FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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NC 351-25

Supporting document 1

Safety assessment – Application A1333

Food derived from purple tomato lines containing event Del/Ros1-N

Executive summary

Background

Application A1333 seeks approval for the sale and use of food derived from tomato lines containing event Del/Ros1-N that have been genetically modified (GM) for purple fruit colour because of increased anthocyanin levels.

Increased anthocyanin levels result from the expression of the *Delila (Del)* and *Rosea1* (*Ros1*) genes from the snapdragon plant *Antirrhinum majus*, which encode transcription factors Del and Ros1. The presence of Del and Ros1 in ripening fruit activates the endogenous anthocyanin biosynthesis pathway.

Tomato lines containing event Del/Ros1-N (Del/Ros1-N purple tomatoes) also express the neomycin phosphotransferase (NPTII) protein from *Escherichia coli*, which is used as a selectable marker.

Food Standards Australia New Zealand (FSANZ) has previously assessed the NPTII protein. This is the first time FSANZ has assessed the Del and Ros1 proteins.

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

Tomatoes have a long history of safe use in the food supply. The tomato fruit is consumed both in the raw or cooked state, and may also be processed into a variety of products such as sauces, pastes and juices.

Molecular characterisation

The genes encoding Del (*Del*), Ros1 (*Ros1*) and NPTII (*nptII*) were introduced into conventional tomato variety MicroTom via *Agrobacterium*-mediated transformation. Detailed molecular analyses indicated that two T-DNA inserts were integrated into the tomato genome in the initial transformation. However, through several rounds of breeding, one of these inserts was segregated

away, resulting in tomato lines containing a single copy of T-DNA (three expression cassettes) at a single genomic insertion site.

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

All three novel proteins (Del, Ros1 and NPTII) were below the limit of detection in Del/Ros1-N purple tomatoes indicating exposure to the proteins from consumption of the purple tomato would be negligible.

There is a history of human consumption of the Del and Ros1 proteins as a component of the edible flowers of snapdragon plants and homologous proteins found in other plants including commonly consumed foods. Bioinformatic analyses showed neither Del or Ros1 had any amino acid sequence similarity with known allergens or toxins of relevance to humans. Both the Del and Ros1 proteins are susceptible to digestion by pepsin and would be thoroughly degraded following ingestion. Taken together this indicates the Del and Ros1 proteins are unlikely to be toxic or allergenic to humans.

An extensive database demonstrating the safety of NPTII exists. Updated bioinformatic analyses provided for this application confirmed that the expressed protein is unlikely to be toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses were performed on Del/Ros1-N purple tomatoes. Statistically significant differences in mean values were found between fruit from tomato lines containing event Del/Ros1-N and the non-GM control for 9 of the 26 analytes evaluated, however these differences were consistent with the normal biological variability that exists in tomato.

Anthocyanins were not detected in the non-GM control, while Del/Ros1-N purple tomatoes were found to contain anthocyanins, as expected. Del/Ros1-N purple tomatoes also contained an increased level of chlorogenic acid (CGA), a secondary metabolite associated with the anthocyanin biosynthesis pathway.

Overall, the compositional data support the conclusion that, other than the intended increase in anthocyanins and associated metabolites, there are no biologically meaningful differences in the levels of key constituents in fruit from Del/Ros1-N purple tomatoes compared to non-GM tomato varieties available on the market.

Nutritional impact

The anthocyanin content of Del/Ros1-N purple tomatoes is elevated compared to the non-GM comparator but within the natural range of variation for anthocyanins in commonly consumed foods. The elevated levels of anthocyanin in Del/Ros1-N purple tomatoes does not raise any safety concern.

Conclusion

No public health and safety concerns were identified in the assessment Del/Ros1-N purple tomatoes. Based on the data provided in the application and other available information, food derived from Del/Ros1-N purple tomato lines is as safe for human consumption as food derived from conventional non-GM tomato varieties.

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List of Abbreviations

Abbreviation	Description
aa	amino acid(s)
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CGA	chlorogenic acid
COMPARE	COMprehensive Protein Allergen Resource
DNA	deoxyribonucleic acid
dw	dry weight
FASTA	fast alignment search tool – all
FAO	Food and Agriculture Organisation of the United Nations
FSANZ	Food Standards Australia New Zealand
FW	fresh weight
g	gram(s)
GM	genetically modified
iPCR	inverse polymerase chain reaction
kb	kilobase pair(s)
kDa	kilodalton(s)
LB	left border
MT	million tons
NCBI	National Centre for Biotechnology Information
ng	nanogram(s)
nt	nucleotide(s)
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RB	right border
RF	reading frame
RNA	ribonucleic acid

Abbreviation	Description
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T-DNA	transfer DNA
µg	microgram(s)
USDA	United States Department of Agriculture
UTR	untranslated region
WGS	whole genome sequencing
WT	wild type

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Norfolk Healthy Produce, Inc to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food from new genetically modified (GM) purple tomato lines containing event Del/Ros1-N, with the OECD Unique Identifier NPS-01201-8. This event results in increased anthocyanin levels in the tomato fruit, which have purple coloured flesh and skin. In this report, tomato lines containing event Del/Ros1-N are referred to as 'Del/Ros1-N purple tomato'.

Increased anthocyanin levels result from the expression of the *Delila (Del)* and *Rosea1* (*Ros1*) genes from the snapdragon plant *Antirrhinum majus*, which encode the transcription factors Del and Ros1. Their presence in ripening fruit activates the endogenous anthocyanin biosynthesis pathway. Del/Ros1-N purple tomato also expresses the neomycin phosphotransferase (NPTII) protein from *Escherichia coli*, which is used as a selectable marker.

FSANZ has previously assessed the NPTII protein. This is the first time FSANZ has assessed the Del and Ros1 proteins.

The applicant is currently seeking a licence for the commercial cultivation of Del/Ros1-N purple tomatoes from the Gene Technology Regulator (GTR[1]).

2 History of use

2.1 Host organism

The host organism is cultivated tomato (*Solanum lycopersisum* (L.)), from the family Solanaceae. The Solenaceae, or nightshade family, also includes potato, eggplant and chilli. Cultivated tomatoes originate from wild tomatoes native to South America and were first domesticated in Mexico (Peralta et al. 2008). Improved varieties were developed by Italian breeders in the 17th and 18th centuries and its popularity as a food crop increased throughout the 19th and 20th centuries.

As well as being a major crop, tomato is also a model species for the study of gene characterisation and fruit development. The tomato genome, which is diploid and consists of 12 chromosome pairs, is well characterised (The Tomato Genome Consortium 2012; Su et al. 2021).

Tomatoes have a long history of safe human consumption and the cultivated tomato is the most consumed fruit in the world (OECD 2016). Total global production was 192.3 MT[2] in 2023 (FAOSTAT 2025), and the top tomato producing countries were China (70.1 MT), India (20.4 MT), Türkiye (13.3 MT) and the United States (12.4 MT). Production of tomatoes in Australia and New Zealand is comparatively minor at 0.322 MT and 0.0517 MT, respectively, in 2023 (FAOSTAT 2025).

Tomatoes are widely consumed in Australia – the per capita consumption in 2021 was 23 kg/person (Australian Processing Tomato Research Council 2022) which is amongst the highest in the world. Tomatoes are consumed in both the raw and cooked state, but may also be processed into sauces, juice, pulp, paste and soup (OECD 2008).

[1] The Office of the Gene Technology Regulator (OGTR) provides administrative support to the Gene Technology Regulator in the performance of functions under the *Gene Technology Act 2000*.
[2] Million tons

2.2 Gene donor organisms

2.2.1 Antirrhinum majus

The *Del and Ros1* genes are derived from the common snapdragon plant, *Antirrhinum majus*, a common perennial flowering plant which is cultivated worldwide. Snapdragon flowers are widely used as edible garnishes (Seo et al. 2020). Both the Del and Ros1 proteins are expressed in the snapdragon flowers (Goodrich et al. 1992; Schwinn et al. 2006).

2.2.2 Escherichia coli

The *nptII* gene encodes the NPTII protein and is derived from *Escherichia coli* Tn5 transposon. *E. coli* is a non-pathogenic, facultative anaerobic bacterium commonly found in the gastrointestinal tract of humans and animals. It is used globally in the commercial manufacturing of products ranging from amino acids and vitamins for food applications, to recombinant human proteins used in pharmaceutical applications, including injectable protein products such as insulin (Riggs 2021).

2.2.3 Other organisms

Genetic elements from several other organisms have been used to produce event Del/Ros1-N (refer to Table 1 and Appendix 1). These genetic elements are non-coding sequences and are used to regulate the expression of the inserted genes.

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 into 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.

3.1 Transformation method

To develop tomato lines containing event Del/Ros1-N, the conventional tomato variety MicroTom was transformed with a plasmid containing the *Del*, *Ros1* and *nptII* expression cassettes located within a transfer DNA (T-DNA) region (Butelli et al. 2008).

MicroTom leaf discs were transformed using *Agrobacterium tumefaciens* strain LBA4404 containing the transformation plasmid pDEL.ROS (Figure 1) using the method described by McCormick (1991). Transformants were selected using media containing kanamycin (to allow selection of plants containing the *nptII* gene) and regenerated using tissue culture.

Four primary transformants (T0 generation) carrying the T-DNA insert from pDEL.ROS were generated and designated as lines C, N, Y and Z. Event N was determined to express the highest levels of anthocyanins and was selected for further characterisation and breeding.

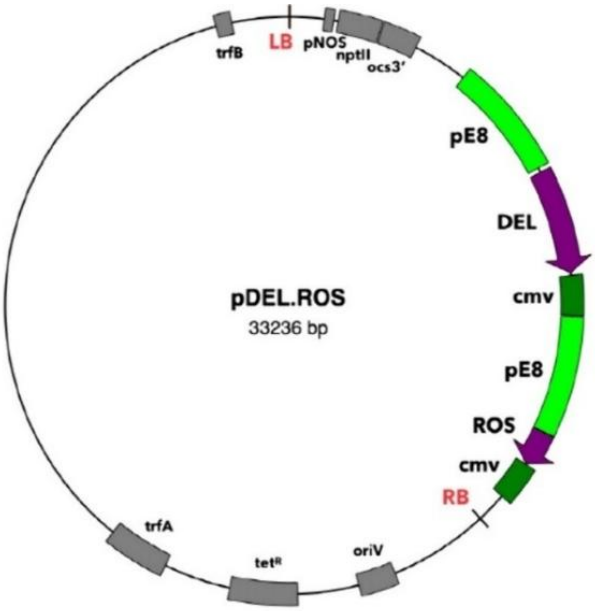

Figure 1. *Map of plasmid pDEL.ROS. The T-DNA region between the left and right border regions (LB and RB) was inserted into the tomato genome. This region contains the nptII, DEL and ROS expression cassettes, as indicated. Both the Del and Ros genes are under the control of the tomato fruit-specific E8 promoter.*

3.2 Detailed description of inserted DNA

The T-DNA from plasmid pDEL.ROS (Figure 1) integrated into the tomato genome includes the *nptII*, *Del* and *Ros1* expression cassettes. The final insert was 12,583 base pairs (bp) long (Figure 2).

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

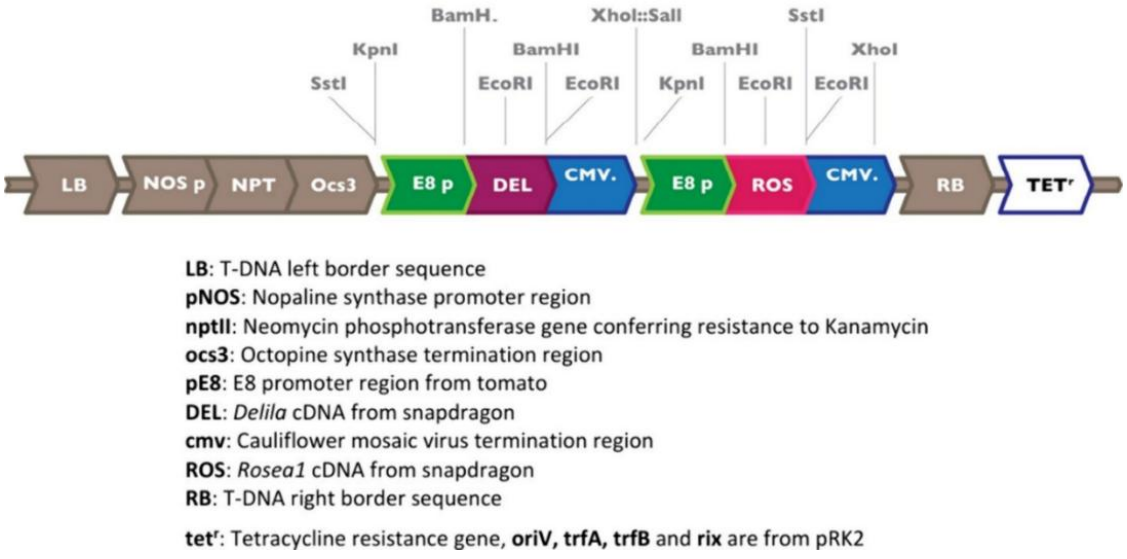

Figure 2. *Schematic of the inserted DNA in event Del/Ros1-N. The 12,583 bp insert contains the nptII, Del and Ros1 expression cassettes.*

Table 1. Expression cassettes contained in the T-DNA of pDEL.ROS

	Promoter	Coding sequence	Terminator	Expected Expression Pattern	Notes
Neomycin phosphotransferase (NPTII)	*Nopaline synthase* promoter region (*A. tumefaciens*)	Neomycin phosphotransferase (*E. coli*)	*Octopine synthase* termination region (*A. tumefaciens*)	Constitutive	Selectable marker (kanamycin resistance)
Delila (Del)	*E8* promoter (tomato)	*Delila* cDNA[3] (snapdragon)	*CMV terminator* region (Cauliflower mosaic virus)	Ripe fruit	Transcription factor (anthocyanin biosynthesis)
Rosea1 (Ros1)	*E8* promoter (tomato)	*Rosea1* cDNA (snapdragon)	*CMV terminator* region (Cauliflower mosaic virus)	Ripe fruit	Transcription factor (anthocyanin biosynthesis)

3.3 Development of the tomato event from the original transformation

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of tomato lines containing event Del/Ros1-N
- ensuring that the Del/Ros1-N event is incorporated into elite lines for commercialisation.

Of the four primary transformants carrying the pDEL.ROS T-DNA insertion (lines C, N, Y and Z), the 'N' event was selected for further development based on anthocyanin levels (this is denoted as 'MicroTom T0' in Figure 3 below).

One individual plant in the T1 generation was crossed to the tomato variety MoneyMaker to generate an F1 generation (Figure 3). Both T and F lineages were selfed for multiple generations using single seed descent to generate the stock lines "Del/Ros1-N in MicroTom" (MicroTom T6) and "Del/Ros1-N in MoneyMaker" (MoneyMaker F9).

In this document, where relevant, tomato plants are referred to using their variety name and generation number, for example, *MicroTom T6* and *MoneyMaker F9*.

MoneyMaker F9 was crossed with several other tomato cultivars, including Goldkrone and VF36 (Figure 3). The applicant notes that the current commercial breeding line is the result of a cross of MoneyMaker F9 x Goldkrone, followed by multiple generations (>7) of selfing and subsequent selection for purple fruit colour and high fruit count per truss (denoted as 'Goldkrone F7+' in Figure 3 and throughout this report).

[3] cDNA = complementary DNA; DNA that has been synthesised by the enzyme reverse transcriptase using messenger RNA (mRNA) as a template. Because mRNA contains only the exons (protein-coding parts of a gene), cDNA represents only the protein-coding portion of the source DNA sequence and excludes the non-coding intron sequences.

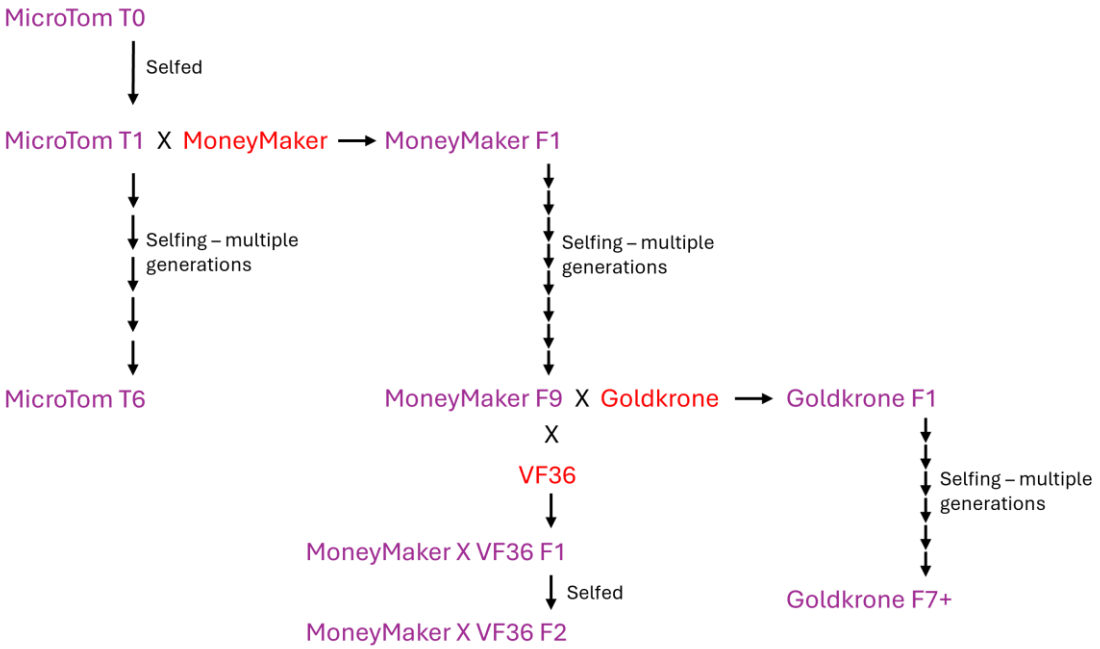

Figure 3. *Breeding pedigree of Del/Ros1-N purple tomatoes.*

The generations analysed for the molecular characterisation and other analyses are listed in Table 2.

Table 2. Del/Ros1-N lines and generations used for various analyses

Analysis	Section	Generation(s) used
Southern blot, qPCR and iPCR to determine copy number of initial transformation	Section 3.4.1	MicroTom T0, MicroTom T1
Insertion copy number, insertion organisation, insertion integrity, site of insertion, genetic stability and the absence of plasmid backbone sequences by whole genome sequencing (WGS)	Section 3.4.1; Section 3.4.2; Section 3.4.3	MicroTom T6, Goldkrone F7+
Segregation analysis	Section 3.4.4	MoneyMaker F9 X VF36
Protein expression; compositional analysis	Section 4; Section 5	MoneyMaker F9

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

A range of analyses were undertaken to characterise the genetic modification in Del/Ros1-N purple tomato lines. These analyses focused on the nature and stability of the inserted DNA and whether any unintended rearrangements or products may have occurred as a consequence of the transformation procedure.

3.4.1 Number of integration site(s)

The initial transformation of MicroTom with pDEL.ROS resulted in two T-DNA insertions at two separate loci in the tomato genome (designated A and B). Following subsequent breeding steps, the T-DNA insert at locus A was lost from the Del/Ros1-N event by segregation, and the lines taken forward for further breeding and commercialisation contain a single T-DNA insertion at locus

B. The analyses used to determine copy number in different generations are summarised below.

In the initial MicroTom T0 event, Southern blot analysis was used to determine the copy number of the T-DNA insert. Genomic DNA from MicroTom T0 was digested with *Bam*HI and probed with *Ros1* cDNA. The results indicated there were two copies of the T-DNA insert present. Copy number was confirmed by quantitative polymerase chain reaction (qPCR) analysis of the *nptII* gene in MicroTom T1. The observed pattern of distribution of *npt*II copy number was consistent with the expected distribution based on integration of two T-DNA copies inserted at two genetically unlinked Mendelian loci (one T-DNA copy per locus) in the tomato genome.

Inverse PCR (iPCR) was performed on MicroTom T1 plants to amplify the sequences flanking both T-DNA inserts. Sequencing of the PCR products confirmed the presence of two insertions at locus A and locus B, on chromosomes 2 and 4, respectively.

DNA from purple fruited MicroTom T6 and MoneyMaker F9 plants was analysed for T-DNA insertions at locus A and locus B by PCR using primers specific to the sequences flanking both loci. The T-DNA insertion at locus A was not detected in either line, indicating it had segregated away during selfing. Using primers from the sequences flanking the original insertion at locus A, a wild type (WT) fragment of DNA of 1.25 kb was amplified from both MicroTomT6 and MoneyMakerF9 as well as from the control non-GM MicroTom DNA. Sequencing of this fragment of DNA confirmed it was the WT sequence from MicroTom with and that the T-DNA insertion at locus A had indeed segregated away rather than being lost by recombination.

The insertion at locus B was confirmed as present in 105 subsequent generations and in outcrosses introducing the purple colour trait into other genetic backgrounds. Whole genome sequencing (WGS) of MicroTom T6 and Goldkrone F7+ confirmed the absence of the insertion at locus A (insertion A) and presence of the insert at locus B (insertion B) in these lines. A summary of which lines and generations contain which insertions is shown in Table 3.

Table 3. Insertion sites in different generations of Del/Ros1-N purple tomato

Line/Generation	Insertion A (Chromosome 2)	Insertion B (Chromosome 4)	Method of Analysis
MicroTom T0 and T1	Present	Present	Southern blot, qPCR, iPCR
MicroTom T6	Absent	Present	WGS, PCR
MoneyMaker F9	Absent	Present	PCR, Sanger sequencing
Goldkrone F7+	Absent	Present	WGS
Other outcrosses	Absent	Present	PCR

3.4.2 Absence of backbone and other sequences

WGS demonstrated that no DNA from the pDEL.ROS plasmid backbone was integrated into the genome in either MicroTom T6 or Goldkrone F7+.

While WGS was not performed directly on MoneyMaker F9, the applicant states that no plasmid backbone sequences are linked to locus B, based on WGS of the MicroTom line. The applicant notes that as both lines are derived from the same parent, the likelihood of any vector backbone sequence being present in lines derived from MoneyMaker F9 is vanishingly small. FSANZ agrees with this statement and notes:

- the majority of instances of plasmid backbone being integrated into the plant genome as a result of *Agrobacterium*-mediated transformation are genetically linked to the insert. The insertion of unlinked plasmid backbone sequences as a result of *Agrobacterium*-mediated transformation is rare (Wu et al. 2006a; Nicolia et al. 2017; Xu et al. 2024).
- WGS analysis of MicroTom T6, which was bred by multiple generations of selfing of the initial transformation event, demonstrates the absence of genetically linked plasmid backbone sequence and supports the absence of unlinked plasmid backbone integration.
- given the breeding pedigree of Del/Ros1-N purple tomatoes (Figure 3), if there was unlinked plasmid backbone present in the initial transformation event, it would have most likely been lost during the subsequent breeding and crossing steps.
- WGS of Goldkrone F7+, which was bred from MoneyMaker F9, did not identify any linked or unlinked plasmid backbone sequences.

3.4.3 Insert integrity and site of integration

WGS of a single tomato plant containing the Del/Ros1-N event (MicroTom generation >6) confirmed the sequence of the T-DNA insertion was as expected, with no mutations, deletions or insertions. Short deletions were identified at either end of the insertion: 52 bp were deleted from the right border (RB) of the T-DNA insertion and 75 bp were deleted from the left border (LB). This type of deletion is common in *Agrobacterium*-mediated transformation (Kleinboelting et al. 2015; Thomson et al. 2024). These deletions only impacted the border regions, not the coding or regulatory sequences of the introduced genes.

The T-DNA insertion also caused a 94 bp deletion of the tomato genome sequence at the site of insertion (Figure 4).

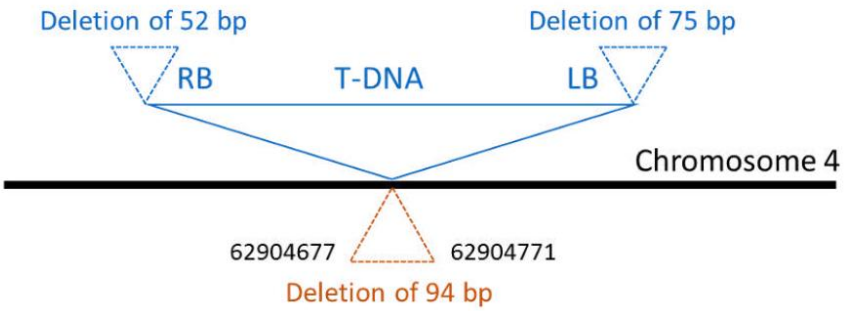

Figure 4: *Schematic representation of the Del/Ros1-N T-DNA insert in chromosome 4 of Del/Ros1-N purple tomatoes.*

3.4.4 Stability of the genetic changes

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over several generations. Genetic stability refers to maintenance of the modification over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.4.1 Genetic stability

The genetic stability of the T-DNA insertion at locus B was assessed by PCR amplification of the T-DNA:genomic junction sequences at the LB and RB in multiple generations and genetic backgrounds, followed by sequencing. The LB was assessed in MicroTom T1 and T6 plants, and RB integrity was assessed in MicroTom T6 plants and Moneymaker F9 plants. Sequencing revealed that the flanking sequences of the insert were identical in MicroTom T1 and T6, and in the F9 generation following outcrossing to MoneyMaker.

These results indicate the insert at locus B has been stably maintained across 5 generations in the MicroTom genetic background, as well as after crossing into the MoneyMaker background and multiple selfing generations. In addition, WGS sequencing analysis further confirmed the genetic stability of the insert between MicroTom T6 and Goldkrone F7+.

The consistency of these results across generations and backgrounds demonstrates the inserted DNA at locus B is stably maintained.

3.4.4.2 Phenotypic stability

Since the inserted T-DNA resides at a single locus in the relevant generations of Del/Ros1-N purple tomatoes, it would be expected to be inherited according to Mendelian principles. Twenty plants from the F2 generation from the MoneyMaker F9 x VF36 cross were analysed by germinating seeds on agar containing kanamycin. The expected segregation ratio, based on Mendelian inheritance principles, was 3:1.

A chi-square (χ^2) analysis was conducted to compare the observed and expected segregation ratio of the kanamycin resistance phenotype. The results (Table 4) demonstrated no statistically significant difference ($p < 0.05$) from those expected for a 3:1 segregation ratio and are consistent with a single genetic locus (locus B) being present.

Table 4. Segregation of the presence of the *nptII* gene, kanamycin resistance phenotype and purple fruit phenotype in the F2 generation of the MoneyMaker F9 x VS36 cross

Genotype/Phenotype	Expected segregation ratio (positive:negative)	Observed number of plants			Statistical anallysis	
		Positive	Negative	Total	χ^2	*P value*
Presence of *nptII* gene	3:1	16	4	20		
Kanamycin resistance	3:1	16	4	20	0.267	0.61
Purple fruit	3:1	16	4	20		

The applicant states the purple trait has been introgressed into dozens of tomato varieties, and in every instance, segregation of the purple phenotype consistent with simple dominant inheritance has been observed, though data has not been collected in every case. Purple tomato lines now in commercial production in the US are >10 generations removed from the original transformation event and no variation in penetrance of the trait has been observed within varieties.

Taken together, the data provided, combined with observations over numerous generations support the conclusion that the inserted DNA is present at a single locus in MoneyMaker F9 and is inherited according to Mendelian principles in subsequent generations.

3.4.5 Reading frame analysis

A bioinformatic analysis of the DNA regions flanking the inserted T-DNA was undertaken to identify whether any novel reading frames (RFs) had been created as a result of the DNA insertion in Del/Ros1-N purple tomatoes, and whether any putative peptides encoded by the identified RFs have the potential for allergenicity or toxicity.

Potential new RFs were investigated by translating the genomic:T-DNA junction sequence at the left and right borders in all six reading frames and detecting initiator of translation codons (methionine). RFs were selected for analysis if they were greater than 30 amino acids (aa) and spanned the genomic:T-DNA junction.

No novel RF sequences of >30 aa were detected at the left genomic border:T-DNA junction. A theoretical 32 aa peptide containing an initiating methionine codon and spanning the right genomic border:T-DNA junction was identified.

Homology searches were conducted on the AllergenOnline database[4] using either the whole 32 aa sequence (Fasta36 search methods) or 8 amino acid epitopes. No hits were detected. BLASTp against the NCBI non-redundant protein database[5] did not identify any hits.

These results demonstrate this putative peptide does not have any homology to known toxins or allergens. Additionally, there is no evidence the sequence is translated into a functional protein as it lacks a nearby promoter or Kozak consensus sequence around the initiating ATG codon. The bioinformatic analysis also demonstrated that no native tomato ORFs were interrupted by the presence of the T-DNA insert on chromosome 4.

Although potential novel RFs within the insert were not specifically analysed, the phenotype of the Del/Ros1-N purple tomato demonstrates expression of the three target proteins (NPTII, Del, and Ros1). Combined with the absence of regulatory elements for other RFs, it is highly unlikely that any RFs other than the expected expression products would be expressed *in planta*.

3.5 Conclusion

Event Del/Ros1-N consists of a single 12,583 bp insertion, integrated into chromosome 4 of the tomato genome. The intended expression cassettes have been inserted with the expected sequence and organisation, except for small deletions in the left and right border regions of the T-DNA sequence. No backbone sequences from the plasmid used in the transformation are present, including antibiotic resistance genes. The inserted DNA is stably inherited and expressed across several breeding generations. Bioinformatic analyses of the novel RFs created by the insertion did not raise any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

In considering the safety of novel proteins it is important to understand that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impact health, because of anti-nutrient properties or triggering of allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects must be considered during the safety assessment including potential toxic, allergenic or anti-nutrient effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects 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.

Three novel proteins are expressed in Del/Ros1-N purple tomatoes: (1) Del and (2) Ros1, which are transcription factors and activate the endogenous anthocyanin biosynthesis pathway in tomato fruit, and (3) NPTII, which serves as a selectable marker.

4.1 Del and Ros1

Both the *Del* and *Ros1* genes in the Del/Ros1-N event are derived from the garden snapdragon (*A. majus*). *Del* encodes a 644 amino acid, ~71 kDa basic-helix-loop-helix (bHLH)-type transcription factor and *Ros1* encodes a 220 amino acid, ~25 kDa R2R3MYB-related transcription factor (Goodrich et al. 1992; Schwinn et al. 2006; Butelli et al. 2008).

[4] Allergen online – http://allergenonline.org/
[5] Basic Local Alignment Search Tool (BLAST) – https://blast.ncbi.nlm.nih.gov/

The sole function of both the Del and Ros1 proteins is as transcriptional activators of target genes involved in anthocyanin biosynthesis (Goodrich et al. 1992; Schwinn et al. 2006; Butelli et al. 2008). In plants, anthocyanins are synthesised via the flavonoid branch of the phenylpropanoid pathway, which also catalyses the production of an array of other secondary metabolites such as chlorogenic acid (CGA), tannins and flavonols (Vogt 2010; Zhang et al. 2015; Figure 5).

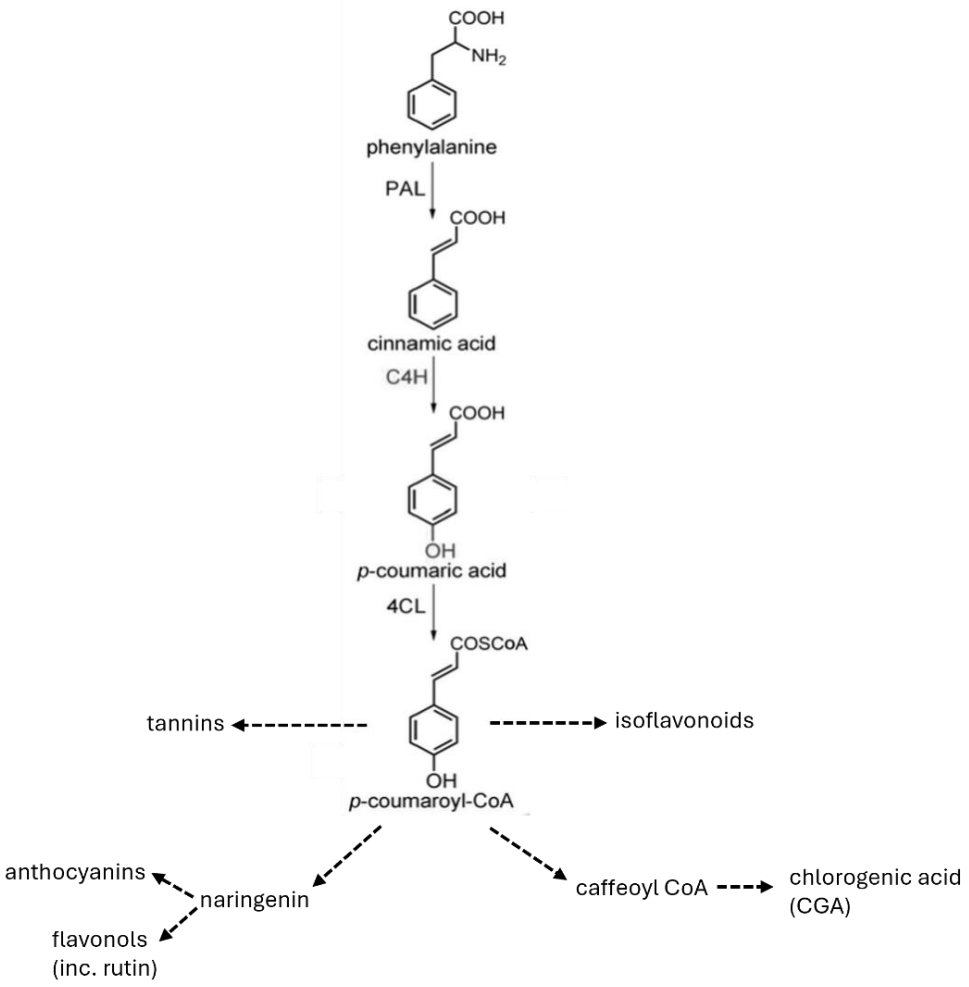

Figure 5. *Schematic view of some branches of the phenylpropanoid pathway in plants, including the branches leading to synthesis of anthocyanins, flavonols, and other secondary metabolites. PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumarate: coenzyme A ligase. Adapted from Xia et al. (2017). Refer to Appendix 2 for a more detailed schematic of anthocyanin biosynthesis.*

When Del and Ros1 are co-expressed in tomato, they, along with an additional endogenous transcription factor, form a complex which acts to upregulate most genes in the endogenous tomato anthocyanin biosynthetic pathway (Butelli et al. 2008; Tohge et al. 2015; Naing and Kim 2018). Del and Ros1 significantly increase the expression of late biosynthetic genes such as flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose 3-O-flavonoid transferase (UFGT) (Butelli et al. 2008; see also Appendix 2).

In ripening tomato fruit, the upregulation of the anthocyanin synthesis pathway as a result of Del and Ros1 expression leads to a strong accumulation of two major anthocyanin pigments: delphinidin 3-O-(coumaroyl) rutinoside-5-O-glucoside and petunidin 3-O-(coumaroyl) rutinoside-5-O-glucoside (Butelli et al. 2008; Figure 6a). This results in purple pigmentation throughout the ripe fruit (Figure 6b). These anthocyanins are naturally found in non-GM tomato leaves, where they help protect against light stress. They are also naturally found in the skin of tomato fruit derived from crosses of *S. lycopersicum* with wild tomato species such as *S. chilense* or *S. cheesmaniae*.

Structurally, the Del and Ros1 proteins are closely related to two endogenous tomato transcription factors – Hoffman's anthocyaninless and ANT1 – which control anthocyanin production in tomato leaves (Mathews et al. 2003; Qiu et al. 2016). In addition, transcription factors, functionally similar to Del and Ros1, control anthocyanin biosynthesis in all angiosperm plants that produce these pigments, including food crops such as pepper, potato, corn, apple, grape, blackberry, strawberry and blood orange (Kobayashi et al. 2002; Borovsky et al. 2004; Ramsay and Glover 2005; Espley et al. 2007; Jung et al. 2009; Lin-Wang et al. 2010).

The applicant reports there is a high degree of similarity between Del and Ros1 and other bHLH (Del) and Myb-type (Ros1) transcription factors present in many other food crops (Goodrich et al. 1992; Aharoni et al. 2001; Deluc et al. 2008; Butelli et al. 2012; Liu et al. 2015; Wang et al. 2017; Zhao et al. 2019; Cao et al. 2020; Albert et al. 2021). This is largely based on phylogenetic protein class clusters, rather than a high level of amino acid similarity amongst these transcription factors. Nonetheless, the evolutionary similarity of Del and Ros1 to a number of other commonly consumed transcription factors, as well as the direct consumption of Del and Ros1 in snapdragon flowers, which have a history of safe use as edible flowers (Rop et al. 2012; González-Barrio et al. 2018), contributes to a weight of evidence for the safety of these proteins.

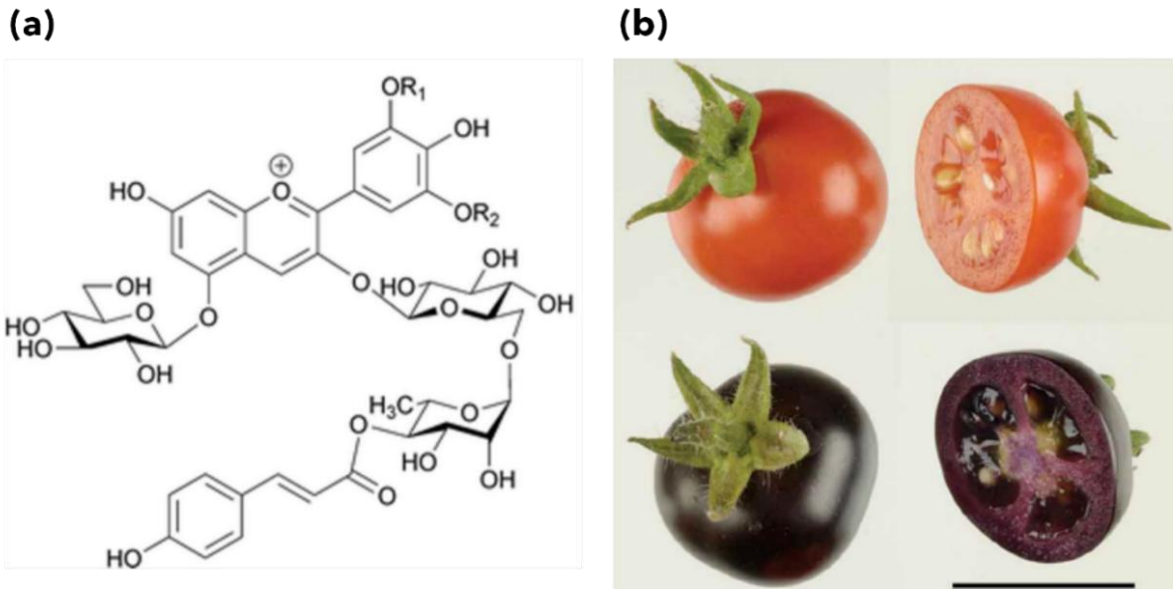

Figure 6. ***(a)*** *Structure of the major anthocyanins produced in tomato leaves of non-GM control plants and in the fruit of purple tomatoes. Delphinidin 3-O-(coumaroyl)rutinoside-5-O-glucoside (Nasunin): R1 and R2 = H; Petunidin 3-O-(coumaroyl)rutinoside-5-O-glucoside: R1 = CH3 and R2 = H.* ***(b)*** *Whole and halved ripe red non-GM (top) and Del/Ros1-N (bottom) tomato lines.*

4.1.1 Expression of Del and Ros1 in Del/Ros1-N purple tomato fruit

Mass spectrometry was used to assess the level of protein expression in ripe fruit from the MoneyMaker F9 generation. Proteins were extracted from test fruits (three replicates) using a phenol extraction method as described by Faurobert et al. (2007). Protein samples were reduced and alkylated with dithiothreitol and iodoacetamide, respectively. Proteins were subsequently digested with trypsin. Aliquots of the reaction corresponding to 360 ng protein were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Orbitrap-Fusion™ mass spectrometer. To determine the limits of detection, two standard proteins (phosphorylase B and bovine serum albumin (BSA)) were spiked in at 0.1, 1, 10 and 100 fmol per 360 ng sample injection.

The mass spectrometry raw data were processed in MaxQuant 1.6[6] to generate peaklists. The peaklists were used for a database search using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the *Solanum lycopersicum* database (UniProt 2015, 35,218 entries) together with a custom database containing the sequences of the two *Antirrhinum* enzymes (Q2VAZ7, Q38736), NPTII, and a database for common contaminants (MaxQuant, 245 entries).

More than 2,700 peptides were detected, but none corresponded to the Del or Ros1 proteins, indicating the expression levels of both proteins were below the limit of detection.[7] The applicant states there is no evidence to suggest that expression levels would change in different tomato genetic backgrounds.

The *E8* promoter that drives expression of both Del and Ros1 is the natural promoter of a hydroxylase enzyme involved in catabolizing alpha-tomatine during ripening. The hydroxylase enzyme can only be detected by heterologous expression (Akiyama et al. 2021), indicating that expression levels from the *E8* promoter are quite weak. This is consistent with the undetectable level of expression seen for Del and Ros1 in Del/Ros1-N purple tomatoes.

4.1.2 Characterisation of Del and Ros1 expressed in bacteria and their suitability for use in safety assessments

Given the very low levels of Del and Ros1 expressed in Del/Ros1-N purple tomato, the applicant also expressed both proteins in *E. coli*, to allow sufficient quantities for analysis to be purified. The equivalence of Del/Ros1-N purple tomato and *E.coli*-derived Del and Ros1 proteins must be established before the safety data generated using the *E. coli*-derived proteins can be applied to Del/Ros1-N purple tomato-derived Del and Ros1. As both the Del and Ros1 proteins are expressed in the plant below the limit of detection (see section 4.1.1), a direct comparison could not be made. However, the applicant provided the results of a series of analytical techniques to characterise *E. coli*-derived Del and Ros1. The results are summarised below.

Sequence. The translated *E.coli*-derived Del and Ros1 sequences are identical to the protein sequences of Del/Ros1-N purple tomato-derived proteins, based on the inserted DNA sequences.

Molecular weight. Purified *E. coli*-derived Del and Ros1 were run on SDS-PAGE then visualised with a GelCode Blue staining reagent. The migration of both proteins indicates that the *E. coli* produced proteins are approximately the same size as expected (~71 kDa for Del and ~26 kDa for Ros1).

Peptide mapping. As part of the digestion assay described in Section 4.1.3, *E. coli*-derived Ros1 was also digested with trypsin and analysed by mass spectrometry. The results of this digestion assay further support the identity of the *E. coli*-derived Ros1 protein.

The results outlined in this section indicate that *E.coli*-derived Del and Ros1 are structurally equivalent to Del and Ros1 derived from Del/Ros1-N purple tomatoes. It can be concluded that *E. coli*-derived Del and Ros1 are suitable surrogates for Del/Ros1-N purple tomato-derived Del and Ros1 for use in the digestibility studies described below.

4.1.3 Safety of the introduced Del and Ros1 proteins

Bioinformatic analyses of Del and Ros1

Bioinformatic analyses, as described in Section 3.4.5, were performed to compare the Del and Ros1 proteins to known allergenic proteins in the AllergenOnline Database (v22, May 2023). No matches between Del or Ros1 and known allergens were identified when searched for 35%

[6] www.maxquant.org
[7] The limits of detection for Del and Ros1 were 4 ng/g FW and 1.5 ng/g FW, respectively.

similarity over ≥ 80 amino acids, nor did any eight amino acid peptide from either sequence match any known allergic epitope.

The Del and Ros1 protein sequences were also compared *in silico* to a toxin protein database. This database is a subset of sequences derived from the UniProt protein database[8], filtered using the keyword "toxin", and contained 109,442 sequences at the date of analysis. A BLASTp algorithm (v2.16.0+: June 25, 2024) was used with PAM30 matrix (more sensitive to highly divergent sequences with low similarity), an E-value threshold of $1x10^{-2}$ (0.001), and a word size of 2 (chosen to increase sensitivity). No matches were identified.

In addition to the bioinformatics toxin search above, the Del and Ros1 sequences were run using BLAST against the ToxinPred 3.0 database of known toxic proteins (Rathore et al. 2024). Using an E-value of 10e-1 resulted in no hits.

Susceptibility of Del and Ros1 to digestion

E. coli-produced Del and Ros1 were incubated with pepsin (10U enzyme/µg protein) for 0-60 min at 37°C. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al. 2004). The extent of digestion was visualised by SDS-PAGE.

For the Del digestion, visual inspection showed there was no intact full-length Del remaining in the reaction mix after 0.5 min. For the Ros1 digestion, the SDS-PAGE did not allow monitoring of the degradation of the Ros1 protein, due to Ros1 and pepsin running as overlapping bands. Instead, the Ros1 degradation was monitored by Orbitrap mass spectrometry. The results showed that Ros1 protein was substantially lost after 10 min and completely lost after 20-60 min digestion with pepsin. These data indicate that both Del and Ros1 would be degraded by gastric enzymes in the human digestive system.

Post-translational modification

Due to the low expression levels, post-translational modification of Del and Ros1 could not be directly evaluated. However, there are limited examples of glycosylation occurring in transcription factors (Jackson and Tjian 1988) given they function in the nucleus, and glycosylation of proteins requires transport to the endoplasmic reticulum (ER). Furthermore, any post-translational modification of the proteins in tomato is likely to be equivalent to that in snapdragon, given the phenotype of the Del/Ros1-N tomatoes suggests the proteins are functional.

4.1.4 Conclusion

The Del and Ros1 proteins are derived from the common snapdragon plant (which has edible flowers) and shares structural and functional similarity to other transcription factors that control anthocyanin biosynthesis in commonly consumed foods (including commercial tomato varieties). Expression studies confirmed the levels of Del and Ros1 in the edible portion of Del/Ros1-N purple tomatoes were below the limit of detection (4 ng/g FW and 1.5 ng/g FW, respectively), which indicates exposure to the proteins from consumption of the tomato would be negligible. A range of characterisation studies were performed on *E. coli*-produced Del and Ros1, which suggested the recombinant proteins were suitable for use in the digestibility analyses. Both the Del and Ros1 proteins were susceptible to pepsin digestion. Bioinformatic analyses showed neither Del or Ros1 had any amino acid similarity with known allergens or toxins of relevance to humans. Taken together, this indicates the Del and Ros1 proteins are unlikely to be toxic or allergenic to humans.

[8] UniProt – https://www.uniprot.org/

4.2 NPTII

The *nptII* gene from transposon 5 of *E. coli* strain K12 encodes the enzyme neomycin phosphotransferase II (NPTII). NPTII is an aminoglycoside 3'-phosphotransferase II enzyme which catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group on aminoglycoside antibiotics, inactivating them. NPTII confers tolerance to the antibiotics kanamycin, neomycin, ribostamycin, geneticin, gentamicin B, butirosin and paromomycin (Beck et al. 1982; Redenbaugh et al. 1994; Padilla and Burgos 2010).

The *nptII* gene is widely used as a selectable marker in the transformation of plants (De Block et al. 1984; Horsch et al. 1984). While the *nptII* gene and its encoded protein are present in event Del/Ros-N, its function is limited to the initial transformation stage (section 3.1), and it has no function in commercial Del/Ros1-N purple tomato lines. FSANZ have previously assessed and approved 12 events containing NPTII.

4.2.1 Expression of NPTII in Del/Ros1-N purple tomatoes

The level of NPTII expression in Del/Ros1-N purple tomatoes fruit was assessed by mass spectrometry, as described in Section 4.1.1. Of the 2,700 peptides detected in the tomato fruit, none corresponded to NPTII, indicating the expression level of NPTII is below the limit of detection.[9]

The *nptII* gene in Del/Ros1-N purple tomatoes is under the control of a *nos* promoter. The applicant indicates that the *nos* promoter drives relatively low gene expression in transgenic plants, resulting in 30-fold lower transcript levels than those generated by the CaMV 35S promoter, and 110-fold lower NPTII activity than with the 35S promoter (Sanders et al. 1987).

4.2.2 Characterisation of NPTII expressed in Del/Ros1-N purple tomatoes

The *nptII* gene prepared by the applicant encodes a protein of 264 amino acids. The protein sequence is 99.6% identical to the NPTII protein sequence from *E.coli* K12, as well as with NPTII sequences previously assessed by FSANZ. Relative to the native *E. coli* NPTII sequence, the predicted sequence of the NPTII protein expressed Del/Ros1-N purple tomatoes contains a single amino acid substitution at position 2 (valine for isoleucine). This substitution is not expected to affect the overall structure, enzyme activity or substrate specificity of the protein.

The NPTII protein was expressed in Del/Ros1-N purple tomatoes fruit at levels below the limit of detection (see Section 4.2.1). Despite this, the expression of NPTII protein in regenerating cells following transformation was sufficient to provide the cells with kanamycin tolerance for the purpose of selecting transgenic transformants, demonstrating that the NPTII protein is functional (see Section 3.1).

4.2.3 Safety of the introduced NPTII

The safety of NPTII has been assessed in 12 previous FSANZ assessments.[10] For these assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans.

The *nptII* gene has a considerable history of use as a selectable marker gene in the development of GM plants (Kumar et al. 2020). Associated with this history of use is a substantial body of evidence to indicate that the presence of NPTII in food derived from GM crops does not pose a

[9] The limit of detection for NPTII was 1.7 ng/g FW
[10] A341, A355, A372, A379, A382, A383, A384, A484, A549, A595, A1029, A1274.

significant risk to human health (Flavell et al. 1992; Nap et al. 1992; Fuchs et al. 1993a; Fuchs et al. 1993b).

Additionally, the safety of NPTII has been evaluated by other regulators, who concluded that using NPTII as a selectable marker in GM plants does not pose a risk to human or animal health or the environment (FDA 1998; EFSA 2004; EFSA 2009; OGTR 2017). Furthermore, humans are already exposed to this protein due to its widespread environmental presence.

Given the NPTII protein expressed in Del/Ros1-N purple tomatoes is sufficiently similar to previous NPTII proteins assessed by FSANZ, no further safety evaluation is required other than the examination of updated bioinformatic searches.

Bioinformatic analyses of NPTII

The applicant submitted updated bioinformatic studies for NPTII that looked for amino acid sequence similarity to known protein allergens and toxins (April 2025). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.2.4 Conclusion

The data evaluated by FSANZ indicates the NPTII expressed in tomato lines containing event Del/Ros1-N is identical to previously assessed NPTII proteins, except for a single amino acid substitution at position 2. Purple Tomato-derived NPTII is functional, as demonstrated by its ability to provide plant cells with kanamycin tolerance following transformation. Updated bioinformatic analyses confirmed that NPTII has no amino acid sequence similarity to known toxins or allergens.

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 antinutrients 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 GM and conventional tomatoes are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Tomato (OECD 2008). The analytes measured included moisture, crude protein, total fat, total dietary fibre, carbohydrate, total sugar, ash, salt (sodium chloride), six sugars, four fatty acids, three minerals, three vitamins, two carotenoids, and one glycoalkaloid (see Figure 7).

In addition, information was provided on the anthocyanin content of Del/Ros1-N purple tomatoes in comparison to other tomato cultivars, as well as an additional phenylpropanoid compound,

chlorogenic acid (CGA).

5.2 Study design

Five MoneyMaker F9 tomato (Del/Ros1-N) plants and five non-GM MoneyMaker (control) plants were grown under controlled conditions in a single glasshouse.[11] Fruit samples were obtained at typical harvest maturity ("red-ripe") from each of the Del/Ros1-N and control plants. From each plant approximately 1 kg of fruit (10 fruits) was collected and represented one sample. In total, five Del/Ros1-N and five control samples were collected. All fruits were harvested at the same stage of ripening and were collected at the same time. Samples were washed with distilled water and sent fresh to an analytical laboratory.

The methods of compositional analysis were accredited through the United Kingdom Accreditation Service and included validated methods from the Association of Official Analytical Collaboration (AOAC) International or other published scientific methods. The analyses were performed through contracted services from Eurofins Laboratories. In-house analysis of carotenoids was also performed using published scientific methods.

A total of 26 analytes common to tomato were assessed in both Del/Ros1-N and control fruit (see Figure 7 for a complete list). In addition, moisture was also measured and used to convert the analyte values from fresh to dry weight, but was not analysed statistically. For each analyte, 'descriptive statistics' (mean and standard error of means) were generated. An unpaired Student's t-test was performed to identify any statistically significant differences between the control and Del/Ros1-N tomatoes. In assessing the significance of any difference, a P-value of 0.05 was used. In cases where a sample had a value below the level of quantification (described as less than X; where X is the lowest quantifiable number for the assay) and other samples had a measurable number, the analytes with a <X value were recorded as zero and analysis of variance was applied to the samples.

Any statistically significant differences were evaluated further to assess whether they were likely to be biologically meaningful. Comparator values were obtained from three sources:

1. The ranges listed for each analyte in the USDA Food Composition Database (now USDA FoodData Central[12], NDB Number 11529) for tomatoes (red, ripe, year-round average).
2. Analyte values in McCance and Widdowson's composition of foods integrated dataset (CoFID) 2019[13] for tomatoes (standard, raw, food code 13-517).
3. Literature values for carotenoids (Holden et al. 1999; Leonardi et al. 2000; Salunke et al. 2012; Martí et al. 2016; Ali et al. 2020) and folate (Iniesta et al. 2009).

These values were consolidated for each analyte, where available, to create an overall range of values for comparison.

5.3 Analysis of key components in fruit

Of the 26 analytes for which mean values were provided, there were 9 for which there was a statistically significant difference ($p < 0.05$) between Del/Ros1-N and the non-GM control: ash, glucose, total sugar, magnesium, potassium, folate, vitamin K, lycopene, and α-tomatine. A summary of these 9 analytes is provided in Figure 8. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application

[11] At the John Innes Centre, Norwich, UK, in 2018.
[12] USDA FoodData Central – https://fdc.nal.usda.gov/
[13] CoFID – https://www.gov.uk/government/publications/composition-of-foods-integrated-dataset-cofid

dossier[14] (pages 29 – 32; 183 – 203).

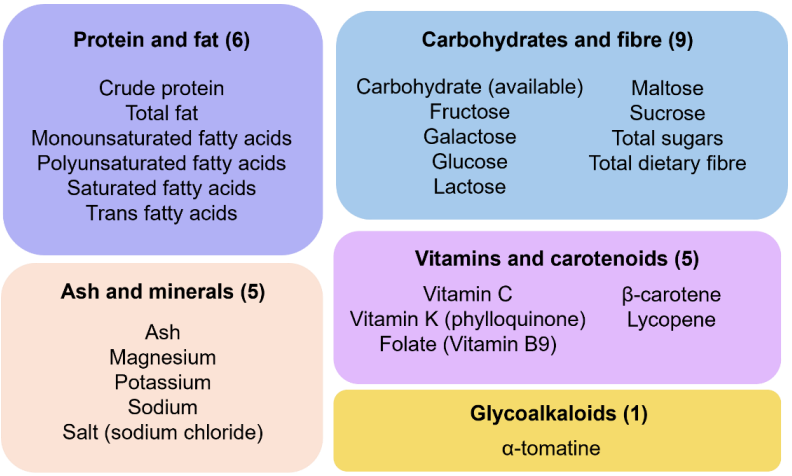

Figure 7. *Analytes measured in Del/Ros1-N purple tomato fruit.*

For most analytes, the observed mean value for Del/Ros1-N fell within the natural variability reported in publicly available literature or other reference ranges (Figure 8). For ash, magnesium and vitamin K (Figure 8b, e, g), the control mean fell slightly below the publicly available range of values. This is likely attributed to the limited sample size (5 plants), stage of ripening and environmental conditions, as well as the specific tomato variety used for this study. The mean value of vitamin K in Del/Ros1-N tomatoes (1.99 µg/100 g; Figure 8g) is also slightly lower than the publicly available range (2.2- 60 µg/100 g). Again, this is likely attributable to limited sample size, stage of ripening and environmental conditions. Given that vitamin K is present in a wide range of foods and the vitamin K content in Del/Ros1-N is minimally lower (0.2 µg/100 g) than the reported range, consumption of Del/Ros1-N is not expected to meaningfully impact vitamin K intakes.

The differences reported here, therefore, are consistent with the normal biological variability that exists in tomato.

5.4 Products and metabolites of the phenylpropanoid biosynthesis pathway

5.4.1 Anthocyanins

The expression of the Del and Ros1 transcription factors in Del/Ros1-N purple tomatoes results in an increase in the level of anthocyanins compared to the non-GM comparator.

Anthocyanins were detected using high-performance liquid chromatography (HPLC) in both peel and flesh of Del/Ros1-N purple tomatoes and the non-GM comparator fruit. Anthocyanins were virtually undetectable in non-GM fruit but averaged 2.83 ± 0.46 mg of anthocyanin per g fresh weight (FW) in hemizygous MicroTom plants containing the Del/Ros1-N event. Other tomato lines containing the Del/Ros1-N event contained lower levels of anthocyanins. For example, Del/Ros1-N purple tomatoes in the MoneyMaker genetic background averaged 0.4 mg anthocyanin per g FW (Butelli et al. 2008). The applicant attributes this variation to the greater amount of water in larger tomato fruit.

The major anthocyanins detected in Del/Ros1-N purple tomatoes were:

[14] The Application dossier can be found on the A1333 webpage – https://www.foodstandards.gov.au/food-standards-code/applications/a1333-food-derived-purple-tomato-lines-containing-event-delros1-n

- Delphinidin 3-(coumaroyl)– rutinoside-5-glucoside (Nasunin),
- Delphinidin 3-(caffeoyl)–rutinoside-5-glucoside,
- Delphinidin 3-(feruloyl)– rutinoside-5-glucoside,
- Petunidin 3-(coumaroyl)–rutinoside-5-glucoside,
- Petunidin 3- (feruloyl)–rutinoside-5-glucoside

as reported by Butelli et al. (2008) and Tohge et al. (2015).

5.4.1 Other metabolites affected as a result of the trait

The upregulation of the anthocyanin biosynthesis pathway (see Section 4.1) could potentially affect the levels of other secondary metabolites involved in or connected to the pathway. The early steps of anthocyanin biosynthesis are common to the general plant phenylpropanoid pathway (see Section 4.1), which is also involved in the synthesis of a number of classes of polyphenolic compounds, including flavonols and lignins (Vogt 2010).

Additional analyses showed the content of a major phenylpropanoid found in Solanaceous species, CGA, was increased in the Del/Ros1-N purple tomato compared to the non-GM control. CGA, which shares the general precursors of the phenylpropanoid pathway with anthocyanins (Clifford et al. 2017), is widely distributed in plants and found at high levels in many foods, including coffee, potato, carrot, apple, strawberry and blueberry (Wang et al. 2022; Su et al. 2025). Some of the highest levels of CGA are found in purple fruits – for example, plums contain 75.9 mg/100g fw; cherries contain 44.7 mg/100g fw[15]. It is also one of the most abundant phenolic compounds in tomato (Clé et al. 2008; D'Orso et al. 2023), though its levels can fluctuate widely depending on ripening stage (Anton et al. 2017), tomato variety (Floare-Avram et al. 2020) and environmental conditions such as exposure to light (Clé et al. 2008). Reported values for CGA in tomatoes range from 1.4 – 3.3 mg/100g fw (Martí et al. 2016).

The average CGA content in Del/Ros1-N purple tomato fruit was reported by the applicant to be 4.6 mg/100g fw, compared to 1.84 mg/100g fw for the control. This difference was attributed to the increased flux through the anthocyanin biosynthesis pathway causing a spillover of flavonoid intermediates, some of which would be directed to CGA synthesis (Zhang et al. 2015; Tohge et al. 2015). Given the much higher levels of CGA found in many commonly consumed foods, the higher level of CGA in Del/Ros1-N purple tomatoes relative to the non-GM control is not a safety concern.

There were no increases in flavonols in the flesh of purple tomatoes. The applicant noted the flavonol rutin was detected by HPLC in the peel of Del/Ros1-N purple tomatoes at a slightly higher level than in the peel of non-GM comparator tomatoes, although these levels were not quantified. As with CGA, rutin levels in tomatoes are highly variable with ripening stage (Anton et al. 2017; Capanoglu et al. 2012), and rutin is known to accumulate in the peel of non-GM tomato varieties (Bovy et al. 2007). As such, this observation, which is based on a small data set, is not biologically meaningful.

5.5 Conclusion

The compositional data indicate there are no biologically meaningful differences in the levels of key constituents in Del/Ros1-N tomatoes when compared with conventional non-GM tomato cultivars already available in agricultural markets. The intended increased levels of anthocyanins and the increase in CGA, a related metabolite, are consistent with what would be expected from the genetic modification (see also Section 6). Apart from the increased levels of anthocyanin and CGA, fruit from Del/Ros1-N purple tomatoes is otherwise compositionally equivalent to fruit from conventional tomato varieties.

[15] Values obtained for CGA from the phenol-explorer database – http://phenol-explorer.eu/contents/polyphenol/467

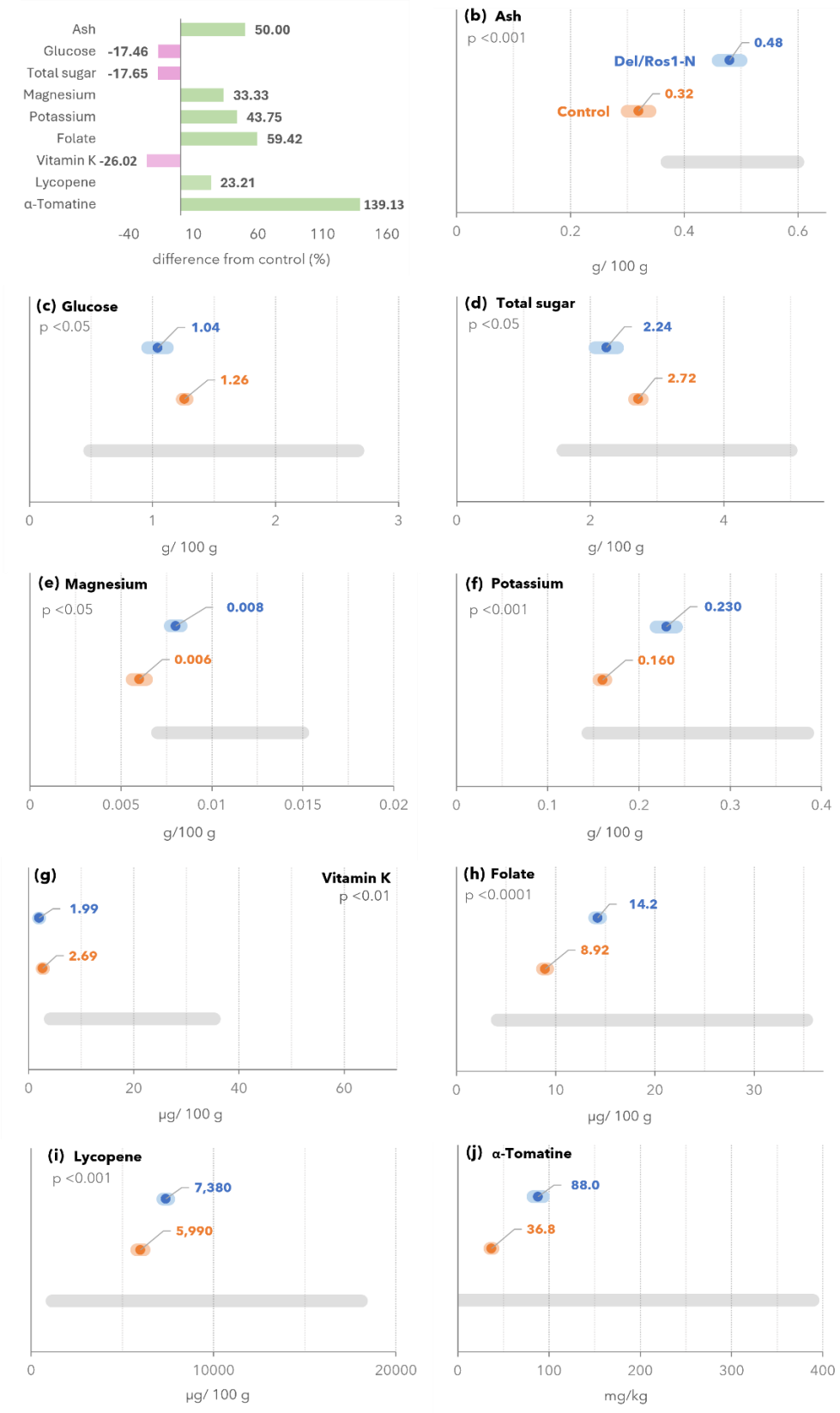

Figure 8. *Visual summary of statistically significant compositional differences between Del/Ros1-N purple tomato and the conventional control tomato. (a) Deviation of the mean Del/Ros1-N value from the mean control value for each of the 9 analytes for which a statistically significant difference was found, expressed as a percentage of the mean control value. (b) – (j) Measured means (dots) ± standard error of means (SEM; coloured bars) for Del/Ros1-N (blue) and the conventional control (orange) for the 9 analytes as labelled. The light grey bars represent the consolidated range of values from the literature and publicly-available databases. 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.

If the compositional analysis indicates biologically meaningful changes in the levels of certain nutrients in the GM food, additional nutritional assessment may assist to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. Evidence indicates that feeding studies using target livestock species will add little to the safety assessment (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014). (Bartholomaeus et al. 2013; Herman and Ekmay 2014; OECD 2003)

In the case of the Del/Ros1-N purple tomato, there is a significant increase in the anthocyanin content of the fruit relative to the non-GM comparator (section 5.4.1). This increase is both expected and intended, due to the nature of the genetic modification. Although there are some commercially available purple-skinned tomatoes, Del/Ros1-N purple tomatoes contain purple pigmentation throughout their skin and flesh and represent a visibly distinct variety compared to other tomatoes on the market.

No reports of adverse effects associated with the consumption of anthocyanins in food have been identified in the scientific literature.

The two major anthocyanins in Del/Ros1-N tomatoes are nasunin and petunidin 3-(coumaroyl)rutinoside-5-glucoside (see Section 5.4.1). Chemically, the anthocyanins in Del/Ros1-N purple tomato are identical to those found in foods including banana, bilberry, blueberry, cranberry, cherry, red grape, raspberries, strawberries, black bean, eggplant, purple potatoes and onion, as well as in the skin of some purple-skinned tomatoes (Azuma et al. 2008; Bhagwat and Haytowitz 2022; Condurache Lazăr et al. 2021; Horbowicz et al. 2008; Lachman et al. 2009; Wu et al. 2006b).

The anthocyanin content of Del/Ros1-N tomatoes is within the ranges present in commonly consumed foods (Figure 9). The increased levels of anthocyanins resulting from upregulation of the anthocyanin biosynthesis pathway are not biologically meaningful, and do not raise any safety concern.

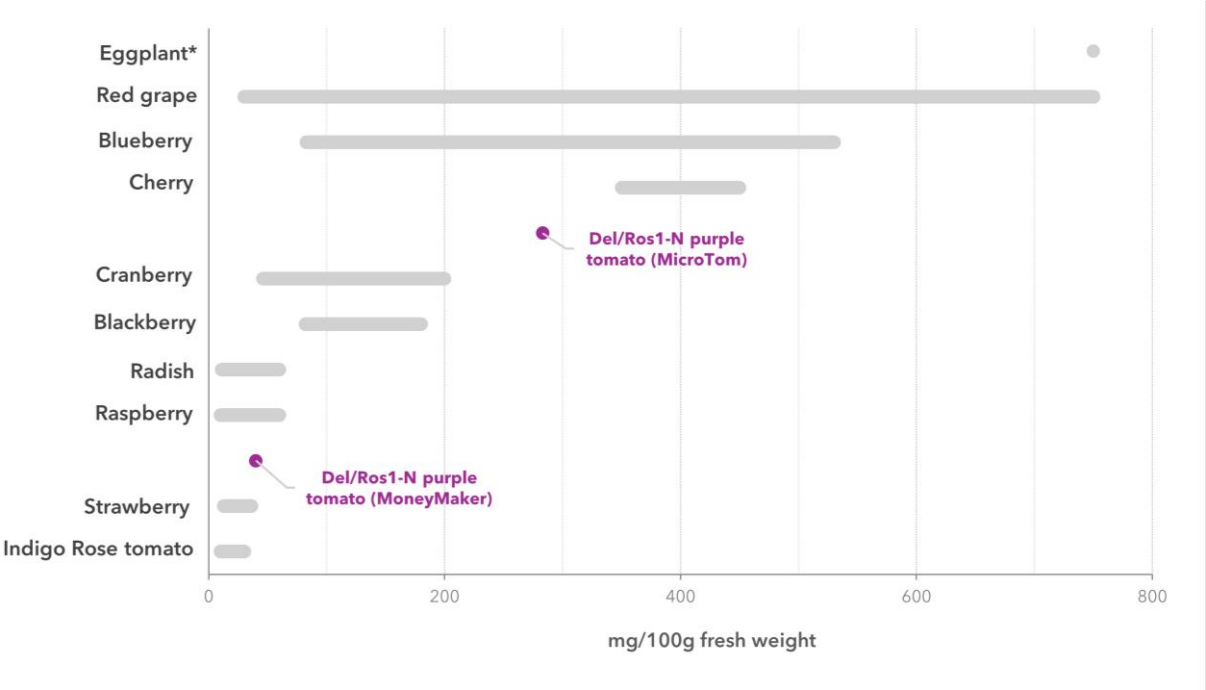

Figure 9. *Anthocyanin content of Del/Ros1-N purple tomatoes (in MicroTom and MoneyMaker genetic backgrounds) compared to the ranges found in other commonly consumed foods. *For eggplant, only a single value, rather than a range, was available. Ranges were provided by the applicant based on a search of the available literature.*

7 Other information

The applicant supplied a summary of a mouse feeding study (from Butelli et al. 2008) as part of the current application. The stated purpose of the feeding study was to investigate whether the levels of anthocyanins in Del/Ros1-N purple tomatoes impacted growth, development or behaviour in a dietary context in laboratory mice. Such feeding studies are not an application handbook requirement, nor considered by FSANZ to be necessary for safety assessment purposes consistent with international regulatory data requirements. However, the study is summarised below as additional supporting information.

WT C57/B16 mice were fed diets supplemented with control (red) non-GM tomato powder or Del/Ros1-N purple tomato powder (10% w/w) and compared to mice fed the standard diet alone (Enriched Standard Diet). Body weight and food consumption were measured twice a week over 11 weeks.

FSANZ notes this study was limited in its scope and is not considered suitable as a toxicity study for regulatory purposes. Any conclusions that can be drawn from this study are therefore limited. The results show that, under the conditions of the study, ingestion of the Del/Ros1-N purple tomato (at 10% w/w in the diet) by WT C57B16 mice did not have a significant effect on body weight or food intake compared to mice ingesting a standard diet.

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Appendix 1

pDEL.ROS-derived genetic elements in T-DNA region

Genetic Element	Description, Source and Reference
Left Border Region	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Zambryski et al. 1982)
***nptII* cassette**	
NOS promoter	Nopaline synthase promoter region from *A. tumefaciens* (Jones et al. 1992)
NPTII coding sequence	Neomycin phosphotransferase gene from transposon Tn5 from *Escherichia coli* that confers kanamycin resistance (van den Elzen et al. 1985)
Ocs 3 terminator	Octopine synthase transcriptional terminator/polyadenylation signal from *A. tumefaciens* (Jones et al. 1992)
***DEL* cassette**	
E8 promoter	Transcriptional promoter from *Solanum lycopersicum* (tomato) activated in the fruit upon ripening (Butelli et al. 2008)
DEL coding sequence	*Delila* cDNA from *Antirrhinum majus* (snapdragon) (Goodrich et al. 1992)
CMV terminator	Cauliflower mosaic virus termination region (Hellens et al. 2000)
***ROS* cassette**	
E8 promoter	Transcriptional promoter from *S. lycopersicum* (tomato) activated in the fruit upon ripening (Butelli et al. 2008)
ROS coding sequence	*Rosea1* cDNA from *A. majus* (snapdragon) (Goodrich et al. 1992)
CMV terminator	Cauliflower mosaic virus termination region (Hellens et al. 2000)
Right Border Region	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Wang et al. 1984)

Appendix 2

Schematic representation of the anthocyanin biosynthesis pathway.

The yellow box encloses flavonols; the purple box encloses anthocyanins. ***PAL****, phenylalanine ammonia lyase;* ***4CL****, 4-coumarate:coenzyme A ligase;* ***C4H****, cinnamate 4-hydroxylase;* ***C3H****, 4-coumarate 3-hydroxylase;* ***CHS****, chalcone synthase;* ***CHI****, chalcone isomerase;* ***F3H****, flavanone-3-hydroxylase;* ***F3'H****, flavonoid-3'-hydroxylase;* ***F3'5'H****, flavonoid-3'5'-hydroxylase;* ***FLS****, flavonol synthase;* ***DFR****, dihydroflavonol reductase;* ***ANS****, anthocyanidin synthase;* ***3-GT****, flavonoid 3-O-glucosyltransferase;* ***RT****, flavonoid 3-O-glucoside-rhamnosyltransferase;* ***AAC****, anthocyanin acyltransferase;* ***5-GT****, flavonoid-5-glucosyltransferase;* ***GST****, glutathione S-transferase;* ***PAT****, putative anthocyanin transporter. Expression of Del and Ros1 results in increased PAL activity and higher total antioxidant capacity.*