additionally, a reduction in the enzyme activity was observed when heated at 37°C for 30-35 min, while no reduction in activity was observed when heated at 25°C. Taken together, the results indicate that DGT-28 EPSPS is heat labile at temperatures ≥ 50°C.

4.2.3.3 14-day acute oral toxicity study

The potential acute toxicity of DGT-28 EPSPS protein (90% purity) was assessed in mice following a single oral exposure. DGT-28 EPSPS protein was administered orally via gavage at doses of 0 or 2000 mg/kg body weight to mice (5/sex/group). The vehicle/negative control was water and the dose volume was 20 ml/kg body weight.

The vehicle control and DGT-28 EPSPS protein formulations were administered on day 1. The mice were fasted prior to and throughout dosing. Mice were housed individually (males) or in pairs (females) under standard laboratory conditions. A 2-week observation period followed dosing. Mice were observed twice daily for animal health and daily for clinical condition. Body weights were recorded on days 1, 2, 3, 5, 8 and 15. All animals were euthanised on day 15 and subject to a gross pathological examination of an extensive range of organs and tissues. The gastrointestinal tracts were prepared for histopathological examination but were later discarded as no microscopic evaluations were required.

All animals survived to study termination. There were no treatment related effects on body weight, clinical or gross observations. It was concluded that the oral LD_{50} of DGT-28 EPSPS protein was greater than 2000 mg/kg body weight.

4.2.4 Conclusion

A range of characterisation studies were performed on DAS1131-derived Cry1Da2 and DGT-28 EPSPS to confirm their identity, structure, biochemistry and function, as well their equivalence to the corresponding proteins produced in a bacterial expression system. Expression of Cry1Da2 in DAS1131 was highest in pollen and lowest in grain, while the expression of DGT-28 EPSPS was highest in leaf and lowest in root. Bioinformatic analyses showed Cry1Da2 and DGT-28 EPSPS did not share any meaningful homology with any known allergens or toxins. Both proteins were heat labile and susceptible to pepsin and pancreatin digestion. Additionally, an acute oral toxicity study in mice did not result in any treatment-related adverse effects. Taken together this indicates that the Cry1Da2 and DGT-28 EPSPS proteins are unlikely to be toxic or allergenic to humans.

4.3 Herbicide metabolites

For EPSPS and glyphosate, the metabolic profiles resulting from the novel protein and herbicide interaction have been established. This has been examined in multiple previous applications to FSANZ. DGT-28 EPSPS has the same mode of action or enzymatic reaction as other natural and characterised modified EPSPS proteins that have been demonstrated to confer glyphosate tolerance. Previous assessments indicate that spraying of DAS1131 with glyphosate would result in the same metabolites that are produced in non-GM corn sprayed with the same herbicide. As no new glyphosate metabolites would be generated in corn event DAS1131, further assessment is not required.

5 Compositional analysis

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analyses of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

5.1 Key components

The key components to be analysed for the comparison of transgenic and conventional corn are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Maize (OECD 2002), and include: proximates (protein, fat, fibre, ash, carbohydrates), amino acids, fatty acids, minerals, vitamins and the anti-nutrients phytic acid, raffinose, furfural and the phenolic acids ferulic acid and *p*-coumaric acid.

5.2 Study design

DAS1131 (F1 generation), a non-GM control of similar genetic background (B104/PH4257),

and a total of 16 non-GM commercial reference lines were grown and harvested from eight field trial sites in the United States and Canada during the 2020 growing season[16]. The sites were representative of corn growing regions suitable for commercial production. The field sites were established in a randomised complete block design with four replicates per site. Each block contained DAS1131, control, and four reference lines selected from BK5883, P0843, XL5858, P0928, P0993, XL5939, MY09V40, XL5828, P1093, BK6076, 6046, P1151, XL6158, 33T56, MPS2H721, and BK6282. Plants were grown under agronomic field conditions typical for each growing region. A herbicide treatment of glyphosate was applied to DAS1131.

At maturity (R6 growth stage), grain was harvested from all plots, with control and reference grain collected prior to glyphosate-treated DAS1131 samples to minimise the potential for contamination. Following harvest, samples were chilled before being transferred to a freezer (<10°C) or dry ice and shipped frozen to an analytical laboratory with full identity labelling. Compositional analyses were performed based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the Analytical Oil Chemists' Society (AOCS), methods specified by the manufacturer of the equipment used for analysis, or other published scientific methods.

A total of 70 analytes in grain were assessed (see Figure 6 for a complete list). For 6 of these analytes (listed in grey in Figure 3) all samples of both DAS1131 and control were below the assay lower limit of quantification (LLOQ) and were therefore not analysed statistically.

For the remaining 64 analytes, ‘descriptive statistics’ (mean, range and 95% confidence interval) were generated. For 62 of these analytes, where both DAS1131 and the control had <50% of samples below the LLOQ, a linear mixed model analysis of variance was applied for combined data and locations, covering the eight replicated field trial sites. The mixed model analysis was also applied to the data from each site separately. Where statistically significant differences were observed in the combined data from all sites, analysis of the data from each site was used to determine if the differences were common to the majority of sites. For the other 2 analytes (Vitamin B2 and β-tocopherol), >50% of either DAS1131 or the control samples were below the LLOQ, Fisher’s exact test was used to assess whether there was a significant difference in the proportion of samples below the LLOQ between the two corn lines across sites.

In assessing the statistical significance of any difference between DAS1131 and the conventional control, a p-value of 0.05 was used. A further adjusted p-value was determined using the false discovery rate (FDR) method, as a consideration of the chance of false positives being observed with the testing due to the multiple analytes being analysed. In cases where the raw p-value was <0.05 but the FDR-adjusted p-value was >0.05, the difference was considered likely to be a false positive.

Any statistically significant differences between DAS1131 and the control were compared to tolerance intervals derived from an in-house database containing compositional analyses from 184 non-GM commercial lines cultivated across 185 unique environments in North and South America, from 2003-2019. Tolerance intervals are expected (with 95% confidence) to contain at least 99% of the values for corresponding analytes of the conventional corn population (Hong et al. 2014). In addition, compositional data from the non-GM reference varieties grown concurrently in the same trial as DAS1131 and the control were combined across all sites and used to calculate an in-study reference range for each analyte. This reference range is useful to define the variability in corn varieties grown under the same agronomical conditions. Finally, the natural variation of analytes from publicly available data

[16] The location of the eight field trial sites: one site in Nebraska, Pennsylvania, Texas, and Ontario; two sites in Iowa and Illinois.

was also considered (Watson 1982; OECD 2002; Lundry et al. 2013; Codex 2019; AFSI 2021). These data ranges assist with determining whether any statistically significant differences are likely to be biologically meaningful.

Key analyte levels (proximates and minerals) were also analysed in forage but the results are not included in this report. It is noted however that, in the combined site analysis, none of the analyte levels in DAS1131 differed significantly from those of the control.

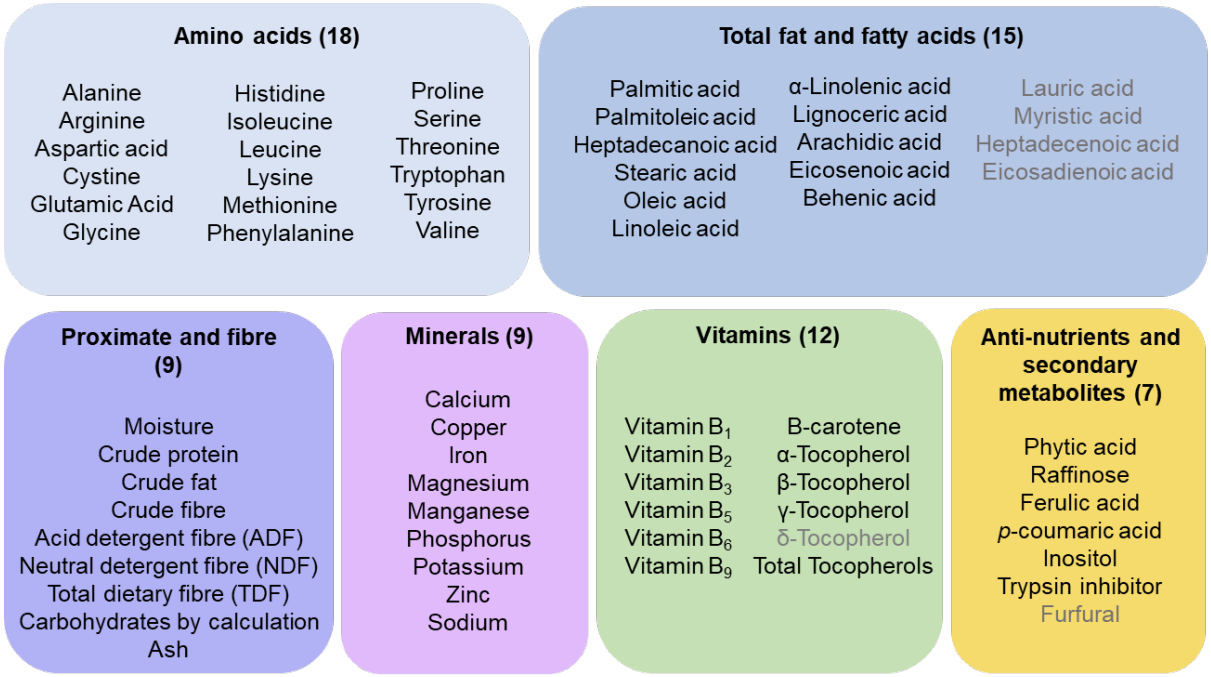

Figure 6: *Analytes measured in DAS1131 grain samples*

5.3 Analyses of key components in grain

Of the 70 analytes measured in grain, mean values were provided for 64 analytes and of these, there were 7 for which there was a statistically significant difference ($p < 0.05$) between herbicide-treated corn line DAS1131 and the control: moisture, palmitic acid, manganese, zinc, calcium, β-carotene and vitamin B5 (pantothenic acid). A summary of these 7 analytes is provided in Figure 7. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier (pages 111 – 135).

Of these 7 analytes for which a statistically significant difference was found, all except manganese and β-carotene had FDR-adjusted p-values of >0.05, suggesting that the differences in these analytes were likely to be false positives. In addition, as can be observed in Figure 7 (panels b-h), the DAS1131 mean for each of these 7 components was within the control range value, indicating that DAS1131 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. For all 7 analytes, including manganese and β-carotene, the observed DAS1131 means fall well within the natural variability represented by the tolerance interval, in-study reference range and publicly available range (purple shaded area, dark grey and light grey bars, respectively, in Figure 7, b-h). The differences reported here are therefore consistent with the normal biological variability that exists in corn.

Overall, the compositional data support the conclusion that no biologically significant

differences exist in the levels of key constituents in DAS1131 when compared to conventional non-GM corn cultivars already available in agricultural markets. Grain from DAS1131 can therefore be regarded as equivalent in composition to grain from conventional non-GM corn.

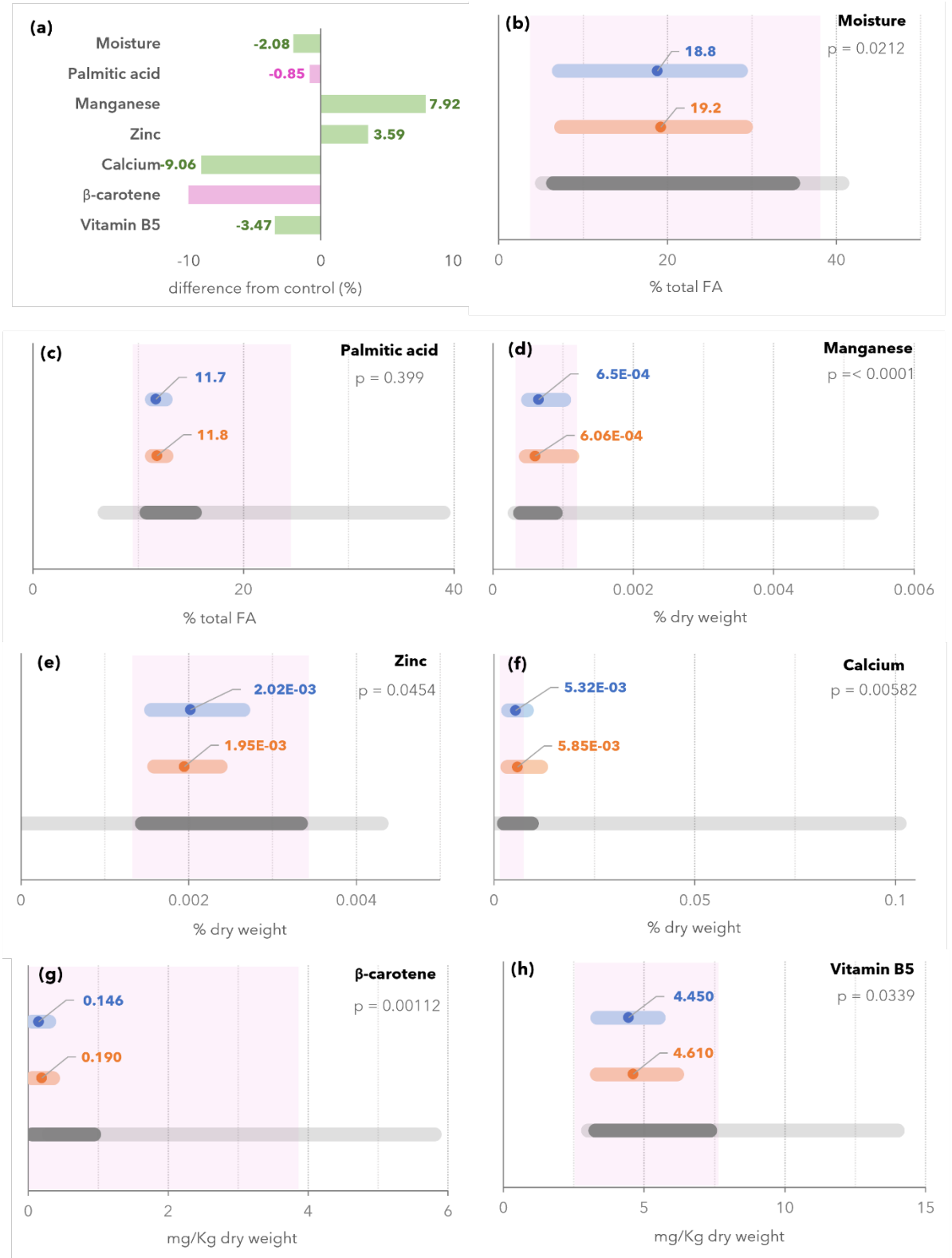

Figure 7. *Visual summary of statistically significant compositional differences between DAS1131 and the conventional control.* **(a)** *Percentage deviation of the mean DAS1131 value from the mean control value for each of the 7 analytes for which a statistically significant difference was found.* **(b) – (h)** *Measured means (dots) and ranges (coloured bars) for DAS1131 (blue) and the conventional control (orange) for the 7 analytes as labelled. The dark and grey bars represent the in-study reference range of values and publicly-available range of values , respectively, for each analyte. The purple shaded range represents the tolerance interval for each analyte. Note that the x-axes vary in scale and unit for each component.*

6 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 wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in section 5.

Where a GM food has been shown to be compositionally equivalent to conventional cultivars, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (OECD 2003; Bartholomaeus et al. 2013). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

DAS1131 is the result of a genetic modification to confer tolerance to the herbicide glyphosate and protection against insect pests, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutrient composition DAS1131 compared with conventional non-GM corn cultivars. The introduction of food derived from DAS1131 into the food supply is therefore expected to have negligible nutritional impact.

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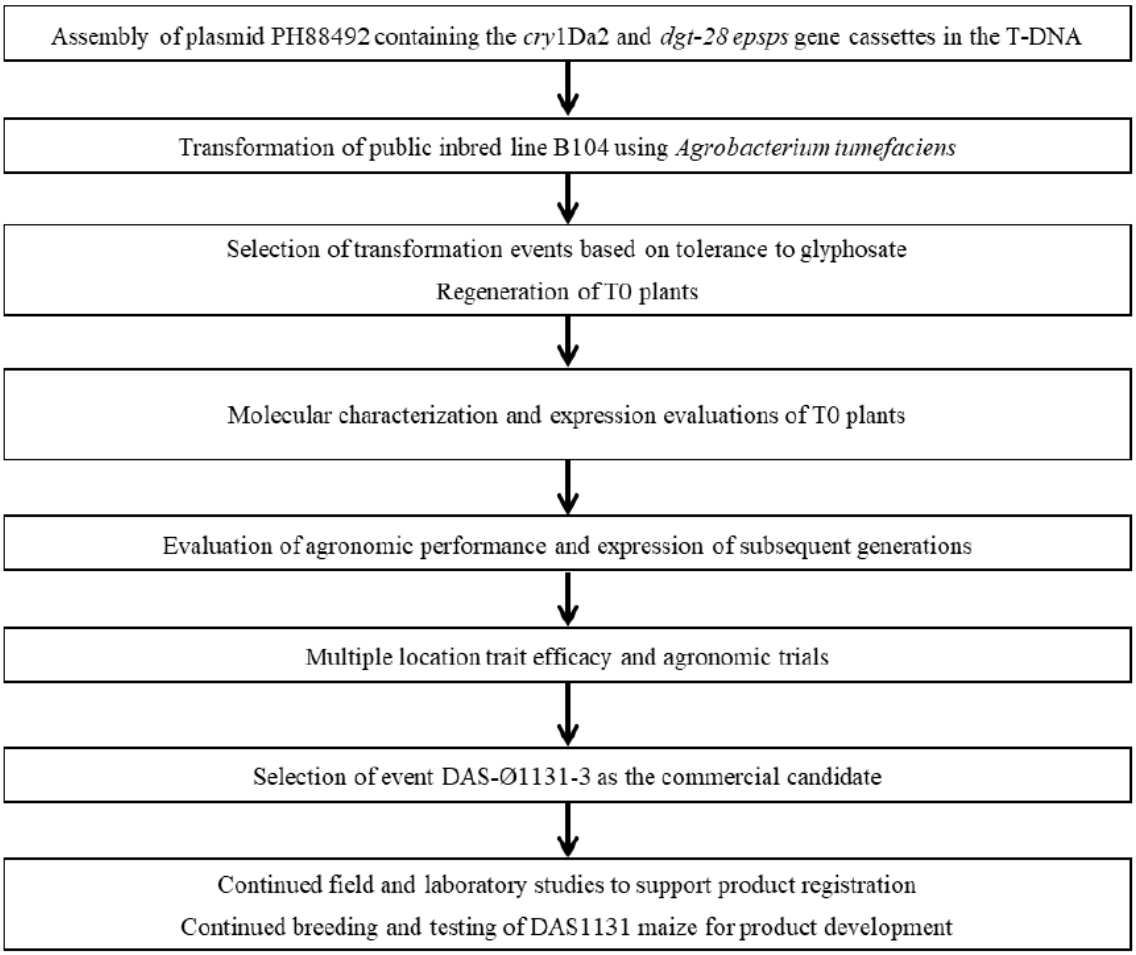
Appendix 1

Flowchart showing the development process used for creation of DAS1131 corn



Appendix 2

PHP88492-derived genetic elements

Genetic element	Relative position	Size (bp)	Description, Source & Reference
Right Border (RB)	1-24	24	T-DNA Right Border from the *Agrobacterium tumefaciens* Ti plasmid (Komari et al. 1996)
Ti Plasmid Region	25-132	108	Sequence from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
Intervening Sequence	133-247	115	DNA sequence used for cloning
*att*B1	248-271	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway cloning system (Hartley et al. 2000; Katzen 2007)
Intervening Sequence	272-316	45	DNA sequence used for cloning
***cry1Da2* cassette**			
*ubi*ZM1 Promoter	317-1,211	895	Promoter region from the *Zea mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 5′ UTR	1,212-1,294	83	5' untranslated region from the *Z. mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 Intron	1,295-2,310	1,016	Intron region from the *Z. mays* ubiquitin gene 1 (Christensen et al. 1992)
Intervening Sequence	2,311-2,349	39	DNA sequence used for cloning
cry1Da2	2,350-4,161	1,812	Chimeric gene comprised of sequences from the *cry1Da2* gene encoding an insecticidal core protein and a segment of the cry1Ab gene, both from Bacillus thuringiensis (US Patent 9890390 (Tan et al. 2018).
Intervening Sequence	4,162-4,194	33	DNA sequence used for cloning
*ubi*ZM1 Terminator	4,195-5,104	910	Terminator region from the *Z. mays* ubiquitin gene 1 US Patent 9688996 (Christensen et al. 1992; Kumar et al. 2017)
Intervening Sequence	5,105-5,269	165	DNA sequence used for cloning
attB2	5,270-5,269	24	Bacteriophage lambda integrase recombination site from the Invitrogen Gateway cloning system (Hartley et al. 2000; Katzen 2007)
Intervening Sequence	5,294-5,310	17	DNA sequence used for cloning
ELP1 (Region 1)	5,311-6,310	1000	Engineered landing pad US Patent 10160975 (Ainley et al. 2018)
ZFN	6,311-6344	34	Zinc finger nucleases target site (Ainley et al. 2013)
ZFN	6,345-6378	34	Zinc finger nucleases target site (Ainley et al. 2013)
ELP1 (Region 2)	6,379-7,378	1000	Engineered landing pad US Patent 10160975 (Ainley et al. 2018)
Intervening Sequence	7,379-7,476	98	DNA sequence used for cloning
ZFN	7,477-7,513	37	Zinc finger nucleases target site (Ainley et al. 2013)

Genetic element	Relative position	Size (bp)	Description, Source & Reference
Intervening Sequence	7,514-7,580	67	DNA sequence used for cloning
***dgt-28 epsps* cassette**			
*ubi*ZM1 Promoter	7,581-8,475	895	Promoter region from the *Z. mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 5' UTR	8,476-8558	83	5' untranslated region from the *Z. mays* ubiquitin gene 1 (Christensen et al. 1992)
*ubi*ZM1 Intron	8,559-9,574	1016	Intron region from the *Z. mays* ubiquitin gene 1 (Christensen et al. 1992)
Intervening Sequence	9,575-9613	39	DNA sequence used for cloning
TraP8	9,614-9,808	195	A chimeric chloroplast transit peptide from *Brassica napus* and *Brassica rapa* - WO Patent 2013116700 (Lira et al. 2013).
Peptide linker	9,809-9,814	6	
dgt-28 epsps	9,614-11,059	1,446	5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene derived from *Streptomyces sviceus* (Griffin et al. 2021)
Intervening Sequence	11,060-11,085	26	DNA sequence used for cloning
*ubi*ZM1 Terminator	11,086-11,995	910	Terminator region from the *Z. mays* ubiquitin gene 1 US Patent 9688996 (Christensen et al. 1992; Kumar et al. 2017)
Intervening Sequence	11,996-12,064	69	DNA sequence used for cloning (synthetic)
ZFN	12,065-12,101	37	Zinc finger nucleases target site (Ainley et al. 2013)
Intervening Sequence	12,102-12,285	184	DNA sequence used for cloning
Left Border (LB)	12,286-12,309	24[1]	T-DNA Left Border from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
Intervening Sequence	12,310-12,328	19	DNA sequence used for cloning
Left Border (LB)	12,329-12,353	25	T-DNA Left Border from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
Intervening Sequence	12,354-12,376	23	DNA sequence used for cloning
Ti Plasmid Region	12,377-12,639	263	Sequence from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
Left Border (LB)	12,640-12,664	25	T-DNA Left Border from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
oriT	13,882-13,933	112	Origin of transfer region from bacteria (Komari et al. 1996)
trfA	14,609-15,757	1,149	Trans-acting replication gene from bacteria (Komari et al. 1996)
spc	16,962-17,750	789	Spectinomycin resistance gene from bacteria (Komari et al. 1996)
Ti plasmid region	17,878-17,986	1091	Sequence from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)
Overdrive	17,987-18,010	24	T-DNA transmission enhancer from the *A. tumefaciens* Ti plasmid (Peralta et al. 1986)

Genetic element	Relative position	Size (bp)	Description, Source & Reference
Ti plasmid region	18,011-18,034	14	Sequence from the *A. tumefaciens* Ti plasmid (Komari et al. 1996)

[1] Shortened by 1 bp on the 3'end