Fusarium Wilt Tropical Race 4 Resistant Banana Event QCAV-4

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Application for Amendment to Standard 1.5.2 - Food Produced Using Gene Technology

Submitted to:

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Part I. Application Handbook Section 3.1

General Requirements (3.1.1)

A. Executive summary

Banana event QCAV-4 (QUT-QCAV4-6) was developed through recombinant DNA techniques to express a banana disease resistance (R) gene that confers resistance to the fungal disease, Fusarium wilt tropical race 4 (TR4) also known as Panama Disease TR4. TR4 is a devastating disease of bananas which kills the commercially important Cavendish banana in addition to many other banana cultivars including Lady finger. The disease was first identified in Australia in the Northern Territory in 1997 where it has subsequently decimated commercial banana production. In 2015, the disease was detected in the major banana-growing region of North Queensland. Despite the implementation of strict biosecurity protocols, the disease continues to spread. QCAV-4 is not intended to replace the current Cavendish banana cultivars growing in Australia but rather to provide a safety net to the Australian banana industry should it be heavily impacted by TR4. Its approval for release in Australia is likely to open opportunities for the GM banana to be grown in overseas countries where TR4 is having or has the potential to have a devastating impact on banana production.

QCAV-4 was created by *Agrobacterium tumefaciens*-mediated transformation of banana (*Musa acuminata* subgroup Cavendish cv Grand Nain) embryogenic cells with plasmid pSAN3 resulting in the introduction of the *MamRGA2* disease resistance (R) gene from the wild banana *Musa acuminata* ssp. *malaccensis* and the *neomycin phosphotransferase* II (*npt*II) gene from *Escherichia coli* as a plant selectable marker.

The resistance of QCAV-4 to TR4 was evaluated in two OGTR-approved field trials from 2012-2015 (DIR107) and from 2018-present (DIR146). Both trials were conducted in the Northern Territory on a commercial banana farm with high TR4 disease pressure. In both trials, the disease incidence in QCAV-4 plants was significantly lower than the non-genetically modified (non-GM) Grand Nain control plants. Further, except for TR4 resistance, both QCAV-4 plants and fruit were agronomically and phenotypically indistinguishable from the non-GM Grand Nain control plants and fruit.

Molecular characterisation of the introduced genetic material in event QCAV-4 showed the presence of a large single insert mapped to a region on chromosome 6 of the banana genome. Nucleotide sequencing revealed that the insert is comprised of (i) three complete copies of the intended T-DNA, and (ii) two truncated portions of the *MamRGA2* expression cassette. Bioinformatic analysis revealed that no open reading frames in chromosome 6 were disrupted by the insertion. While seven unintended open reading frames (ORFs) resulted from the insertion, none contained the required regulatory elements necessary for expression of mRNA and protein biosynthesis and this was confirmed by RNA-Seq. Analysis of the predicted amino acid sequences from these new ORFs showed that none had the potential to encode a protein with any significant amino acid sequence similarity to known toxins or allergens. Using Southern blot analysis, the introduced genetic material was shown to be stably inherited over five generations of plants. Further, assessment of transgene expression levels showed that MamRGA2 is providing resistance to TR4 in event QCAV-4 and that the resistance phenotype trait was stable and inherited across multiple generations.

Western blot analysis using a monoclonal mouse anti-MamRGA2 antibody was used to measure the levels of MamRGA2 in fruit and peel tissue collected from QCAV-4 plants, representing the two tissue types with potential pathways of dietary exposure. MamRGA2 protein could not be detected in either fruit or peel tissue (limit of detection: 1-2 ng). Based on the published 2020/21 average Australian annual banana consumption of 16 kg, the maximal exposure to MamRGA2 was therefore calculated to be lower than 8.3 μg/day (assuming 100% of the

Australian Cavendish market was replaced with event QCAV-4). Data on the consumption of banana peel in Australia is unavailable but is considered to be marginal in comparison resulting in even lower exposure to the MamRGA2 protein from QCAV-4 peel consumption.

A "weight-of-evidence" approach was followed to assess potential hazards associated with the MamRGA2 protein expressed in QCAV-4. This assessment considered the (i) intra-species source of the *MamRGA2* transgene, (ii) the ubiquitous presence of highly similar R proteins in other food crops and their history of safe consumption in common food crops including banana, (iii) the lack of significant amino acid sequence similarity with known toxins and allergens, and (4) the rapid digestibility of MamRGA2 in simulated gastric fluid containing pepsin. Based on these considerations, it was concluded that further hazard characterisation by animal toxicity testing was not warranted.

The NPTII amino acid sequence expressed in QCAV-4 is nearly identical (99.6%) to that expressed in several GM events already assessed as safe by regulatory bodies in Australia and overseas. Therefore, its safety assessment was limited to (i) an updated bioinformatics comparison of its amino acid sequence to known protein toxins and allergens and (ii) the detection and quantification of the amount of NPTII protein present in edible parts of QCAV-4. Bioinformatic searches found no similarity of NPTII to known or putative protein toxins and allergens. Western immunoblot analysis using a commercially available NPTII-specific antibody revealed the presence of NPTII in both fruit and peel samples collected from event QCAV-4. Using quantitative enzyme-linked immunosorbent assay (ELISA), the average concentration of NPTII in fresh ripe fruit and peel from QCAV-4 was 3.1 and 4.5 ppm, respectively. Based on the published 2020/21 average Australian annual banana consumption of 16 kg, the human dietary exposure to NPTII was calculated at 49.6 mg per year (or 136 µg/day) (assuming 100% of the Australian Cavendish market was replaced with event QCAV-4). Dietary exposure to NPTII through the consumption of QCAV-4 banana peel was difficult to establish because of the lack of reliable data on the consumption of this tissue in Australia. If consumption of banana peel was similar to fruit (16 kg/per/year), the exposure would be 72 mg per year (or 197 µg/day).

Changes in the composition of food derived from QCAV-4 were considered as part of the "weight-of-evidence" approach to examine if there were unintended consequences of the genetic modification in QCAV-4. The levels of proximates (moisture, fat, protein, ash, carbohydrates, and energy), minerals (magnesium, manganese, potassium), and vitamins (ascorbic acid and vitamin B6) in banana fruit and peel tissue were compared between samples collected from both event QCAV-4 and its non-GM counterpart. While there were some statistical differences in the levels of some of the analytes between QCAV-4 and non-GM control, the mean values for proximates, vitamins, and minerals from fruit and peel were mostly within the compositional variation reported in the literature. Further, no consistent pattern indicated that expression of the *MamRGA2* and *npt*II transgenes impacted the nutritional composition of QCAV-4. We conclude from this analysis that event QCAV-4 is substantially equivalent to conventional Grand Nain banana for the levels of all proximates, vitamins, and minerals reported.

Except for resistance to TR4, the analysis of event QCAV-4 presented in this submission has not revealed any biologically relevant differences to the non-GM counterpart, nor could it identify any health and safety concerns, and supports the conclusion that fruit and peel tissue derived from event QCAV-4 is substantially equivalent and as safe as conventional Grand Nain banana. Collectively, results of the molecular characterisation, agronomic assessment and composition analysis support this application for amendment to the *Australia New Zealand Food Standards Code* to allow inclusion of QCAV-4 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

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(f) Nature of the applicant's business: Higher education and research

(g) Details of other individuals, companies or organizations associated with the application:

C. Purpose of the application

This application seeks to amend the *Australia New Zealand Food Standards Code* to allow for the inclusion of banana event QCAV-4 in **Standard 1.5.2**-*Food Produced Using Gene Technology*.

QUT has developed and field tested a Grand Nain banana event that expresses a wild banana gene that confers resistance against the devastating fungal disease known as Fusarium wilt tropical race 4 (TR4). The banana event described in this application has the unique OECD code QUT-QCAV4-6 and is referred to as QCAV-4 in this submission. Should the Australian banana industry become heavily impacted by TR4, QCAV-4 would provide a safety net for banana growers and consumers.

D. Justification for the application

QUT has developed QCAV-4, a new banana event created using genetic engineering techniques and containing the *MamRGA2* gene from a wild diploid banana (*Musa acuminata* ssp. *malaccensis*) and the *neomycin phosphotransferase* II (*npt*II) gene from *Escherichia coli* as a plant selectable marker. *MamRGA2* is a disease resistance (R) gene which, when transferred to Cavendish bananas, confers resistance to the disease known as Fusarium wilt TR4.

D.1. Costs and benefits

Bananas are Australia's largest horticultural industry and highest selling supermarket product with more than five million bananas eaten every day (https://abgc.org.au/our-industry). The sweet dessert Cavendish banana is the most popular banana in Australia and accounts for around 97% of production. During 2020/21, 95% of Australian households purchased bananas with an average yearly per capita consumption of 16 kg (Hort Innovation, 2022). Although mainly consumed as a fresh fruit, some bananas are fried as banana chips, used in smoothies and for baking. Green (starchy, unripe) bananas have also been used to make banana flour for use in baking and in smoothies. There are also some instances where the banana peel has been used in baking and to make smoothies. The vast majority of the country's banana production is located in the north Queensland regions of Kennedy, Tully, Innisfail, Atherton Tableland, Mossman, Lakeland and Hopevale (https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/crop-growing/banana-industry). In November 2018, Pinnacle Agribusiness released the Banana Enterprise Performance Comparison

(BA16009), funded by Hort Innovation (Hort Innovation, 2018). This report stated that the farm gate value from the 2016/17 production of 414,000 tonnes of fresh bananas was $679 million which delivered a $1.3 billion contribution to the economy. The same study also showed the industry supported more than 18,000 full-time and part-time jobs (including supply chain) in Australia and is the major economic driver and employer in Tully, Innisfail, Mareeba, Kennedy, Lakeland and Carnarvon. According to the Hort Innovation Banana Strategic Investment Plan 2022-2026, during 2019/2020, 381,676 tonnes of bananas were produced with a farmgate value of $596.2 million (Hort Innovation, 2021).

Fusarium wilt TR4 is a devastating disease of bananas caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cubense*. TR4 kills the commercially important Cavendish in addition to many other banana cultivars including Lady finger. The disease was thought to have originated in Southeast Asia but was first identified in Australia in the Northern Territory (NT) in 1997. Since that time, it has spread rapidly throughout the banana growing regions of the NT to the point where there is now only one large commercial plantation in operation. In 2015, the disease was detected in North Queensland where over 90% of Australia's bananas are grown. Despite the implementation of strict but expensive biosecurity protocols, the disease continues to spread. There are no long-term effective control strategies for TR4 and no consumer-acceptable resistant banana cultivars available. In nature, wild bananas with small bunches bearing fruit with large, hard seeds have been found that are resistant to TR4. We have identified an R gene (*MamRGA2*) in one such wild banana (*Musa acuminata* ssp. *malaccensis*) and have used modern biotechnology tools to transfer this gene from this wild banana to the commercially cultivated Cavendish banana cultivar, Grand Nain to create QCAV-4. The availability of a TR4 resistant, consumer acceptable banana cultivar such as QCAV-4 should significantly reduce the economic, social, and environmental impacts of this disease as outlined below.

Economic

Fusarium wilt TR4 is already having a dramatic economic impact on the Australian banana industry. The Northern Territory commercial banana industry has nearly collapsed as a direct result of TR4. With the arrival of TR4 into north Queensland in 2015, there have been numerous very significant negative economic impacts. These include:

- the impact on the farms of growers where TR4 has been recorded. This has, in one instance, resulted in the shutting down of the entire farm and, in other instances, the quarantining of significant areas and the implementation of extensive physical biosecurity measures.
- the impact on growers whose farms are currently not affected. This includes the very significant cost of implementing the extensive biosecurity measures.
- the impact on the Australian Banana Growers Council (ABGC) where a large component of the ABGC levy funds collected from growers has been diverted to TR4 control, management, and education through the Queensland Government & ABGC jointly funded TR4 Program in place through to 2023.
- the impact on the Queensland and Australian Governments with the commitment of significant funds to control and manage TR4.

It is estimated collectively that the cost of TR4 to the Australian industry will be approximately $138 million per year (Cook *et al*., 2015). The availability of a TR4 resistant banana cultivar should significantly reduce this impact and the financial quantum.

Social

The banana industry is a major horticulture industry in Australia and particularly in the Tully Valley, Innisfail and Atherton areas. The industry is essentially based on family-owned and operated farms with extensive additional local employment. The arrival of TR4 has had a severe impact on these enterprises beyond the economic impact.

Naturally, there has been understandable concern about the future of the industry and the viability of the family farms and enterprises. This has been further exacerbated by the imposition of the extensive biosecurity protocols which have resulted in greater isolation of farms and growers.

The availability of a TR4 resistant Cavendish banana should have a major positive influence regarding concerns about the viability of the industry and of individual farms. Further, while certain biosecurity measures should permanently remain in place as best practice, the availability of a TR4 resistant cultivar provides the opportunity to relax the more extreme measures.

Environmental

One of the negative impacts of the biosecurity measures implemented to control TR4 on banana farms is the use of chemical disinfectants. The availability and adoption of a TR4 resistant cultivar by farmers should result in a reduction in the use of these environmentally-unfriendly disinfectants.

D.2. Impact on international trade

According to the Hort Innovation Banana Industry Export Market Development Strategy 2023 (Hort Innovation, 2023), almost all the bananas produced in Australia are grown for domestic consumption with only limited export sales. Relative to the volume of national banana production of around 380,000 tonnes in 2019/2020, banana exports have been minimal and opportunistic. In many years, there have been virtually no recorded exports. Total exports in the 2016/17 year were 138 tonnes or 0.04 per cent of Australia's total production. The current trade is largely sporadic. Australian exporters are usually filling short-term market gaps when there is a market supply failure from other countries. Considering the small size of the Australian banana industry on a global scale, the approval of QCAV-4 bananas for human consumption in Australia is likely to have no impact on international trade.

E. Information to support the application

This application consists of 2 parts containing information in accordance with the following checklists:
Part I: General requirements (3.1.1)
Part II: Foods produced using gene technology (3.5.1) main document, Part 2 information. Supplement form molecular analysis.

F. Assessment procedure

QUT is anticipating that this application will be considered under the General Procedure for Administrative Assessment process by Food Standards Australia New Zealand.

G. Confidential commercial information (CCI)

CCI has not been included in this submission document.

Release of Information
QUT is submitting the information in this application for review by Food Standards Australia New Zealand (FSANZ) for amendment to the Food Standard 1.5.2 - Food Produced Using Gene Technology. QUT holds proprietary rights to the extent allowable by law to all such information and, by submitting this information, QUT does not authorise its release to any third party except to the extent it is duly requested under the Freedom of Information Act 1982 (FOI Act) or in compliance with the responsibility of FSANZ to publish documents required under Sections 8, 8(A), 8(C) and 8(D) of the FOI Act; and this information is responsive to the specific aforementioned

request. Accordingly, except as specifically stated above, QUT does not authorise the release, publication or other distribution of this information (including website posting or otherwise), nor does QUT authorise any third party to use, obtain, or rely upon this information, directly or indirectly, as part of any other application or for any other use, without QUT's prior notice and written consent. Submission of this information does not in in any way waive QUT's rights (including rights to exclusivity and compensation) to such information.

H. Other confidential information

No additional confidential material is included in this submission document.

I. Exclusive capturable commercial benefit (ECCB)

QUT commenced this project nearly 20 years ago through an ARC Discovery Project which led to the identification of the RGA2 gene from *Musa acuminata* ssp. *malaccensis* as a possible TR4 resistance gene and the subsequent transfer of this gene into Cavendish. The next phase involved field trialling of the GM lines in the Northern Territory which involved an ARC Linkage Project supported by an industry partner. The third phase involved a greatly expanded field trial in the Northern Territory through the support of a CRC-P Project. The development of QCAV-4 has been strongly supported by industry with the expectation that QUT would develop and make available a TR4 resistant Cavendish banana. This application is the culmination of those 20 years of development and delivering on the expectation of a TR4 resistant Cavendish. The major outcome of approval of this application will be the opportunity for Australian banana growers to produce Cavendish bananas in Australia with minimal threat from TR4.

QUT will benefit from the approval of the application in a number of important ways:

· QUT will have delivered on its commitment to develop and commercialise a TR4 resistant Cavendish banana for Australian banana growers
· QUT is likely to receive a royalty stream from the production and sale of QCAV-4 fruit in Australia
· It is possible that, with approval in Australia, QCAV-4 will garner greater interest internationally as a strategy to continue to produce Cavendish in regions affected by TR4.

The greatest benefit from the approval of this application will accrue to Australian banana growers. QUT ultimately intends to make QCAV-4 available to all commercial banana growers in Australia. TR4 is already widespread in the Northern Territory where it is now considered endemic. Commercial production of Cavendish bananas in the Northern Territory is already very significantly impacted by TR4 and therefore, in the short term, growers in that region would derive most benefit from adopting QCAV-4. However, TR4 is present and spreading, be it slowly, in north Queensland. It is therefore possible that banana growers in that region will, in the future, also adopt QCAV-4 with perhaps those already affected being early adopters.

QUT is already committed to providing non-exclusive licenses for the commercial exploitation of QCAV-4 in Australia to a number of other industry groups. QUT will endeavor to make QCAV-4 available to other groups through likely non-exclusive licenses to Australian banana micropropagation operations. It is likely that QUT will receive a royalty stream from the commercial exploitation of QCAV-4.

QUT owns the intellectual property rights to QCAV-4.

J. International and other National Standards

An application has been submitted to the Australian Office of the Gene Technology Regulator (OGTR) for a licence

for a dealing involving intentional release (DIR) of QCAV-4 plants into the environment – commercial release.

The Codex Alimentarius Commission Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants (CAC/GL 45-2003) is applicable to the assessment of this application to amend the Australia New Zealand Food Standards Code to allow for the inclusion of event QCAV-4 in Standard 1.5.2 - Food Produced Using Gene Technology.

K. Statutory declaration

Statutory Declaration Act 1959

[redacted]

make the following declaration under the *Statutory Declaration Act 1959*:

1. the information provided in this application fully sets out the matters required
2. the information provided in this application is true to the best of my knowledge and belief
3. no information has been withheld that might prejudice this application, to the best of my knowledge and belief

I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the *Statutory Declaration Act 1959*, and I believe that the statements in this declaration are true in every particular.

[redacted]

JUSTICE OF THE PEACE (QUALIFIED) QUEENSLAND GOVERNMENT Reg.No.: 129101 DEPT. OF JUSTICE & ATTORNEY-GENERAL

L. Checklists provided with the application

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory requirements**
☒	1	**A Form of application** ☒ Application in English ☒ Executive Summary (separated from main application electronically) ☒ Relevant sections of Part 3 clearly identified ☒ Pages sequentially numbered ☒ Electronic copy (searchable) ☒ All references provided
☒	3	**B Applicant details**
☒	3	**C Purpose of the application**
☒	3	**D Justification for the application** ☒ Regulatory impact information ☒ Impact on international trade
☒	5	**E Information to support the application** ☒ Data requirements
☒	5	**F Assessment procedure** ☒ General ☐ Major ☐ Minor ☐ High level health claim variation
☒	5	**G Confidential commercial information** ☐ CCI material separated from other application material ☐ Formal request including reasons ☐ Non-confidential summary provided
☒	6	**H Other confidential information** ☐ Confidential material separated from other application material ☐ Formal request including reasons
☒	6	**I Exclusive Capturable Commercial Benefit** ☒ Justification provided
☒	7	**J International and other national standards** ☒ International standards ☒ Other national standards
☒	7	**K Statutory Declaration**
☒	8	**L Checklist/s provided with application** ☒ 3.1.1 Checklist ☒ All page number references from application included ☒ Any other relevant checklists for Chapters 3.2-3.7

Foods produced using gene technology (3.5.1)		
Check	**Page No.**	**Mandatory requirements**
☒	16	A.1 Nature and identity
☒	16	A.2 History of use of host and donor organisms
☒	18	A.3 Nature of genetic modification
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☒	35	B.2 New proteins
☐	N/A	B.3 Other (non-protein) new substances
☐	N/A	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
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☐	N/A	D Other information

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Abbreviations, acronyms and definitions

Abbreviation	Definition
AI	Adequate intake
AP	Alkaline phosphatase
A. tumefaciens	*Agrobacterium tumefaciens*
APH(3')II	amino-glycoside-3'-phosphotransferase II
AUG	Start codon
BLAST	Basic Local Alignment Search Tool
BMP	Best management practice
bp	Base pair
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
CaMV35S	35S promoter from CaMV
cat	Chloramphenicol acetyl transferase gene
CC	Coiled-coil domain
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DW	Dry weight
EAR	Estimated average requirement
E. coli	*Escherichia coli*
ECS	Embryogenic cell suspension
ELISA	Enzyme-linked immunosorbent assay
EPA	United States Environmental Protection Agency
ETI	Effector-triggered immunity
FARRP	Food Allergy Research and Resource Program at the University of Nebraska-Lincoln
FDA	United States Food and Drug Administration
FSANZ	Food Standards Australia New Zealand
FW	Fresh weight
GM	Genetically modified
GN212-12	Wild-type Grand Nain control banana cell line
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPF	Insoluble protein fraction
kb	Kilobase
LB	Left border
LOD	Limit of detection
LOQ	Limit of quantification
LRR	Leucine-rich repeat domain
Ma	*Musa acuminata*
Mam	*Musa acuminata* ssp. *malaccensis*
MamRGA2	*RGA2* gene from *Musa acuminata* ssp. *malaccensis*
MaRGA2	Endogenous *RGA2* homolog present in *Musa acuminata*
NBS	Nucleotide-binding site
NBS-LRR	Nucleotide binding site/leucine rich repeat

NGS	Next generation sequencing
NIASA	Nursery Industry Accreditation Scheme Australia
NLR	CC-NBS-LRR type plant resistance protein
Nos	Nopaline synthase gene
*npt*II	*Neomycin phosphotransferase* II gene
NPTII	Neomycin phosphotransferase II protein
OECD	Organisation for Economic Cooperation and Development
OGTR	Office of the Gene Technology Regulator
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PE	Paired-end
PPB	Parts per billion
PPM	Parts per million
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
PTM	Post-translational modification
QBAN	Quality Banana Approved Nursery
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
QUT-QCAV4-6	OECD identifier for QCAV-4
RB	Right border
RDI	Recommended daily intake
RGA2	Resistance gene analogue 2 isolated from *Mam*
RNA	Ribonucleic acid
R gene	Resistance gene
R protein	Resistance protein
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid
SPF	Soluble protein fraction
TC	Tissue culture
TIR	Toll/interleukin receptor
TR4	Fusarium wilt tropical race 4
TSP	Total soluble protein
USDA-APHIS	United States Department of Agriculture-Animal and Plant Health Inspection
UTR	Untranslated region
WT	Wild-type

Study reports submitted

Study report ID	Study report title
QUT2023-1	Nucleotide sequence analysis of the inserted DNA and host genomic flanking regions in event QCAV-4
QUT2023-2	Stability of the DNA insert in banana event QCAV-4 across multiple generations
QUT2023-3	Field performance of banana event QCAV-4
QUT2023-4	Concentrations of MamRGA2 and NPTII proteins in fruit and peel tissues from event QCAV-4 and dietary exposure estimations
QUT2023-5	Differential messenger RNA (mRNA) expression of *MamRGA2*, *nptII* and the endogenous *MaRGA2* in event QCAV-4
QUT2023-6	Safety assessment of the seven new ORFs identified in event QCAV-4
QUT2023-7	*In silico* assessment of MamRGA2 and NPTII for allergenicity and toxicity
QUT2023-8	Characterisation of the MamRGA2 protein derived from an E. coli expression system and its equivalence to MamRGA2 expressed in event QCAV-4
QUT2023-9	*In vitro* assessment of the MamRGA2 protein lability to gastric enzymatic digestion and its thermal stability
QUT2023-10	Nutrient composition of fruit and peel tissues harvested from banana event QCAV-4 and non-GM Grand Nain control GN212-12

Part II. Application Handbook Section 3.5.1

The following information is provided to support an application for a new genetically modified food. The details presented are in accordance with Section 3.5.1. of the FSANZ Application Handbook as of the 1st of July 2019.

A. Technical information on the food produced using gene technology

A.1. Nature and identity of the GM food

A.1(a) Description of the GM organism from which the new GM food is derived

QCAV-4 is a line of genetically modified banana (*Musa acuminata* subgroup Cavendish cv Grand Nain) with resistance to the disease Fusarium wilt tropical race 4 (TR4) caused by the fungal pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4. QCAV-4 was created using *Agrobacterium*-mediated transformation of a banana embryogenic cell suspension (ECS) with plasmid vector pSAN3. The vector pSAN3 contains two expression cassettes, one for expression of the *npt*II plant selectable marker gene and one for expression of the banana disease resistance gene *MamRGA2* to confer resistance to TR4.

A.1(b) Name, line number and OECD unique identifier

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants", QCAV-4 has been provisionally assigned the unique identifier QUT-QCAV4-6.

A.1(c) Name the food will be marketed under

A trade name for the banana fruit harvested from QCAV-4 has not yet been selected.

A.2. History of use of the host and donor organisms

A.2(a) Donor organisms from which the genetic elements are derived

The donor DNA in the insert of QCAV-4 consists of both coding and non-coding genetic elements from plasmid pSAN3. The following section relates to any known pathogenicity, toxicity or allergenicity of relevance to the food.

A.2(a)(i) Musa acuminata ssp. malaccensis

Musa acuminata ssp*. malaccensis* (Mam) was the source of the *MamRGA2* transgene (Peraza-Echeverria *et al*., 2008, 2009). Mam is a wild seeded diploid banana that does not occur naturally in Australia and is not grown commercially due to the lack of edible flesh in the fruit and the presence of numerous hard seeds (Figure 1). It is grown in some germplasm collections in Australia for conservation purposes and is used as a parent in overseas breeding programs to introgress disease resistance traits into new cultivars. Mam is one of five banana subspecies that are known to have prominent roles in the domestication of bananas, many of which are widely consumed today (Sardos *et al.,* 2022). It is the foundation maternal parent of Cavendish based on chloroplast genome analysis (Dale *et al*., unpublished data).

Figure 1. Representative picture of Cavendish (top) and Mam (bottom) fruit.

Taxonomy
ORDER: Zingiberales
FAMILY: Musaceae
GENUS: *Musa*
SPECIES: *Musa acuminata* ssp. *malaccensis*
COMMON NAME: wild banana, malaccensis

A.2(a)(ii) Escherichia coli

Escherichia coli (family *Enterobacteriaceae*) non-pathogenic strain K12 was the source of the *npt*II gene (Beck *et al.,* 1982). *E. coli* is a facultative anaerobic bacterium, with most strains harmlessly colonising the gastrointestinal tract of humans and animals as common microbiota. However, some strains occur which are pathogenic and can cause colitis, urinary infections, or food poisoning (Braz *et al.*, 2020). None of the genetic elements derived from *E. coli* that are present in the pSAN3 transformation vector are associated with causing disease.

Taxonomy
ORDER: Enterobacteriales
FAMILY: Enterobacteriaceae
GENUS: *Escherichia*
SPECIES: *E. coli*
STRAIN: K-12

A.2(a)(iii) Other donor organisms

The well-known plant pathogens Cauliflower mosaic virus (CaMV) and *Agrobacterium tumefaciens* were used as sources of gene regulatory sequences. These sequences include the promoter and 3' untranslated region (UTR) derived from the 35S RNA of CaMV (CaMV35S) as well as the nopaline synthase (Nos) promoter and 3' UTR region from *A. tumefaciens*. CaMV predominantly infects members of the *Brassicaceae* family although some variants infect some solanaceous plants (Bak and Emerson, 2020). *A. tumefaciens* is a common soil-borne bacterium with a wide host range of plant species (Nester, 2015). Since none of these regulatory sequences encode proteins and none of the source organisms infect humans, they are of little relevance to assessing toxicity or allergenicity.

A.2(b) Description of the host organism into which the genes were transferred

Taxonomy
ORDER: Zingiberales
FAMILY: Musaceae
GENUS: *Musa*
SPECIES: *Musa acuminata*
COMMON NAME: Banana, Cavendish, Grand Nain

A.2(b)(i) Its history of safe use for food

Based on archeological and linguistic studies, cultivated bananas were believed to be initially domesticated by farmers in Southeast Asia about 7,000 years ago, and subsequently introduced into other regions of the world by transmigrants or travelers (Heslop-Harrison and Schwarzacher, 2007; Perrier *et al*., 2011; Simmonds and Shepherd, 1955). Bananas are now a major food crop globally and are grown and consumed in more than 100 countries throughout the tropics and sub-tropics. Domesticated bananas are cultivated mainly for their edible fruit and are not known to cause disease or show toxicity in humans or animals and have a long history of safe use as a food. In rare cases, some susceptible individuals are allergic to bananas, which is almost always associated with an allergy to latex. Worldwide, however, the prevalence of allergy to the consumption of banana amongst the population is uncommon (0.04-1.2%) (Suriyamoorthy *et al*., 2022).

A.2(b)(ii) The parts of the organism typically used as food

In Australia, the fruit is the most commonly consumed part of the banana plant, with dessert bananas the number one selling supermarket product with more than five million bananas eaten daily. The banana peel and flower are also sometimes eaten in Australia, but to a much lesser extent than the fresh fruit. In other countries, parts of the banana plant that are also eaten include the corm, pseudostem and leaves (Pereira and Maraschin, 2015).

A.2(b)(iii) The types of products likely to include the food or food ingredient

Although mainly consumed as a fresh fruit, some bananas can be (i) cooked (eg. fried, dehydrated, baked, steamed, boiled), (ii) processed into flour, and (iii) used in smoothies. Although less common, there are also some instances where the banana peel has been used for baking and to make smoothies. Commercially available products containing banana include banana chips (through frying), dried banana (after dehydration), banana bread and frozen banana.

A.2(b)(iv) Special processing is required to render food derived from the organism safe to eat

There is no special processing required to render food derived from banana plants safe to eat.

A.3. The nature of the genetic modification

A.3(a) Description of the method used to transform the host organism

Event QCAV-4 banana plants (*Musa acuminata* subgroup Cavendish cv Grand Nain) were generated by centrifugation-assisted *Agrobacterium*-mediated transformation (Khanna *et al*., 2004) using plasmid pSAN3. Briefly, embryogenic cell suspensions (ECS) were generated from immature male flowers and transformed using *Agrobacterium tumefaciens* strain AGL1. Following transformation, cells were layered on glass fibre filter disks and maintained on BL solid media (25-100 mg/L kanamycin; 200 mg/L timentin) for 3 months with monthly sub-culturing. During this 3-month period, the kanamycin concentration was sequentially increased from 25 mg/L

(month 1) to 50 mg/L (month 2) and finally 100 mg/L (month 3). Pre-embryogenic cells on filter disks were then transferred to solid M3 media (100 mg/L kanamycin; 200 mg/L timentin) for 3 months with monthly sub-culturing. Plants were regenerated from embryos in M4 media and rooted in MS media (both containing 100 mg/L kanamycin and 200 mg/L timentin). All tissue culture media constituents and other experimental parameters are described in Khanna *et al*. (2004). The GM banana plants were subsequently multiplied by micropropagation and acclimatised to soil. The presence or absence of residual *Agrobacterium* in GM plants was tested by PCR using primers specially designed to amplify a 738 bp region of the *Agrobacterium* VirC operon (Haas *et al*., 1995).

A.3(b) Description of the potentially introduced genetic material

Event QCAV-4 was created using *Agrobacterium*-mediated transformation of a banana ECS with plasmid pSAN3 (Figure 2). pSAN3 was assembled in the pCAMBIA-2200 (Hajdukiewicz *et al*., 1994) backbone by directional cloning and contains two expression cassettes (Table 1). Cassette 1, already present in pCAMBIA-2200 allows the expression of the *npt*II (accession #AAF65391/AF234313) plant selectable marker gene (Beck *et al.,* 1982) and is controlled by upstream and downstream CaMV35S regulatory elements. Cassette 2 allows the expression of the *MamRGA2* (accession #EU616673) gene derived from *M. acuminata* ssp*. malaccensis* (Peraza-Echeverria *et al.,* 2008, 2009) which confers resistance to Fusarium wilt tropical race 4 (TR4) and is under the control of the 5' and 3' *Agrobacterium* nopaline synthase (Nos) regulatory elements. Following the creation of pSAN3, its entire sequence of 13,084 bp was confirmed by Illumina next generation sequencing (NGS) and compared to the available sequence of pCAMBIA-2200. A total of 18 minor nucleotide changes were observed between pSAN3 and pCAMBIA-2200 (Table 2). These changes could have resulted from the cloning of Cassette 2 into pCAMBIA-2200 or may have already been present in the original pCAMBIA-2200 vector. Importantly, 13 out of the 18 reported changes (shaded light green) were outside the T-DNA region of pSAN3 and therefore not expected to be integrated in event QCAV-4. Of these 13 changes, one T to C substitution occurred in the ORF of the chloramphenicol acetyl transferase (cat) bacterial selectable marker gene resulting in a N51S substitution. The growth of recombinant pSAN3 *E. coli* and *Agrobacterium tumefaciens* was not affected by this change. The remaining 12 minor changes were in non-coding regions of the pCAMBIA-2200 vector backbone.

Five nucleotide changes were identified within the T-DNA of pSAN3. One G to A substitution occurred within the *npt*II ORF and did not result in any amino acid change. Two changes occurred in the CaMV35S promoter controlling *npt*II and did not appear to result in a change of expression considering the growth of QCAV-4 on kanamycin-containing selection media. The remaining two changes were two inconsequential deletions in the multiple cloning site (MCS - intervening sequence) of pCAMBIA-2200. Importantly, the sequence of *MamRGA2* in pSAN3 was identical to the original *MamRGA2* sequence (EU616673).

A.3(c) Molecular characterisation of the genetic modification in event QCAV-4

The molecular characterisation of event QCAV-4 was performed using a range of traditional and modern molecular and sequencing techniques. The absence of plasmid backbone sequence integrations was initially investigated by PCR. Southern blot analyses (Southern, 1975) were then used initially to investigate the number of T-DNA insert copies in the genome of QCAV-4 and subsequently to investigate the multi-generational stability of the introduced genetic material. Finally, next generation whole genome sequencing (Illumina and PacBio) and bioinformatic analysis were used to determine the nucleotide sequence of the entire insert in QCAV-4 along with its 5' and 3' flanking genomic sequences, examine the integrity of the inserted genetic material and confirm the absence of plasmid backbone integration in QCAV-4.

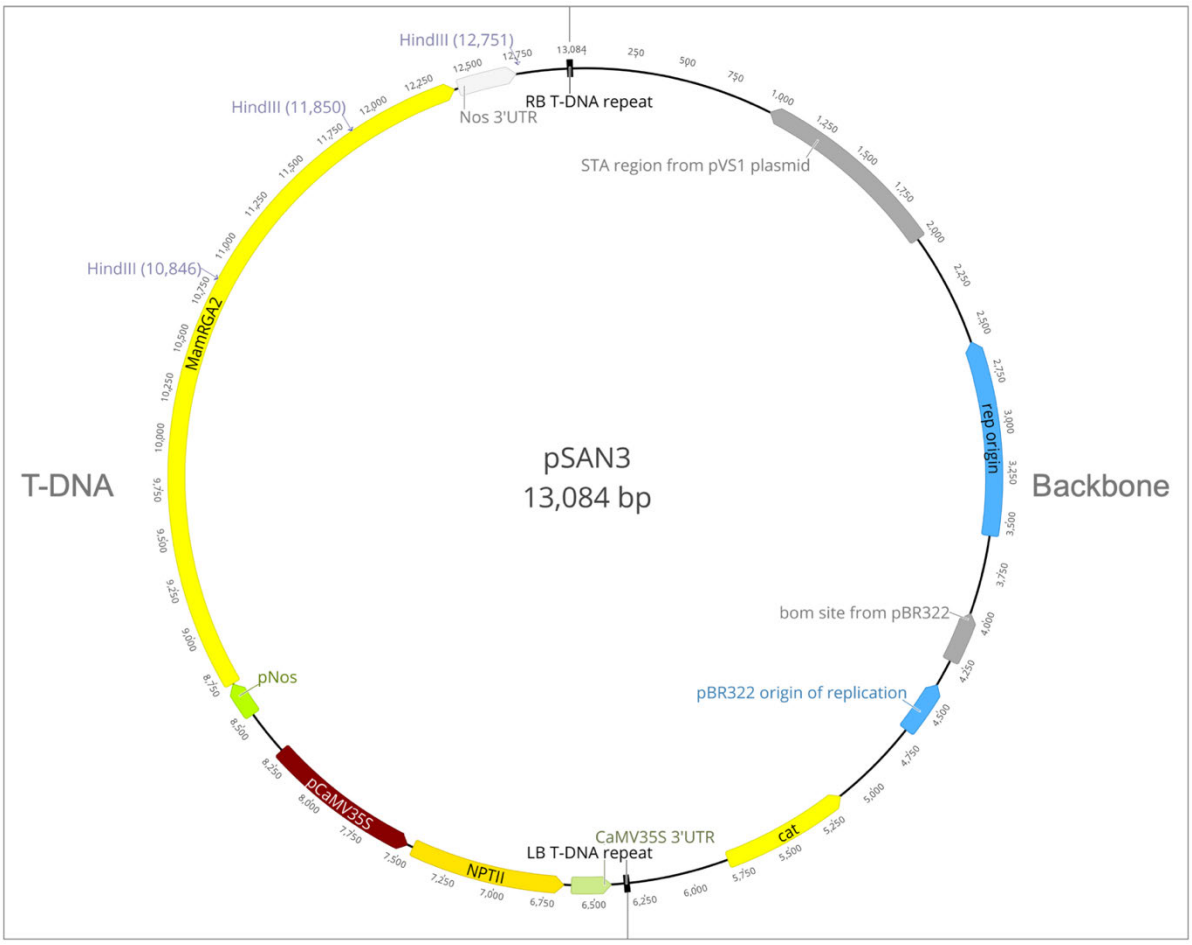

Figure 2. Plasmid map of pSAN3.

Table 1. Genetic elements contained on the T-DNA of pSAN3 from left border (LB) to right border (RB)

Expression cassette	Genetic element	Position on pSAN3	Size (bp)	Origin	Intended function	Accession number	Reference
	LB T-DNA repeat	1-26	26	Nopaline Ti-plasmid pTiC58 of *Agrobacterium tumefaciens* C58 and contained on pCAMBIA-2200	Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the genome of the host plant	AF234313	Holsters *et al.,* 1980; Hajdukiewicz *et al.,* 1994
	Ti plasmid LB proximal sequence	27-92	66	pTiC58 and contained on pCAMBIA-2200	Buffer for truncations during insertion	AF234313	Hajdukiewicz *et al.,* 1994
NPTII	CaMV35S 3' UTR	93 -296	204	3' UTR sequences derived from the 35S RNA of Cauliflower mosaic virus (CaMV)	Transcription termination and polyadenylation of the *npt*II gene	AF234313	Guerineau *et al.,* 1988
	Intervening sequence	297-333	37	pCAMBIA-2200	Sequence used for DNA cloning	AF234313	Hajdukiewicz *et al.,* 1994
	*npt*II-coding sequence	334-1,131	798	Transposon Tn5 of *Escherichia coli* strain K12 amplified from pIG121Hm	Encodes the neomycin phosphotransferase type II (NPTII) protein providing resistance to kanamycin	AAF65391/AAA85506	Beck *et al.,* 1982
	Intervening sequence	1,132-1,161	30	pCAMBIA-2200	Sequence used for DNA cloning	AF234313	Hajdukiewicz *et al.,* 1994
	CaMV35S promoter	1,162-1,969	808	Promoter sequences derived from the 35S RNA of CaMV	Drives transcription of downstream *npt*II gene	AF234313	Odell *et al.,* 1985
	Intervening sequence	1,970-2,201	232	pCAMBIA-2200	Sequence used for DNA cloning	AF234313	Hajdukiewicz *et al.,* 1994
MamRGA2	Nos promoter	2,202-2,385	184	Promoter sequences derived from the *A. tumefaciens* nopaline synthase gene	Drives transcription of downstream *MamRGA2* gene		Bevan *et al.,* 1983
	Intervening sequence	2,386-2,391	6		Sequence used for DNA cloning		
	MamRGA2-coding sequence	2,392-6,090	3,699	Banana (*Musa acuminata* ssp. *malaccensis accession 850*)	Encodes the NBS-LRR MamRGA2 protein for *Fusarium oxysporum* f. sp. *cubense* tropical race 4 resistance	EU616673/ACF21694	Peraza-Echeverria *et al.,* 2008, 2009
	Intervening sequence	6,091-6,109	19		Sequence used for DNA cloning		
	Nos 3' UTR	6,110-6,411	302	3' UTR sequences derived from the *A. tumefaciens* nopaline synthase gene	Transcription termination and polyadenylation of the *MamRGA2* gene		Bevan *et al.,* 1983; Depicker *et al.,* 1982
	Ti plasmid RB proximal sequence	6,412-6,676	265	pTiC58 and contained on pCAMBIA-2200	Buffer for truncations during insertion	AF234313	Hajdukiewicz *et al.,* 1994
	RB T-DNA repeat	6,677-6,702	26	Nopaline Ti-plasmid pTiC58 of *Agrobacterium tumefaciens* C58 and contained on pCAMBIA-2200	Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the genome of the host plant	AF234313	Holsters *et al.,* 1980

Table 2. Nucleotide sequence changes occurring in the creation of pSAN3

pSAN3 element	pCAMBIA-2200			pSAN3	
	Position	Change	Old	Position	New
Backbone	2,228	Substitution	T	2,228	C
	2,280/2,281	Insertion	-	2,281	G
	5,054	Deletion	T	-	-
	5,067	Deletion	C	-	-
	5,071/5,072	Insertion	-	5,071-5,074	CGCC
chloramphenicol acetyl transferase (*cat*)	5,646	Substitution	T	5,649	C
Backbone	5,933	Deletion	T	-	-
	5,942-5,944	Deletion	AAG	-	-
	6,015	Substitution	C	6,014	G
	6,016	Substitution	G	6,015	C
	6,020-6,021	Deletion	GT	-	-
	6,055	Deletion	G	-	-
*npt*II	7,342	Substitution	G	7,338	A
pCaMV35S	7,565/7,566	Insertion	-	7,562	G
	7,942-7,943	Deletion	TA	-	-
Intervening sequence	8,500	Deletion	C	-	-
	8,528	Deletion	C	-	-
Backbone	8,842/8,843	Insertion	-	13,032	T

A.3(c)(i) Absence of plasmid backbone sequence in QCAV-4

PCR was used to analyse event QCAV-4 for the presence or absence of integrated vector backbone sequences. Multiple primer sets were designed on the backbone sequence of pSAN3/pCAMBIA-2200 and when these primers were used in PCR reactions with genomic DNA extracted from leaf tissue of QCAV-4 as template, no amplicons were generated. In contrast, when plasmid DNA from pSAN3 was used as a positive control template, amplicons of the expected size were generated.

A.3(c)(ii) Insert copy number, nucleotide sequence analysis and integrity

The number of T-DNA copies present in event QCAV-4 was initially assessed by Southern blot analysis (Southern 1975) and revealed multiple integrations (Dale *et al.*, 2017). To analyse these integrations in greater detail, a whole genome sequencing approach (Illumina 150 bp PE) was used in combination with a bioinformatics mapping analysis to (i) confirm the absence of vector backbone sequences, (ii) identify their chromosomal locations and (iii) characterise their organisational structure (study report QUT2023-1). Mapping of all 700 million sequencing reads obtained from Illumina whole genome sequencing of event QCAV-4 against the sequence of pSAN3 confirmed the absence of vector backbone sequences. In addition, the presence of one T-DNA left border (LB)-genome junction and one genome-T-DNA right border (RB) junction as well as multiple T-DNA junctions (one RB/RB, one RB/LB) indicated the presence of a single and large integration site on chromosome 6 of the banana genome. Long-read PacBio sequencing was then used to further characterise the integration site. A total of ~75 Gbp of data was obtained in CLR mode (4.9 million reads with a read length N50 of 17,973 bp). This corresponded to approx. 42x coverage of the Cavendish genome at the haplotype level. The T-DNA sequence of pSAN3 was used to filter out long-reads from the total genomic pool. Approximately 80 long-reads which mapped onto the T-DNA sequence were then assembled using the Flye plugin in Geneious Prime® version 2022.2.1. A single 26,849 bp T-DNA-containing insertion locus (which will be referred to as the "insert" in the rest of this application) was assembled and together with 4,211 bp and 3,576 bp of 3' and 5' flanking chromosome 6 sequences, respectively, was further refined using the QCAV-4 genomic Illumina short reads (previously generated using Novaseq 6000) to correct all Flye assembly errors (short indels). Banana genome (*M. acuminata* DH-Pahang version 4 on Banana Genome Hub, http://banana-genome-hub.southgreen.fr/) nucleotide BLAST analysis using the two flanking sequences either side of the insert determined its location between position 35,127,849 and 35,127,965 of chromosome 6 and in the intergenic region between two protein kinase domain-containing proteins

(Macma4_06_g29410.1 and Macma4_06_g29420.1). Comparative analysis of the insertion locus in QCAV-4 with its parent non-GM line (GN212-12) showed that a 116 bp deletion in one of the chromosome 6 loci resulted from the integration of the insert in QCAV-4. Deletions of this nature are a common feature of *Agrobacterium*-mediated transformation (Latham *et al*., 2006). Importantly, the insertion into the described chromosome 6 location did not interrupt any known ORF.

Comprehensive analysis of the insert sequence in event QCAV-4 revealed a structure comprised of three identical copies of the 6,702 bp pSAN3 T-DNA. These are identified by blue arrows labelled T-DNA1, T-DNA2 and T-DNA3 in Figure 3. In addition, a 6,668 bp hybrid fragment of the *MamRGA2* expression cassette had recombined in opposite directions and inserted between T-DNA2 and T-DNA3 (identified by the green box in Figure 3). Seven newly identified ORFs (red arrows) were created as a result of the insert in QCAV-4. Finally, several rearrangements have occurred at each of the following locations:

1. Between the 3' genome flanking region and T-DNA1 at position 4,212 to 4,273 (Figure 3, G-LB orange box).
2. Between T-DNA1 and T-DNA2 at position 10,905 to 10,942 (Figure 3, RB-RB orange box).
3. Large *MamRGA2* expression cassette rearrangement at position 17,594 to 24,261 (Figure 3, green box, LB and RB-LB orange boxes) including a 3,042 bp antisense portion of the *MamRGA2* ORF, a 2,672 bp sense portion of the MamRGA2 ORF and its Nos 3'UTR and a 142 bp sense portion of the CaMV35S promoter.
4. Between T-DNA3 and the 5' genome flanking region at position 30,868 to 31,060 (Figure 3, RB-G orange box).

A.3(c)(iii) Occurrence of unintended ORFs

To investigate the possibility of novel ORFs resulting from the presence of the insert in QCAV-4, an ORF analysis was conducted in Geneious to look for potential start-to-stop ORFs within the entire insert and including both the 3' 4,211 bp and 5' 3,576 bp sequences spanning the insert. This analysis examined each of three possible reading frames in both orientations for potential ORFs capable of encoding sequences of 30 or more amino acids. Seven new and unintended ORFs resulting from the presence of the insert in QCAV-4 were identified from this analysis (Figure 3, red arrows and Figure 4). To address the transcriptional potential (mRNA expression) of the seven newly identified ORFs described above, a two-stage approach was undertaken as follows.

1. The upstream (5') and downstream (3') sequences of each of these sequences were scrutinised for the presence of both 5' and 3' regulatory elements which could result in the transcription of these ORFs. *In silico* analyses of plant promoter-like sequences and transcription factor binding sites using tools available on PlantCARE (Lescot *et al*., 2002) and the TSSP Prediction of PLANT Promoters algorithm accessible from Softberry (Solovyev *et al.*, 2010) as well as searches for 3' UTR-like sequences on POLYAH [Softberry] (Salamov and Solovyev, 1997) were inconclusive (study report QUT2023-6) because of the high number of small motif sequences returned from these searches. The significance of these small motifs in the context of gene expression is difficult to assess. In a 2014 review, Hernandez-Garcia and Finer state that "… the small size of motif sequences that are recognized during genome-wide analyses using current prediction algorithms, frequently leads to the identification of a tremendous number of putative elements. Moreover, the presence of DNA sequence motifs alone is not sufficient to identify functional protein binding sites, which is highly influenced by other several factors. Many copies of a short sequence motif can be present in large genomes; however, pending position and accessibility, only a small portion of those copies may be functional and enable *in vivo* protein binding". Further, the likelihood of the presence and adequate functional location of upstream and downstream *cis*-regulatory elements working in conjunction to promote the expression of these new ORFs is very remote (Porto *et al*., 2014).

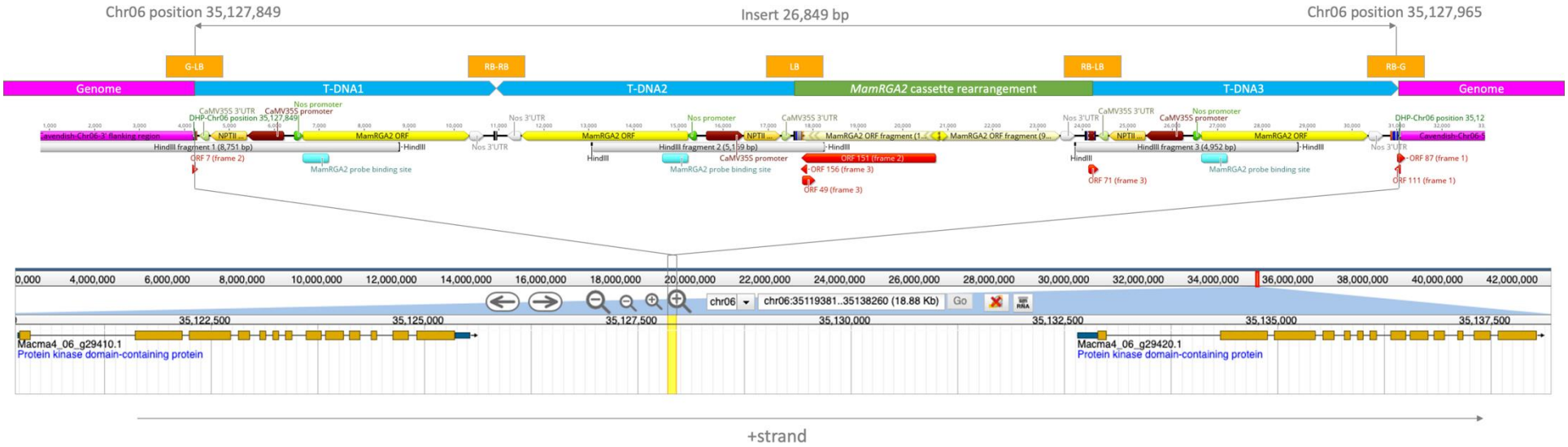

Figure 3. General organisation of the insert in event QCAV-4. A T-DNA insertion of 26,849 bp was assembled from PacBio long-reads and corrected with Illumina short-reads and located on banana chromosome 6. The single insertion is located in the anti-sense direction between positions 35,127,849 and 35,127,965 creating a 116 bp deletion of the original chromosome 6 locus in the intergenic region between two protein kinase domain-containing proteins (Macma4_06_g29410.1 and Macma4_06_g29420.1) and therefore did not interrupt any known open reading frame. The insert contains three full, intact and functional copies of the 6,702 bp T-DNA (T-DNA 1 to 3, blue arrows) as well as two fragmented portions of the *MamRGA2* expression cassette recombined in opposite directions and inserted between T-DNA2 and T-DNA3 (green box). The two genome-T-DNA and three inter T-DNA junctions contain various levels of rearrangement and are indicated with orange boxes. Seven new ORFs larger than 30 amino acids were identified (red arrows) in the inter T-DNA regions only. No evidence of any vector backbone sequence was detected. **Please note:** the insert sequence and diagrams provided are oriented in the antisense of their real orientation on Chromosome 6.

```
>ORF_111_(frame_1)
MHVMLYSWIRRGREDDSGGSIRITHYYGQFKLKAGANSH*

>ORF_87_(frame_1)
MCYSDRSSRVVFPAPPNPTIEHHMHSGIIENKNLKFSTEKCFVIVRRLVHKTENVK*

>ORF_7_(frame_2)
MDRHLKSRIRFWFKQQWPRQLNNTLRCKQIDA*

>ORF_151_(frame_2)
MWVCVSDDFDVKRITREITEYATNGRFMDLTNLNMLQVNLKEEIRGTTFLLVLDDVWNEDPVKWESLLAPLDAGGRGSVVIVTTQSKKVADVTGTMEPYVLEE
LTEDDSWSLIESHSFREASCSSTNPRMEEIGRKIAKKISGLPYGATAMGRYLRSKHGESSWREVLETETWEMPPAASDVLSALRRSYDNLPPQLKLCFAFCAL
FTKGYRFRKDTLIHMWIAQNLIQSTESKRSEDMAEECFDDLVCRFFFRYSWGNYVMNDSVHDLARWVSLDEYFRADEDSPLHISKPIRHLSWCSERITNVLED
NNTGGDAVNPLSSLRTLLFLGQSEFRSYHLLDRMFRMLSRIRVLDFSNCVIRNLPSSVGNLKHLRYLGLSNTRIQRLPESVTRLCLLQTLLLEGCELCRLPRS
MSRLVKLRQLKANPDVIADIAKVGRLIELQELKAYNVDKKKGHGIAELSAMNQLHGDLSIRNLQNVEKTRESRKARLDEKQKLKLLDLRWADGRGAGECDRDR
KVLKGLRPHPNLRELSIKYYGGTSSPSWMTDQYLPNMETIRLRSCARLTELPCLGQLHILRHLHIDGMSQVRQINLQFYGTGEVSGFPLLELLNIRRMPSLEE
WSEPRRNCCYFPRLHKLLIEDCPRLRNLPSLPPTLEELRISRTGLVDLPGFHGNGDVTTNVSLSSLHVSECRELRSLSEGLLQHNLVALKTAAFTDCDSLEFL
PAEGFRTAISLESLIMTNCPLPCSFLLPSSLEHLKLQPCLYPNNNEDSLSTCFENLTSLSFLDIKDCPNLSSFPPGPLCQLSALQHLSLVNCQRLQSIGFQAL
TSLESLTIQNCPRLTMSHSLVEVNNSSDTGLAFNITRWMRRRTGDDGLMLRHRAQNDSFFGGLLQHLTFLQFLKICQCPQLVTFTGEEEEKWRNLTSLQILHI
VDCPNLEVLPANLQSLCSLSTLYIVRCPRIHAFPPGGVSMSLAHLVIHECPQLCQHVPGTFGHP*

>ORF_156_(frame_3)
MRFLPEVSACPWHIWSSMNALSCVSMSLAHLVIHECPQP*

>ORF_71_(frame_3)
MPLPTVVPKMDPHPRGASWKKKTFQPRLQSKWIDVNMLEQLWRIYCGVNKLTLRQLNNTLRTFLMY*

>ORF_49_(frame_3)
MTKCARDMLTQLRAFMDDQMCQGHADTSGRKRMDSWASDDVQGAEGAEALQVCRQYLQVWTINDVQNLKRSKVSPLLFLFAGEGYELWTLADL*
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Figure 4. Amino acid sequences of the seven newly identified ORFs in QCAV-4.

2. Based on the results from the functional motifs analysis above, an RNA-Seq approach was used to definitively assess the expression of these new ORFs. Both leaf and root tissue were collected from three 12-week-old plants from both the non-GM control (GN212-12) and QCAV-4 as well as one ripe non-GM control and one ripe QCAV-4 fruit collected from the field. High quality RNA was extracted from each of these tissues and Illumina RNA-Seq data generated. Each data set was mapped separately in Geneious to a 34,636 bp sequence containing the 26,849 bp sequence of the insert and the 3' 4,211 and 5' 3,576 bp chromosome 6 flanking sequences, to "trap" any 150 bp PE read originating from a sequence transcribed from the insert. The analysis showed that none of the newly identified seven ORFs were transcribed in the three types of QCAV-4 tissue investigated (leaf, root and ripe fruit). For more details on this analysis refer to study report QUT2023-6.

Bioinformatic analyses of the new ORFs were also conducted to investigate amino acid sequence similarities with known allergenic and toxic proteins and the results are reported in section B.1.(d)(ii) and section B.1.(d)(iii)), respectively, and available in study reports QUT2023-6.

A.3(d) Description of how QCAV-4 was obtained from the original transformant

Domesticated bananas are vegetatively propagated and, as such, the progeny from a parent plant is essentially genetically identical to each other and the parent plant. The initial QCAV-4 plant was generated by transformation of a banana ECS and regeneration into a plant through tissue culture (Figure 5). QCAV-4 has subsequently been maintained in tissue-culture and is regularly sub-cultured using standard protocols and growth media. To generate large numbers of QCAV-4 plants for field trials (2012-2015; 2018-present) or other analyses (Figure 5), *in vitro* plantlets are grown in media containing specific plant hormones to induce multiplication, a process known as micropropagation (Smith *et al.,* 2001).

The plant tissue-culture (TC) facilities at QUT where event QCAV-4 was initially generated and is currently maintained have been granted accreditation to the Quality Banana Approved Nursery (QBAN) Scheme which has

been recognised as the Australian banana industry's scheme to provide clean planting material to banana industry members. Administration and auditing of the technical aspects of the scheme, leading to QBAN accreditation are now incorporated into the Nursery Industry Accreditation Scheme Australia (NIASA) program. NIASA is the national nursery industry's Best Management Practice (BMP) program for production nurseries, growth media manufacturers and greenlife markets. Facilities are subject to an annual audit to ensure compliance with industry best practice.

A.3(e) Stability of the inserted DNA across multiple generations

A.3(e)(i) Pattern of inheritance of the inserted DNA

Domesticated bananas are vegetatively propagated and, as such, the progeny from a parent plant is essentially genetically identical to each other and the parent plant. Bananas are also a perennial crop. When a banana is planted, the plant crop grows from meristematic tissue at the basal corm. After the development and maturity of a bunch on the plant crop, the plant crop pseudostem dies and another new pseudostem, known as the first ratoon, grows from a different meristem on the basal corm. This process can be repeated through numerous ratoons, and commercially in Australia through more than 10 ratoons before replanting (OGTR, 2023).

The stability of the insert in event QCAV-4 was investigated in several different "generations" of GM banana plants using Southern blot analysis (study report QUT2023-2). The samples used for this analysis are shown in Table 3 and Figure 5. The first-generation samples are derived from plants maintained in tissue culture and designated GN212-12 and 121-12. GN212-12 is a non-GM control plant derived from the banana cell line (GN212-12) used to generate GM banana event QCAV-4, while sample 121-12 is the original mother plant of event QCAV-4. Samples 20236, 20246 and 20265 represent three QCAV-4 plants (clones) in tissue culture that were initiated from meristems of plants growing in the field under OGTR licence DIR107 (2012-2015). Samples 20236, 20265 and 20267 represent three QCAV-4 banana plants (clones) currently growing in a field trial in the Northern Territory under OGTR licence DIR146. Samples were taken from each of these three plants growing in three successive "generations": the plant crop, first ratoon and second ratoon. Leaf tissue was collected from the banana plants, genomic DNA extracted and digested with the restriction enzyme *Hin*dIII. Evidence of genetic stability was indicated based on consistent banding patterns between the original QCAV-4 plants maintained in tissue culture and the three successive generations of QCAV-4 plants in the field.

Table 3. Sample information for generational stability analysis

Sample ID	Sample Details	Origin of sample
GN212-12	Non-GM Grand Nain control	Tissue culture
121-12	QCAV-4 original mother plant	
20236	QCAV-4 (Ex-field trial 1 plants)	
20246		
20265		
20236	QCAV-4 - Plant crop	Field trial 2
20265		
20267		
20236	QCAV-4 - 1st ratoon	
20265		
20267		
20236	QCAV-4 - 2nd ratoon	
20265		
20267		

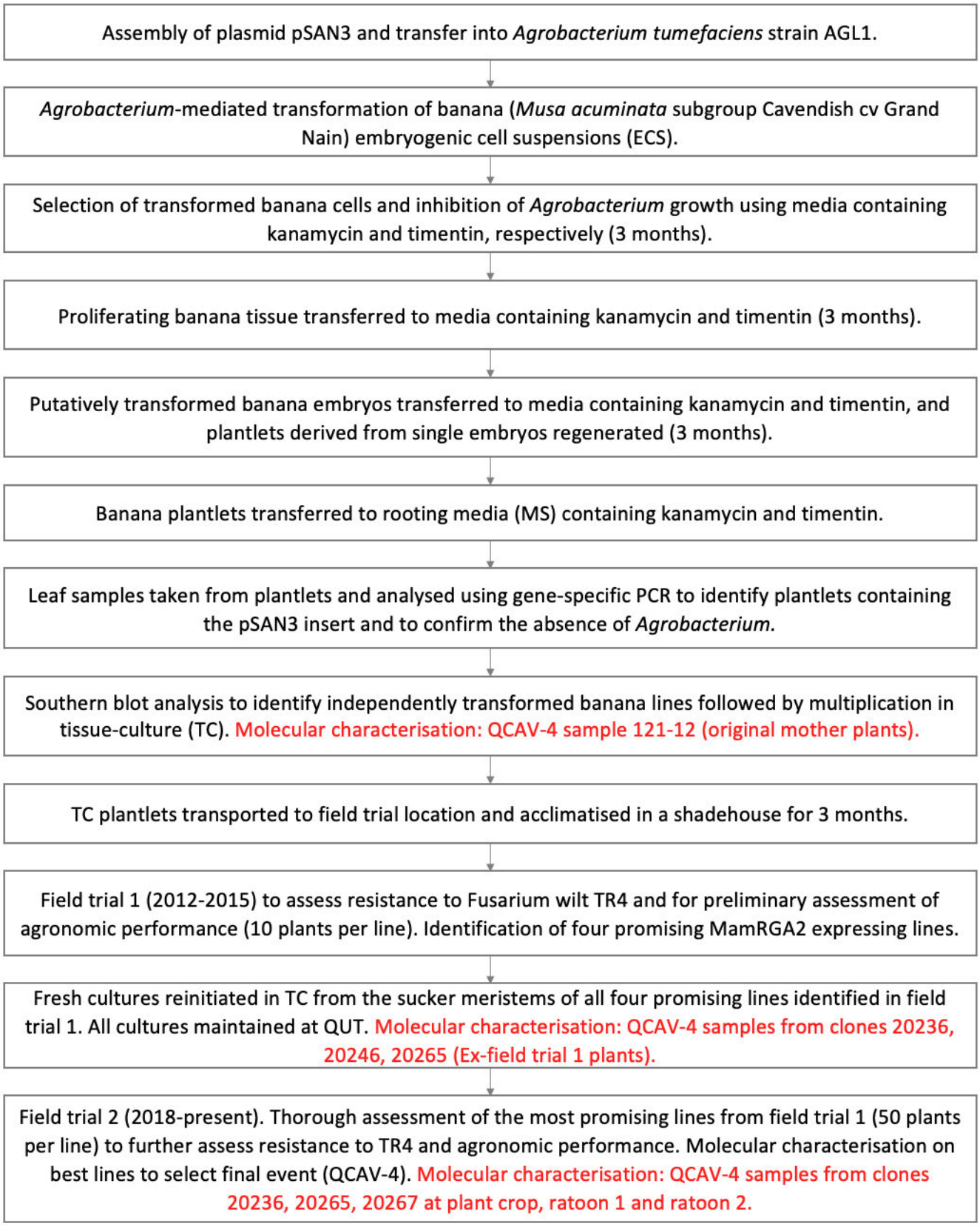


Figure 5. Development process of banana event QCAV-4 and sample identification.

A simplified organisation of the insert in QCAV-4 is shown in Figure 6A with the expected *Hin*dIII restriction digestion products indicated along with the fragment sizes and *MamRGA2* probe hybridisation specificity. Although wild-type Grand Nain bananas contain three endogenous *RGA2* homologs (*MaRGA2*) to which the *MamRGA2* probe hybridises, these can be differentiated from *MamRGA2* due to their unique predicted *Hin*dIII restriction fragment sizes of 3,453 bp, 3337 bp and 3,313 bp.

As shown in Figure 6B, the *MamRGA2* probe hybridises to the three predicted *Hin*dIII restriction fragments of 8,751 bp, 5,169 bp and 4,952 bp in all QCAV-4 samples (lanes 2-14), as well as to undigested pSAN3 (lane 15). Further, the presence of the endogenous homologs of RGA2 in Grand Nain bananas is shown by hybridisation of the probe to the expected fragment sizes of 3,453 bp, 3,337 bp and 3,313 bp (the shorter two fragments co-migrate as a more intensely staining single band) in all banana samples.

The observation of consistent hybridisation patterns across each of the different banana "generations" confirmed stable integration of the inserted DNA in event QCAV-4.

A.3(e)(ii) Pattern of inheritance and expression of the phenotype over several generations

Event QCAV-4 (formerly referred to as a line of RGC2 in OGTR licence DIR107, a line of RGA2 in OGTR licence DIR146 and as RGA2-4 in Dale *et al*., (2017)) has been evaluated for resistance to TR4 in two OGTR-approved field trials conducted in Australia (study report QUT2023-3). The first trial was conducted from 2012-2015 (DIR107 issued in 2011) while the second trial commenced in 2018 and is ongoing (DIR146 issued in 2016). Both trials were conducted on a commercial banana farm in the Northern Territory with high TR4 disease pressure.

The first field trial included 10 replicates each of five independently transformed MamRGA2 expressing lines (QCAV-4 and four additional lines) as well as several non-GM control cultivars (Dale *et al*., 2017). Plants were assessed for TR4 infection during the 3-year duration of the trial by the presence of typical internal and external disease symptoms and by a combination of fungal isolation and PCR-based assays. By the end of the trial, 87.5% of the non-GM Grand Nain control plants were either dead or infected by TR4. In contrast, the infection rate in the five transgenic MamRGA2 lines after 3 years ranged from 0-67% with QCAV-4 showing a 20% infection rate. To investigate the basis of the resistance, quantitative RT-PCR (qRT-PCR) was used to assess the levels of transgene expression. Using primers that specifically amplified *MamRGA2* RNA, a strong correlation was observed between *MamRGA2* RNA expression and the degree of TR4 protection. Using primers that would amplify both *MamRGA2* RNA and endogenous *MaRGA2* RNA, the most resistant line was again the highest expressor of *RGA2* RNA (*MamRGA2* + endogenous *MaRGA2*) whereas the other three lines that were TR4 resistant also showed moderate to high levels of *RGA2* RNA expression (*MamRGA2* + endogenous *MaRGA2*). In contrast, the most susceptible transgenic *MamRGA2* line had the lowest expression levels of *RGA2* (*MamRGA2* + endogenous *MaRGA2*). The results of this field trial have been published in *Nature Communications* (Dale *et al*., 2017).

In the second (ongoing) field trial, 50 replicates of QCAV-4 and the three additional promising *MamRGA2* lines identified in field trial 1, in addition to 50 non-GM control banana plants, was planted in 2018 and assessment for TR4 resistance is continuing. The presence of TR4 in plants showing typical disease symptoms is confirmed using PCR-based assays. The disease incidence in the QCAV-4 plants after five generations (plant crop and four ratoons) was 2% whereas 66% of non-GM control plants were infected (Figure 7).

These results provide strong evidence, based on the differing levels of expression of the transgene across five different transgenic lines and controls, that MamRGA2 is providing resistance to TR4 in event QCAV-4 and that the phenotype trait was stable and inherited across multiple generations.

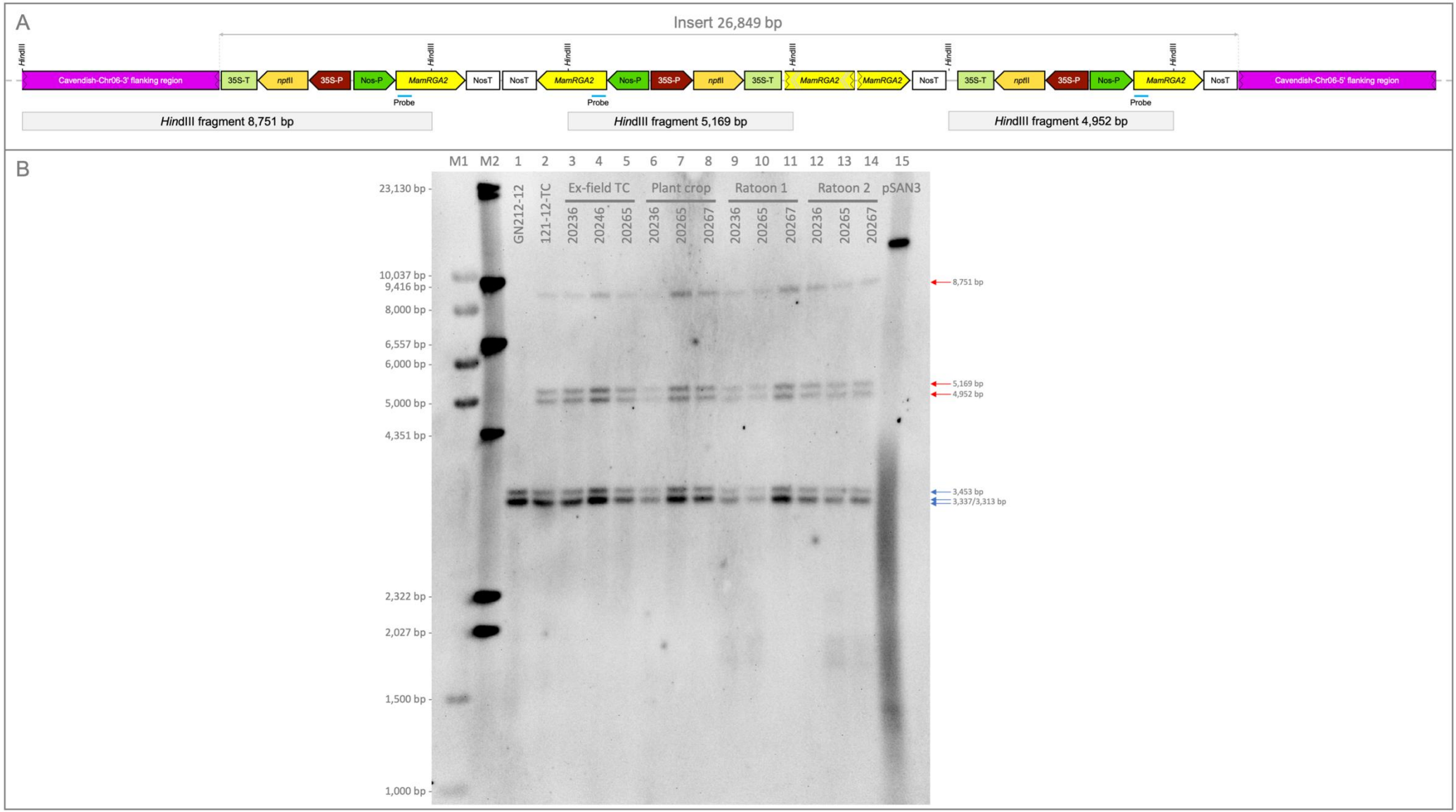


Figure 6. Stability of the T-DNA insert in event QCAV-4 demonstrated by Southern blot analysis. A. Simplified representation of the organisation of the insert in QCAV-4. The relevant positions of *Hin*dIII restriction sites as well as predicted *Hin*dIII restriction fragments are shown (grey boxes) along with the hybridisation position of the chosen *MamRGA2* DIG-labelled DNA probe. B. Southern blot analysis of wild-type (GN212-12) and different generations of QCAV-4 (121-12, 20236, 20246, 20265 and 20267) plants. Genomic DNA from each sample was digested with *Hin*dIII and hybridised with the *MamRGA2* probe. Red arrows indicate *Hin*dIII DNA fragments originating from the insert while blue arrows indicate endogenous *MaRGA2* fragments. M1 = Bioline HyperLadder 1kb; M2 = Sigma-Aldrich DNA Molecular Weight Marker II, DIG-labeled; pSAN3 = linearised plasmid pSAN3.

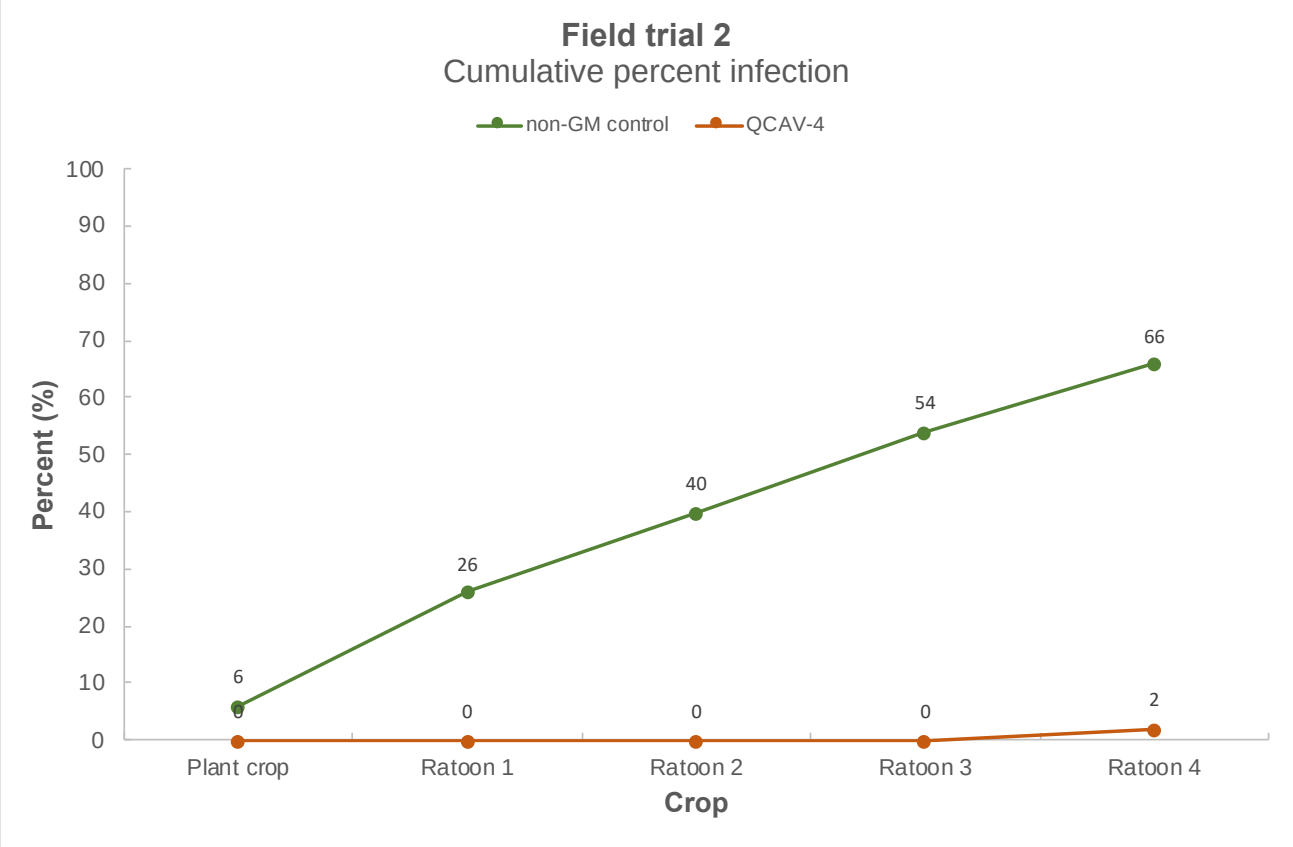


Figure 7. Progression of TR4 infection in non-GM control plants and QCAV-4 over 5 crop cycles.

A.3(f) Conclusions on the nature of the genetic modification in event QCAV-4

Molecular characterisation of the introduced genetic material within QCAV-4 demonstrated the presence of a 26,849 bp, pSAN3-derived, T-DNA-containing single insertion locus in an intergenic region of chromosome 6, resulting in a 116 bp deletion at the insertion site. The insertion into the described chromosome 6 location did not interrupt any known ORF. Sequence analysis of the insert and it 5' and 3' flanking regions revealed the presence of three identical copies of the pSAN3 T-DNA, a 6,668 bp hybrid fragment of the MamRGA2 expression cassette recombined in opposite directions and several minor rearrangements at T-DNA/T-DNA and T-DNA/genome junctions. Seven new and unintended ORFs resulted from the presence of the insert in QCAV-4 but, using a combination of bioinformatic and RNA-Seq analysis, none had the potential to be expressed. Finally, Southern blot analysis demonstrated that the insert was stably inherited over five generations.

B. Information related to the safety of the GM food

B.1. Characterisation and safety assessment of new substances

B.1(a) Biochemical function and phenotypic effects of newly expressed proteins in QCAV-4

B.1(a)(i) Biochemical function and phenotypic effects of MamRGA2

MamRGA2 is a CC-NBS-LRR type plant resistance (R) protein encoded by an R gene isolated from a wild seeded diploid banana (*Musa acuminata* ssp. *malaccensis*) (Peraza-Echeverria *et al*., 2008, 2009; Dale *et al*., 2017) reported to be resistant to infection by TR4 (Ploetz, 2018; Peraza-Echeverria *et al*., 2009). R genes are one component of a plant's two-layered innate immunity system for defence against pathogens (Jones and Dangl,

2006; Fick *et al*., 2022). The first layer comprises membrane-anchored receptors, called pattern recognition receptors (PRR), that can detect conserved molecules on pathogens known as pathogen-associated molecular patterns (PAMPs). This detection results in the initiation of weak defences known as PAMP-triggered immunity (PTI). Many specialised pathogens can overcome PTI by secreting effector molecules which suppress PTI. In response to these pathogens, plants have evolved a second layer of plant immunity whereby intracellular receptors (R proteins) that are encoded by R genes, can recognize the pathogens effector and initiate a suite of very strong defences, a process known as effector-triggered immunity (ETI).

There are a number of different classes of R proteins based on the organisation of their domains (Zhang *et al*., 2022). The largest class of R proteins contain a central nucleotide binding site (NBS) and a C-terminal leucine-rich repeat domain and are known as NLRs. The N-termini of NLRs are different but commonly consist of a Toll/interleukin receptor (TIR) domain or coiled-coil (CC) domain. In plants, the CC and TIR are signaling domains, while the NBS and LRR domains perform regulatory and sensor functions. R proteins are maintained in an inactive state at low levels in the cell but following recognition of a pathogen effector either directly or indirectly, the NLRs typically form complexes called resistosomes with associated proteins to activate strong defence pathways (Burdett *et al*., 2019). Sequence and structural similarities between R proteins from different plant species and similarities in observed defence responses demonstrate that R proteins generally activate conserved pathways in plants (Baker *et al*., 1997; Feys and Parker, 2000).

As indicated in section A.3.(e)(ii) above, event QCAV-4 has been evaluated for resistance to TR4 in two OGTR-approved field trials conducted in Australia. Except for TR4 resistance (conferred by transfer of MamRGA2), both QCAV-4 plants and fruit were phenotypically equivalent to non-GM Grand Nain control plants and fruit.

B.1(a)(ii) Biochemical function and phenotypic effects of NPTII

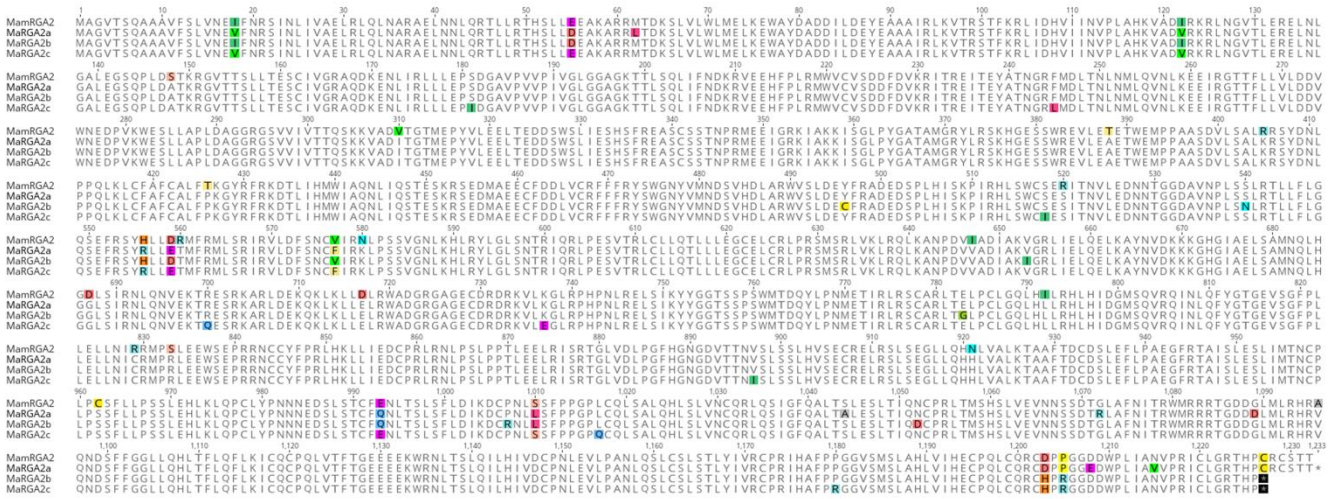
Event QCAV-4 expresses the protein neomycin phosphotransferase II (NPTII, EC 2.7.1.95), also referred as amino-glycoside-3'-phosphotransferase II [APH(3')II], as a plant selectable marker. NPTII, encoded by the *npt*II gene derived from the transposon Tn5 of *E. coli* strain K12, catalyses the ATP-dependent phosphorylation of the 3'-hydroxyl group of the amino-hexose portion of certain aminoglycosides including kanamycin thereby inactivating the antibiotics (Beck *et al.,* 1982). NPTII is the most widely used selectable marker system in plants.

As indicated previously, in two field trials of QCAV-4, except for TR4 resistance, both QCAV-4 plants and fruit were phenotypically equivalent to non-GM Grand Nain control plants and fruit. This indicates that there were no phenotypic effects of NPTII.

B.1(a)(iii) Tissue specificity and concentrations of MamRGA2 in QCAV-4

MamRGA2 is a 1,232 amino acid protein with a predicted molecular weight of 139.6 kDa. As mentioned previously, the triploid Cavendish banana genome contains three alleles of endogenous *MaRGA2* which are translated into peptides with more than 97.3% amino acid identity to MamRGA2 (Dale *et al*., 2017). For the detection and quantification of MamRGA2 expression in edible parts of event QCAV-4, an antibody-based approach was used. Because of the large number of CC-NBS-LRR proteins in banana and the high likelihood of cross-reactivity, a mouse anti-MamRGA2 monoclonal antibody (designated 17F07) was generated by Maine Biotechnology Services (MBS, BBI Solutions, Portland, ME) for the purpose of specifically detecting MamRGA2 in event QCAV-4 while avoiding cross-detection of the endogenous MaRGA2 protein as well as other R proteins. Similarities between MamRGA2 and endogenous MaRGA2 amino acid sequences are shown in Figure 8.

To assess the limit of detection (LOD) of the 17F07 antibody, Western blot analysis was done using semi-purified *E. coli*-expressed MamRGA2 (6x-His, 142 kDa) which represented about 11% of the total protein concentration



Sequence	MamRGA2	MaRGA2a	MaRGA2b	MaRGA2c
MamRGA2		97.5	97.6	97.3
MaRGA2a	97.5		98.4	98.4
MaRGA2b	97.6	98.4		98.2
MaRGA2c	97.3	98.4	98.2	

Figure 8. Protein alignment showing differences between MamRGA2 and endogenous MaRGA2 sequences. Top: Global alignment with Blosum62 cost matrix generated in Geneious, dissimilar amino acids are highlighted in color. Left: percent identity matrix.

based upon densitometry (study report QUT2023-8). A serial doubling dilution of MamRGA2 protein solution was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and incubated with the 17F07 antibody. This analysis revealed an MamRGA2 LOD between 1 and 2 ng (Figure 9A). The limit of quantification (LOQ) was subsequently calculated to be around 45-88 ppm of total soluble protein (TSP) using a Western blot and serially diluted MamRAG2 protein spiked into ripe banana fruit protein extracts (Figure 9B). Extrapolated to the mass of dry and fresh banana tissue, the LOQ was calculated to be 0.8-1.5 ppm and 190-370 ppb, respectively.

Finally, TSP extracts were made from ripe fruit flesh (35 µg) and peel tissues (15 µg) collected from non-genetically modified GN212-12 plants as well as QCAV-4 and used for SDS-PAGE electrophoresis followed by Western immunoblot analysis (Figure 10). MamRGA2 could not be detected in any of the edible plant parts of event QCAV-4 tested and therefore its concentration is believed to be below the lower LOQ of 190 ppb in fresh banana fruit tissue.

B.1(a)(iv) Estimated maximum human dietary exposure to MamRGA2

In Australia, 95% of households purchase bananas with an average yearly per capita consumption of 16 kg for 2020/21 (Hort Innovation, 2022). Since bananas are almost exclusively consumed as fresh fruit and the amount of MamRGA2 protein present in edible parts of event QCAV-4 was established below the lower LOQ of 190 ppb (or 190 µg/kg), individuals consuming banana fruit in Australia would be exposed to less than 3.04 mg of MamRGA2 protein per year (or less than 8.3 µg/day), assuming that 100% of the Australian Cavendish market was replaced with event QCAV-4. Data on the consumption of banana peel in Australia is unavailable but is considered to be marginal in comparison resulting in even lower exposure to the MamRGA2 protein from QCAV-4 peel consumption.

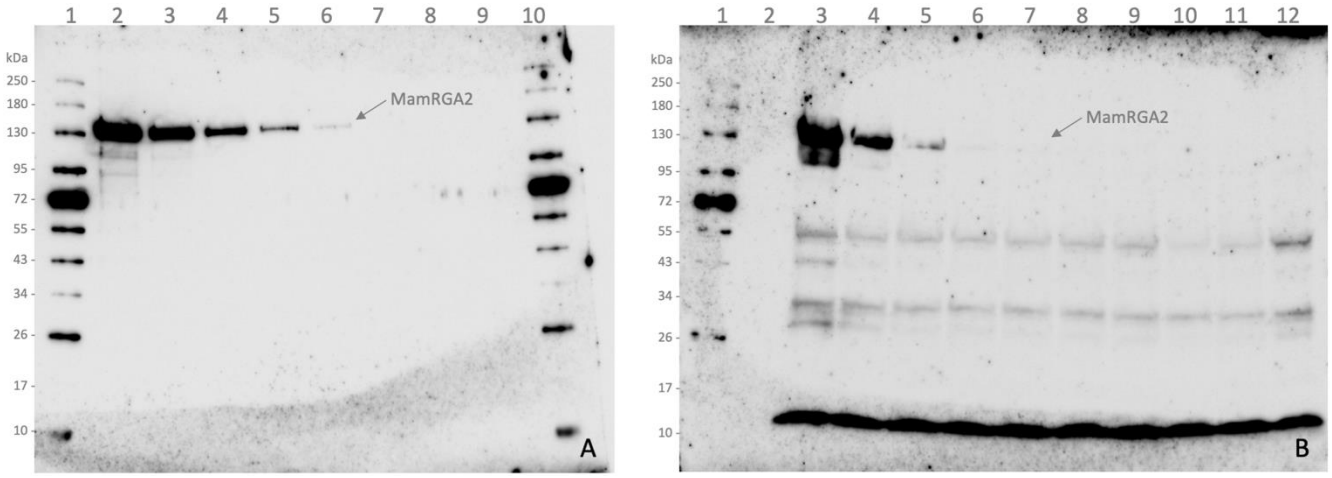


Figure 9. Protein detection efficacy by Western immunoblotting. A: Limit of detection (LOD) of *E. coli*-expressed MamRGA2 (139.6 kDa). Lanes 1 and 10, Colour Pre-stained Protein Standard, Broad Range (10-250 kDa) (Cat. #P7719S, New England Biolabs) and lanes 2-9*, E. coli*-expressed MamRGA2 at 31.3 ng, 15.6 ng, 7.8 ng, 3.9 ng, 1.9 ng, 0.98 ng, 0.49 ng and 0.24 ng, respectively. B: Limit of quantitation (LOQ) of *E. coli*-expressed MamRGA2 spiked into ripe banana fruit total soluble protein (TSP). Lane 1, Colour Pre-stained Protein Standard, Broad Range (10-250 kDa); lane 2, empty; lanes 3-11, 35 µg TSP from wild-type GN212-12 fruit spiked with 25 ng, 12.5 ng, 6.3 ng, 3.1 ng, 1.6 ng, 0.8 ng, 0.4 ng, 0.2 ng and 0.1 ng, respectively, and lane 12, 35 µg TSP from wild-type GN212-12 fruit (unspiked). Primary custom monoclonal mouse anti-MamRGA2 antibody 17F07 (MBS, BBI Solutions), 1:1,000 followed by secondary goat-anti-mouse IgG-HRP (Cat. #62-6520, ThermoFisher), 1:5,000, chemiluminescent substrate development and exposure time of 2 h. Low molecular weight band (~15 kDa) due to non-specific binding of the secondary IgG.

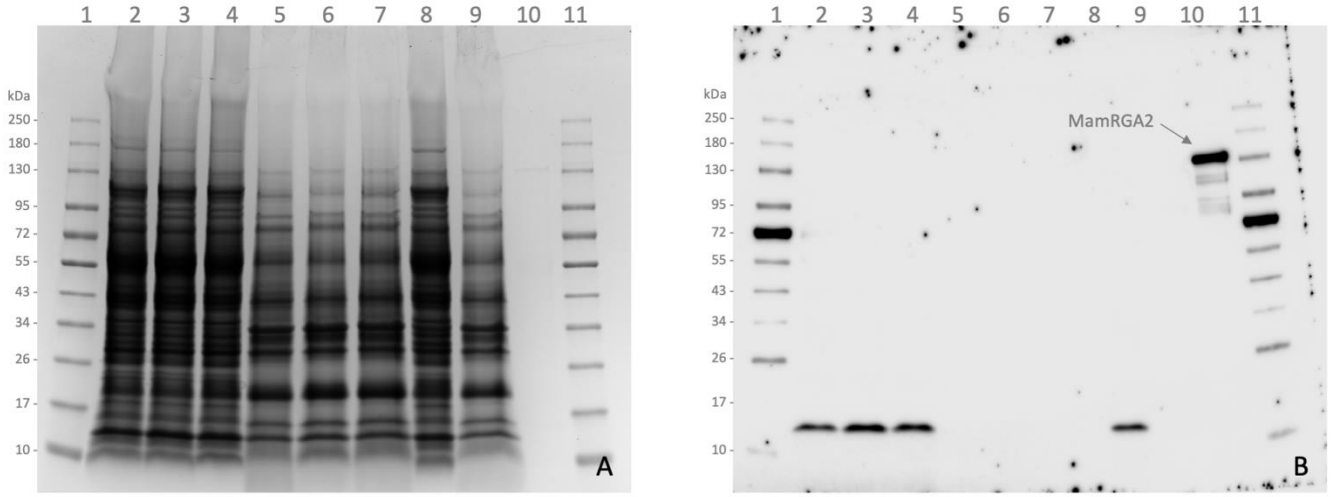


Figure 10. Detection of MamRGA2 in banana fruit tissue of event QCAV-4. A: 4-20% Mini-PROTEAN TGX Precast Protein Gel, Coomassie brilliant blue R-250 stain (Cat. #0472-25G, Amresco) and B: Western immunoblot. Detection with primary custom monoclonal mouse anti-MamRGA2 antibody 17F07 (MBS, BBI Solutions), 1:1,000 followed by secondary goat-anti-mouse IgG-HRP (Cat. #62-6520, ThermoFisher), 1:5,000, chemiluminescent substrate development and auto optimal exposure time of 436.7 s (~7.3 min). Lane 1 and 11, Colour Pre-stained Protein Standard, Broad Range (10-250 kDa) (Cat. #P7719S, New England Biolabs); lanes 2-4, 35 µg total soluble protein (TSP) from ripe fruit of QCAV-4 plants 20224, 20229 and 20230; lane 5-7, 15 µg TSP from ripe peel of QCAV-4 plants 20234, 20236 and 20246; lane 8-9, TSP from ripe fruit (35 µg) and peel (15 µg) of wild-type GN212-12 (20077), respectively; lane 10, *E. coli*-expressed MamRGA2 (15.6 ng, 139.6 kDa). Low molecular weight band (~15 kDa) due to non-specific binding of the secondary IgG.

B.1(a)(v) Tissue specific differential messenger RNA (mRNA) expression of MamRGA2

Because the MamRGA2 protein remained below our LOD in edible tissues (fruit and peel) of event QCAV-4, the presence and amount of mRNA from the *MamRGA2* transgene, the three endogenous *MaRGA2* alleles and the *npt*II selectable marker gene was investigated using RNA-Seq data from event QCAV-4 and the non-GM control GN212-12. Details of this analysis can be found in study report QUT2023-5.

The expression of the endogenous *MaRGA2* alleles was negligible in both GN212-12 and QCAV-4 with the expression levels considered as "noise" in comparison to *MamRGA2* and *npt*II (Table 4). As expected, no reads from the seven libraries of GN212-12 tissues mapped to *MamRGA2*. Although a very small number of reads (22) mapped to *npt*II, these were likely due to the potential presence of endogenous bacteria in the tissue. When the reads from QCAV-4 were analysed, *MamRGA2* was expressed at highest levels in roots (24x), followed by leaves (20x) and to negligible levels in fruit tissue (1x). In the fruit of QCAV-4, *MamRGA2* expression was about 3.2-fold higher than the endogenous *MaRGA2*. The expression of the *npt*II selectable marker gene was highest in the fruit of QCAV-4 and about 1,000x higher than the expression of the *MamRGA2* transgene.

In summary, the abundance of *MamRGA2* transcripts in the fruit of QCAV-4 was relatively low compared to other tissues examined and was of a similar order of magnitude to the endogenous *MaRGA2* counterpart. These results are consistent with previous analyses presented in section B.1(a)(iii) (and study report QUT2023-4) which demonstrated that the amount of MamRGA2 protein in edible portions of QCAV-4 is very low (below the LOD). In contrast, the relative amounts of transcripts originating from the *npt*II selectable marker gene were much higher which is not unexpected considering the use of the strong, constitutive CaMV35S promoter controlling *npt*II expression.

Table 4. Tissue specific RNA-Seq differential analysis in non-GM control GN212-12 versus QCAV-4

Genotype	Tissue	Number of reads mapped to		
		Transgene		Endogenous
		MamRGA2	***npt*II**	***MaRGA2***
GN212-12 (non-GM control)	Leaf*	0	15	2
	Root*	0	7	450
	Fruit^	0	0	1,019
QCAV-4	Leaf*	55,482	1,199,830	7
	Root*	69,077	648,468	902
	Fruit^	2,835	2,905,092	892

*Averaged from data sets from three plants, ^single plant dataset

B.1(a)(vi) Tissue specificity and concentrations of NPTII in QCAV-4

Since the NPTII amino acid sequence expressed in QCAV-4 is nearly identical (99.6%) to the one expressed in several GM events already assessed by FSANZ (Beck *et al.*, 1982), its safety assessment was limited to two types of analysis, (i) an updated bioinformatics comparison of its amino acid sequence to known protein toxins (see section B.2(b)(ii)) and allergens (see section B.2(c)(ii)) and (ii) the detection and quantification of the amount of NPTII protein present in edible parts of the plant by Western blot analysis and quantitative enzyme-linked immunosorbent assay (ELISA).

Western immunoblot analysis using a commercially available NPTII-specific antibody (study report QUT2023-4) revealed a band of the expected molecular weight for NPTII (29.1 kDa) in both fruit and peel samples collected from event QCAV-4 (Figure 11, lane 2-7) but absent from the wild-type GN212-12 (Figure 11, lane 8-9).

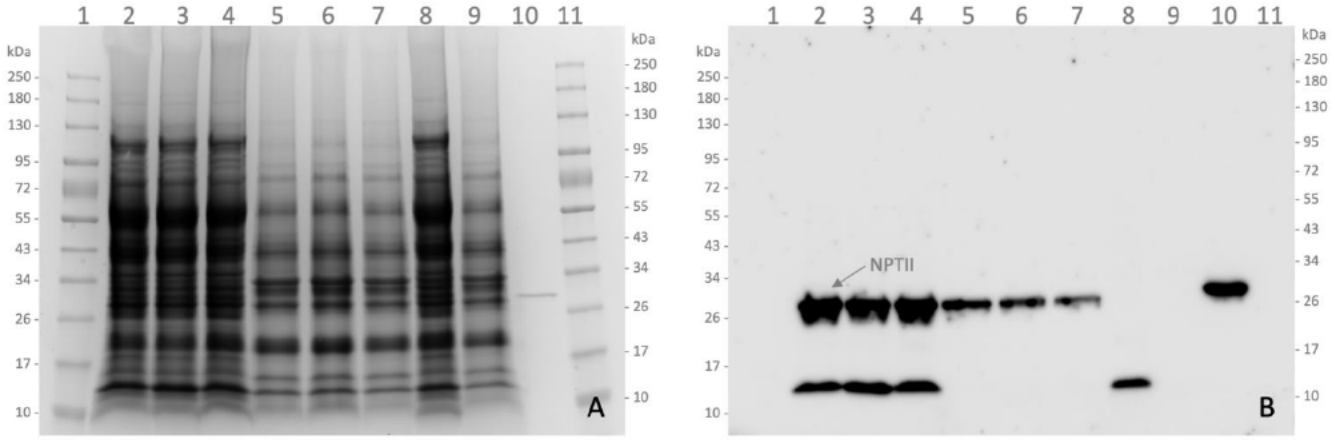


Figure 11. Detection of NPTII in edible banana tissue of event QCAV-4. A: 4-20% Mini-PROTEAN TGX Precast Protein Gel, Coomassie brilliant blue R-250 stain (Cat. #0472-25G, Amresco) and B: Western immunoblot. Detection with primary anti-NPTII detection antibody (Cat. #PSP 73000, Agdia), 1:1,000 followed by secondary goat-anti-mouse IgG-HRP (Cat. #62-6520, ThermoFisher), 1:5,000, chemiluminescent substrate development and auto optimal exposure time of 30 min. Lanes 1 and 11, Colour Pre-stained Protein Standard, Broad Range (10-250 kDa) (Cat. #P7719S, New England Biolabs); lanes 2-4, 35 µg total soluble protein (TSP) from ripe fruit of QCAV-4 plants 20224, 20229 and 20230; lanes 5-7, 15 µg TSP from ripe peel of QCAV-4 plants 20234, 20236 and 20246; lanes 8-9, TSP from ripe fruit (35 µg) and peel (15 µg) of wild-type GN212-12 plant 20077, respectively; lane 10, 113 ng of NPTII protein standard (Cat. #LST 73000, Agdia). Low molecular weight band (~15 kDa) due to non-specific binding of the secondary IgG.

To estimate human dietary exposure to the NPTII enzyme expressed in QCAV-4, its concentration in edible tissues of the plant was determined by quantitative enzyme-linked immunosorbent assay (ELISA) (study report QUT2023-4). These analyses were performed on the same ripe fruit and peel samples analysed previously by Western blotting. On average, the concentration of NPTII in fresh ripe fruit and peel samples was 3.1 and 4.5 ppm, respectively (Table 5).

Table 5. NPTII concentration measured in event QCAV-4 edible tissues by ELISA

Tissue	Sample	NPTII ng/mg (DW)			NPTII % TSP	NPTII (ng/mg FW)*
		Rep.	Average	SD		
Fruit	20224	11.5	12.4	0.6	0.08	3.1
	20229	12.6			0.07	
	20230	12.9			0.09	
Peel	20234	19.0	18.1	0.7	0.15	4.5
	20236	18.0			0.10	
	20246	17.2			0.15	

Average NPTII background readings recorded from respective non-GM GN212-12 plant 20077 tissues were subtracted from all QCAV-4 readings. DW = dry weight, FW = fresh weight, SD = standard deviation, Rep. = replicate, *Assume 25% dry matter content.

B.1(a)(vii) Estimated maximum human dietary exposure to NPTII

As per section B.1(a)(iv), human dietary exposure to NPTII was calculated based on an average yearly per capita consumption of fresh banana fruit of 16 kg for 2020/21 (Hort Innovation, 2022) at 49.6 mg per year (or 136 µg/day). This scenario again assumes a 100% shift of the Australian Cavendish market to event QCAV-4. Dietary exposure to NPTII through the consumption of QCAV-4 banana peel is difficult to establish because of the lack of

reliable data on the consumption of this tissue in Australia. If consumption of banana peel was similar to the fruit flesh (16 kg/per/year) then the exposure would be 72 mg per year (or 197 μg/day).

B.1(b) Prior history of human consumption

B.1(b)(i) History of exposure to MamRGA2 through food

Musa acuminata ssp*. malaccensis* (*Mam*) was the source of the *MamRGA2* transgene (Peraza-Echeverria *et al*., 2008, 2009). *Mam* is a wild seeded diploid banana that is found in germplasm collections in Australia but is not grown commercially or consumed either in Australia or elsewhere due to the lack of edible flesh in the fruit and the presence of numerous hard seeds. It is maintained in some field germplasm collections for conservation and breeding purposes only. *Mam* is one of five banana subspecies that are known to have prominent roles in the domestication of bananas many of which are widely consumed today (Sardos *et al.,* 2022). It is the foundation maternal parent of Cavendish based on chloroplast genome analysis (Dale *et al*., unpublished data).

MamRGA2 is a CC-NBS-LRR (NLR) type plant resistance (R) protein. This class of R proteins are ubiquitous in the plant kingdom and are abundant in all plants for which they have been studied including the major dicot and monocot food crops such as tomato, bean, chickpea, cassava, maize, potato, sorghum, rice, soybean and wheat (Figure 12) (Baggs *et al*., 2017; Liu *et al*., 2021). NLRs are also abundant in banana. Chang *et al*. (2020) identified a total of 98 NBS-LRR proteins in the haploid banana genome (1n gamete). More recently, 128 NBS-LRR loci have been identified in the current version of the Double Haploid Pahang (AA) banana genome (1n gamete) while in-house analysis at QUT has revealed at least 356 haplotype resolved NLRs (3n gametes) in the Cavendish (*Musa acuminata* (*Ma*) AAA) genome. Further, the triploid Cavendish banana genome contains three alleles of endogenous *RGA2* (referred to as *MaRGA2*) with a minimum of 98.6% nucleotide identity to *MamRGA2* and translating into peptides with more than 97.3% amino acid identity to MamRGA2 (Dale *et al*., 2017; Figure 8). In addition, *RGA2* homologs are present in all banana cultivars analysed in our laboratory including the Musaceae close relative, *Ensete ventricosum* (Dale *et al*., unpublished data).

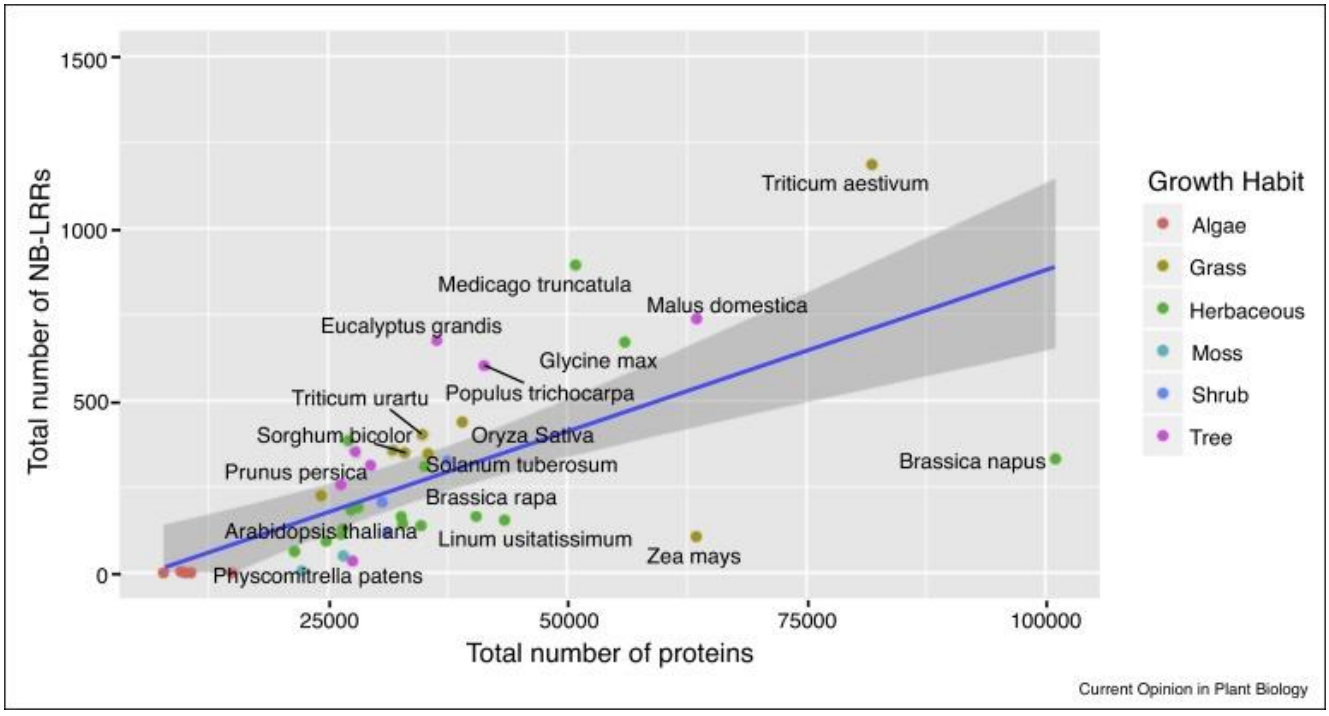


Figure 12. Ubiquitous nature of NLRs in plants and algae. Adapted from Baggs *et al*. (2017).

In summary, the evidence supporting a history of safe use of MamRGA2 includes (i) the presence of RGA2 homologous sequences in all banana cultivars, (ii) the prevalence of NLRs similar to MamRGA2 in foods commonly consumed in diets worldwide and (iii) centuries of consumption of Cavendish bananas with NLRs very similar to MamRGA2.

B.1(b)(ii) History of exposure to NPTII through food

NPTII has been used as a plant selectable marker for many internationally approved GM food crops grown on millions of hectares globally since 1996 (Kumar *et al*., 2020; Verma *et al*., 2021). As such, there has been a long history of approval of GM crops containing the *npt*II marker gene and expressing the NPTII protein. The US Food and Drug Administration (FDA) was the first regulatory agency to consider the food safety of the *npt*II marker gene and NPTII protein and approved its presence in food as an additive in 1994 (FDA, 1994). In guidance to the industry the FDA concluded that this gene is safe to use as a selectable marker in the development of transgenic crops (FDA, 1998). The US EPA has issued an exemption from the requirement of a tolerance for the NPTII protein (US-EPA, 1994), which means that NPTII is safe at any level expressed in GM crops. Further, the European Food Safety Authority concluded that the use of the *npt*II gene as a selectable marker in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment (EFSA 2004, 2009). There is no evidence that the NPTII protein is toxic or allergenic, and food derived from GM canola, corn and cotton with the *npt*II gene has been approved for sale in Australia (OGTR, 2017).

B.1(c) Post-translational modification analysis of newly expressed proteins in event QCAV-4

Glycosylation is an essential post-translational modification (PTM) of eukaryotic secretory and membrane proteins which involves the covalent attachment of oligosaccharides most commonly to asparagine (N-linked) or serine/threonine (O-linked) amino acids (Rademacher *et al*., 1988). Protein N-glycosylation is initiated in the endoplasmic reticulum (ER) (Nagashima *et al*., 2018). As several known protein allergens are glycosylated, it has been postulated that glycosyl groups may contribute to their allergenicity (Altmann, 2007). Conversely, many glycosylated proteins are not allergenic, therefore, glycosylation should only be considered in the context of the overall weight-of-evidence presented (Ladics, 2019).

The glycosylation status of a protein is typically evaluated by SDS-PAGE followed by glycoprotein staining (Roth *et al*., 2012). As this laboratory analysis requires relatively large quantities of the target protein (e.g. micrograms) and the levels of MamRGA2 protein in edible QCAV-4 banana tissue are below the limit of detection for this type of assay, glycosylation could not be directly evaluated. As such, a bioinformatics approach was used to assess the potential for glycosylation of MamRGA2.

In silico analysis using NetNGlyc-1.0 (Gupta and Brunak, 2002) identified seven potential N-glycosylation consensus sites (defined as [Asp-X-Thr/Ser] where X can be any amino acid) on the amino acid sequence of the MamRGA2 protein expressed in event QCAV-4. However, this analysis also revealed the absence of an N-terminal ER signal peptide, a pre-requisite for glycosylation (Pattison and Amtmann, 2009). The absence of a signal peptide was further confirmed using two additional machine learning models, SignalP-6.0 (Teufel *et al*., 2022) and TargetP-2.0 (Almegro Armenteros *et al*., 2019). Therefore, although MamRGA2 contains several potential N-glycosylation acceptor sites, the lack of a signal peptide suggests that it is unlikely to transit into the ER and therefore unlikely to be glycosylated.

Based on the high level of similarity between the nucleotide and amino acid sequences of *MamRGA2* and the three endogenous Cavendish *MaRGA2* homologs (see B.1(b)(i)), there is a high probability that the MamRGA2 protein would be processed similarly to MaRGA2 in QCAV-4 and that novel PTM would therefore also be unlikely in MaRGA2.

B.1(d) Bioinformatic analysis of newly created ORFs

B.1(d)(i) Identification of new ORFs

As described in Section A.3(c)(iii) of this document, seven new unintended ORFs resulted from the presence of the insert in QCAV-4. None contained the required regulatory elements necessary for expression of mRNA and protein biosynthesis and this was confirmed by RNA-Seq. In the below section, a bioinformatic analysis was used to investigate the potential similarity of these seven new ORFs to known allergenic and toxic proteins.

B.1(d)(ii) Potential allergenicity of newly created ORFs

Although both bioinformatic and RNA-Seq analysis demonstrated that RNA expression (and hence protein synthesis) from the seven newly created unintended ORFs is highly unlikely (Section A.3(c)(iii)), their allergenicity potential was still investigated. *In silico* analyses were performed to compare the predicted amino acid sequences of each ORFs (Figure 4) to known allergenic proteins in the Food Allergy Research and Resource Program (FARRP) dataset, which is available through www.AllergenOnline.org (University of Nebraska). The version of the database used (v21) contains 2,233 protein (amino acid) sequence entries that are categorised into 913 taxonomic-protein groups of unique proven or putative allergens (food, airway, venom/salivary and contact) and celiac protein sequences from 430 species. Three types of analyses were performed for this comparison on October 27, 2022:

(a) Full-length sequence search. FASTA36 alignments were performed comparing the entire amino acid sequence query of each ORF to the database entries. Significant homology was determined when there was (i) greater than 50% homology between query protein and database entry and (ii) the E-value was less than 10^{-4}.

(b) 80-mer sliding window search. FASTA36 alignments were performed comparing all contiguous 80 amino acids within each ORF query to the database entries. Matches were identified if there was greater than 35% homology (E-value < 10^{-4}).

(c) 8-mer exact match search. A FASTA alignment was performed comparing contiguous 8 amino acids within the ORF to the database entries. Matches were identified if there was 100% homology. The 8-mer exact match search identifies regions of 8 amino acid identity between the queried ORF sequence and known or suspected allergens in the database.

Full-length sequence (E-value < 10^{-4}), 80-mer sliding window (35% homology with E-value < 10^{-4}) and 8-mer exact match searches identified no sequence similarity between any of the seven new ORFs and known allergens in the FARRP database indicating that if any were to be expressed in QCAV-4, they would not be of any allergenicity concern (Table 6).

B.1(d)(iii) Potential toxicity of newly created ORFs

To assess the potential for toxicity of the newly created ORFs, potential structural similarities shared between the predicted amino acid sequences and sequences in a protein toxin database were evaluated using the Basic Local Alignment Search Tool (BLAST) available within the Geneious program. A toxin BLAST database was created in Geneious from a subset of sequences derived from the UniProt Knowledgebase, comprised of 568,002 manually annotated and reviewed sequences from Swiss-Prot and 226,771,948 automatically annotated, un-reviewed sequences from TrEMBL, that were selected using a keyword search on toxins (KW-0800). At the time of the analysis, August 29, 2022, the collection contained a total of 92,851 sequences, including 7,523 reviewed sequences from Swiss-Prot and 85,328 un-reviewed sequences from TrEMBL.

For six out of the seven newly identified ORFs, the BLAST (blastp) search using the BLOSUM45 similarity scoring matrix and their respective amino acid sequences as queries did not return any accessions of biological significance from the toxin database with an E-value acceptance criteria lower than 1 x 10^{-5}. However, following this analysis, newly created ORF 151 matched three protein accessions that contain the keyword "toxin" in UniProt Knowledgebase (Table 6 and 7). Of these three proteins, two were from *Triticum aestivum* (wheat) and one was from *Hordeum vulgare* (barley). ORF 151 originates in a large, fragmented portion of the *MamRGA2* ORF present in the insert of QCAV-4 (Figure 3) and spans 993 amino acid of MamRGA2 (219-1211) with 99.1% identity. To assess the significance of these findings, an identical search into the toxin database was made with the amino acid sequence from MamRGA2 (Section B.2(c)) and SvVNT1, another CC-NBS-LRR type resistance gene (*Rpi-vnt1*) product derived from *Solanum venturi* and that provides protection against foliar late blight in potato (*Solanum tuberosum*). As predicted, the blastp search using SvVNT1 as query returned the identical three sequences as the blastp search using ORF 151 or MamRGA2 as query (Table 7). SvVNT1 is expressed in the Innate® Hibernate (event Y9) and Innate® Acclimate (event X17) potatoes developed by J.R. Simplot Co. that have been approved for food, feed and cultivation in the USA and Canada since 2017. Both events were also approved for food that same year in Australia and New Zealand and for both food and feed in 2020 in the Philippines.

In summary, using conservative search criteria, the amino acid sequences of the proteins encoded by the seven new ORFs showed no significant sequence similarity to any proteins known, or suspected, to be of mammalian toxicological concern.

Table 6. Location and identity of the seven new ORFs resulting from the insert in QCAV-4

ORF ID	Nucleotide location[a]	Frame	Strand	Length (bp)	Length (amino acid)	Molecular weight (kDa)	Deducted amino acid sequence	Allergenicity hits[b]	BLAST toxicity hits[c]
ORF 111	31,078 → 30,959	1	Sense (+)	120	39	4.5	MHVMLYSWIRRGREDDSGGSIRITHYYGQFKLKAGANSH*	None	None
ORF 87	31,004 → 31,174	1	Antisense (-)	171	56	6.5	MCYSDRSSRVVFPAPPNPTIEHHMHSGIIENKNLKFSTEKCFVIVRRLVHKTENVK*	None	None
ORF 7	4,188 → 4,286	2	Antisense (-)	99	32	4.1	MDRHLKSRIRFWFKQQWPRQLNNTLRCKQIDA*	None	None
ORF 151	20,724 → 17,749	2	Sense (+)	2976	991	112.8	MWVCVSDDFDVKRITREITEYATNGRFMDLTNLNMLQVNLKEEIRGTTFLLVLDDVWNEDPVKWESL LAPLDAGGRGSVVIVTTQSKKVADVTGTMEPYVLEELTEDDSWSLIESHSFREASCSSTNPRMEEIG RKIAKKISGLPYGATAMGRYLRSKHGESSWREVLETETWEMPPAASDVLSALRRSYDNLPPQLKLCF AFCALFTKGYRFRKDTLIHMWIAQNLIQSTESKRSEDMAEECFDDLVCRFFFRYSWGNYVMNDSVHD LARWVSLDEYFRADEDSPLHISKPIRHLSWCSERITNVLEDNNTGGDAVNPLSSLRTLLFLGQSEFR SYHLLDRMFRMLSRIRVLDFSNCVIRNLPSSVGNLKHLRYLGLSNTRIQRLPESVTRLCLLQTLLLE GCELCRLPRSMSRLVKLRQLKANPDVIADIAKVGRLIELQELKAYNVDKKKGHGIAELSAMNQLHGD LSIRNLQNVEKTRESRKARLDEKQKLKLLDLRWADGRGAGECDRDRKVLKGLRPHPNLRELSIKYYG GTSSPSWMTDQYLPNMETIRLRSCARLTELPCLGQLHILRHLHIDGMSQVRQINLQFYGTGEVSGFP LLELLNIRRMPSLEEWSEPRRNCCYFPRLHKLLIEDCPRLRNLPSLPPTLEELRISRTGLVDLPGFH GNGDVTTNVSLSSLHVSECRELRSLSEGLLQHNLVALKTAAFTDCDSLEFLPAEGFRTAISLESLIM TNCPLPCSFLLPSSLEHLKLQPCLYPNNNEDSLSTCFENLTSLSFLDIKDCPNLSSFPPGPLCQLSA LQHLSLVNCQRLQSIGFQALTSLESLTIQNCPRLTMSHSLVEVNNSSDTGLAFNITRWMRRRTGDDG LMLRHRAQNDSFFGGLLQHLTFLQFLKICQCPQLVTFTGEEEEKWRNLTSLQILHIVDCPNLEVLPA NLQSLCSLSTLYIVRCPRIHAFPPGGVSMSLAHLVIHECPQLCQHVPGTFGHP*	None	3[d]
ORF 156	17,852 → 17,733	3	Sense (+)	120	39	4.4	MRFLPEVSACPWHIWSSMNALSCVSMSLAHLVIHECPQP*	None	None
ORF 71	24,139 → 24,339	3	Antisense (-)	201	66	7.9	MPLPTVVPKMDPHPRGASWKKKTFQPRLQSKWIDVNMLEQLWRIYCGVNKLTLRQLNNTLRTFLMY*	None	None
ORF 49	17,755 → 18,026	3	Antisense (-)	282	93	10.6	MTKCARDMLTQLRAFMDDQMCQGHADTSGRKRMDSWASDDVQGAEGAEALQVCRQYLQVWTINDVQN LKRSKVSPLLFLFAGEGYELWTLADL*	None	None

[a]Location on the supplied insert sequence including 5' and 3' flanking genomic sequence. **Please note:** the insert sequence and diagrams provided are oriented in the antisense of their real orientation on Chromosome 6.

[b]Full-length sequence (E-value < 10^{-4}), 80-mer sliding window (35% homology with E-value < 10^{-4}) and 8-mer exact match searches in the FAARP database. Analysis done on August 29th, 2022.

[c]Amino acid sequences queried against the BLAST toxin database with an E-value cutoff of 1 x 10^{-5}. Analysis done on October 27th, 2022.

[d]Details described in Table 7.

Table 7. BLAST results using ORF 151 as query sequence against the toxin database

Event	Query	Query match					
		Start-end	Identity	E-value	Accession	Description	Organism
QCAV-4	ORF 151	299-695	24.4% in 397 aa overlap	$7.7x10^{-18}$	A0A3B6FZY2	NB-ARC domain-containing protein	*Triticum aestivum* (wheat)
		250-640	24.7% in 391 aa overlap	$5.1x10^{-13}$	A0A287K383	Uncharacterized protein	*Hordeum vulgare* (barley)
		1,367-1,813	25.5% in 447 aa overlap	$6.7x10^{-7}$	A0A3B6SRU1	rRNA N-glycosidase	*Triticum aestivum* (wheat)
QCAV-4	MamRGA2	303-695	24.7% in 393 aa overlap	$1.0x10^{-17}$	A0A3B6FZY2	NB-ARC domain-containing protein	*Triticum aestivum* (wheat)
		175-640	25.2% in 466 aa overlap	$4.8x10^{-16}$	A0A287K383	Uncharacterized protein	*Hordeum vulgare* (barley)
		1,239-1,813	24.9% in 575 aa overlap	$4.2x10^{-11}$	A0A3B6SRU1	rRNA N-glycosidase	*Triticum aestivum* (wheat)
Innate	SvVNT1	175-630	28.7% in 456 aa overlap	$6.0 x10^{-39}$	A0A287K383	Uncharacterized protein	*Hordeum vulgare* (barley)
		306-674	28.5% in 369 aa overlap	$5.5x10^{-30}$	A0A3B6FZY2	NB-ARC domain-containing protein	*Triticum aestivum* (wheat)
		1,267-1,753	23.6% in 487 aa overlap	$1.4x10^{-11}$	A0A3B6SRU1	rRNA N-glycosidase	*Triticum aestivum* (wheat)
		139-260	36.8% in 122 aa overlap	$3.1x10^{-9}$	A0A3B6FZY2	NB-ARC domain-containing protein	*Triticum aestivum* (wheat)

B.2. Characterisation of newly expressed proteins

B.2(a) Source of the protein

The two newly expressed proteins in event QCAV-4 are detailed in the following sections.

B.2(a)(i) MamRGA2

The recognition of pathogen-secreted effectors by R proteins such as MamRGA2 is one of the most well studied mechanisms in plant defense responses (Cui *et al.,* 2015). Most known disease resistance (R)-proteins contain a nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domain (Zhang *et al.,* 2022). The N-termini of NLRs are different but commonly consist of a Toll/interleukin receptor (TIR) domain or coiled-coil (CC) domain. MamRGA2 (accession #ACF21694) is a 1232 amino acid CC-NBS-LRR resistance (R) protein encoded by the *MamRGA2* R gene found in *M. acuminata* ssp. *malaccensis* with a predicted molecular weight of 139.6 kDa (Figure 13).

```
   1 MAGVTSQAAA VFSLVNEIFN RSINLIVAEL RLQLNARAEL NNLQRTLLRT HSLLEEAKAR
  61 RMTDKSLVLW LMELKEWAYD ADDILDEYEA AAIRLKVTRS TFKRLIDHVI INVPLAHKVA
 121 DIRKRLNGVT LERELNLGAL EGSQPLDSTK RGVTTSLLTE SCIVGRAQDK ENLIRLLLEP
 181 SDGAVPVVPI VGLGGAGKTT LSQLIFNDKR VEEHFPLRMW VCVSDDFDVK RITREITEYA
 241 TNGRFMDLTN LNMLQVNLKE EIRGTTFLLV LDDVWNEDPV KWESLLAPLD AGGRGSVVIV
 301 TTQSKKVADV TGTMEPYVLE ELTEDDSWSL IESHSFREAS CSSTNPRMEE IGRKIAKKIS
 361 GLPYGATAMG RYLRSKHGES SWREVLETET WEMPPAASDV LSALRRSYDN LPPQLKLCFA
 421 FCALFTKGYR FRKDTLIHMW IAQNLIQSTE SKRSEDMAEE CFDDLVCRFF FRYSWGNYVM
 481 NDSVHDLARW VSLDEYFRAD EDSPLHISKP IRHLSWCSER ITNVLEDNNT GGDAVNPLSS
 541 LRTLLFLGQS EFRSYHLLDR MFRMLSRIRV LDFSNCVIRN LPSSVGNLKH LRYLGLSNTR
 601 IQRLPESVTR LCLLQTLLLE GCELCRLPRS MSRLVKLRQL KANPDVIADI AKVGRLIELQ
 661 ELKAYNVDKK KGHGIAELSA MNQLHGDLSI RNLQNVEKTR ESRKARLDEK QKLKLLDLRW
 721 ADGRGAGECD RDRKVLKGLR PHPNLRELSI KYYGGTSSPS WMTDQYLPNM ETIRLRSCAR
 781 LTELPCLGQL HILRHLHIDG MSQVRQINLQ FYGTGEVSGF PLLELLNIRR MPSLEEWSEP
 841 RRNCCYFPRL HKLLIEDCPR LRNLPSLPPT LEELRISRTG LVDLPGFHGN GDVTTNVSLS
 901 SLHVSECREL RSLSEGLLQH NLVALKTAAF TDCDSLEFLP AEGFRTAISL ESLIMTNCPL
 961 PCSFLLPSSL EHLKLQPCLY PNNNEDSLST CFENLTSLSF LDIKDCPNLS SFPPGPLCQL
1021 SALQHLSLVN CQRLQSIGFQ ALTSLESLTI QNCPRLTMSH SLVEVNNSSD TGLAFNITRW
1081 MRRRTGDDGL MLRHRAQNDS FFGGLLQHLT FLQFLKICQC PQLVTFTGEE EEKWRNLTSL
1141 QILHIVDCPN LEVLPANLQS LCSLSTLYIV RCPRIHAFPP GGVSMSLAHL VIHECPQLCQ
1201 RCDPPGGDDW PLIANVPRIC LGRTHPCRCS TT
```

Figure 13. Amino acid sequence of MamRGA2. Individual coiled-coil (CC), nucleotide binding site (NBS), and leucine rich repeat (LRR) domains are indicated.

B.2(a)(ii) NPTII

NPTII (accession #AAF65391) is a 265 amino acid protein encoded by the *npt*II plant selectable marker gene derived from *E. coli* with a predicted molecular weight of 29.1 kDa (Figure 14). The NPTII protein encoded by pSAN3 is 99.6% similar to the previously approved original sequence from Beck *et al.* (1982).

```
  1 MGIEQDGLHA GSPAAWVERL FGYDWAQQTI GCSDAAVFRL SAQGRPVLFV KTDLSGALNE
 61 LQDEAARLSW LATTGVPCAA VLDVVTEAGR DWLLLGEVPG QDLLSSHLAP AEKVSIMADA
121 MRRLHTLDPA TCPFDHQAKH RIERARTRME AGLVDQDDLD EEHQGLAPAE LFARLKARMP
181 DGEDLVVTHG DACLPNIMVE NGRFSGFIDC GRLGVADRYQ DIALATRDIA EELGGEWADR
241 FLVLYGIAAP DSQRIAFYRL LDEFF
```

Figure 14. Amino acid sequence of NPTII.

The safety of NPTII has been assessed on numerous previous occasions and is well documented in peer reviewed scientific literature (Fuchs *et al.,* 1993). In all instances it has been concluded that NPTII is non-toxic to humans and has limited potential as a food allergen. A comprehensive review from the European Food Safety Authority concluded that the use of the *npt*II gene as a selectable marker in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment (EFSA 2004, 2009). Further, NPTII has been used as a plant selectable marker for many internationally approved GM food crops grown on millions of acres globally since 1996 (Kumar *et al*., 2020; Verma *et al*., 2021 and food derived from GM canola, corn and cotton with the *npt*II gene has been approved for sale in Australia on multiple occasions (OGTR, 2017). Since the NPTII amino acid sequence expressed in QCAV-4 is almost identical (99.6%) to the one expressed in several GM events already assessed by FSANZ (Beck *et al*., 1982 and Figure 15), its safety assessment in the section below was limited to an updated bioinformatics comparison of its amino acid sequence to known protein toxins and allergens.

```
                          10        20        30        40        50        60
                   ----:----|----:----|----:----|----:----|----:----|----:----|
NPTII-AAF65391-pSAN3 MGIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNE
NPTII-pCAMBIA-2200   MGIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNE
NPTII-AAA85506-Beck  M-IEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNE
                          70        80        90        100       110       120
                   ----:----|----:----|----:----|----:----|----:----|----:----|
NPTII-AAF65391-pSAN3 LQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADA
NPTII-pCAMBIA-2200   LQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADA
NPTII-AAA85506-Beck  LQDEAARLSWLATTGVPCAAVLDVVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADA
                          130       140       150       160       170       180
                   ----:----|----:----|----:----|----:----|----:----|----:----|
NPTII-AAF65391-pSAN3 MRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMP
NPTII-pCAMBIA-2200   MRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMP
NPTII-AAA85506-Beck  MRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGLAPAELFARLKARMP
                          190       200       210       220       230       240
                   ----:----|----:----|----:----|----:----|----:----|----:----|
NPTII-AAF65391-pSAN3 DGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADR
NPTII-pCAMBIA-2200   DGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADR
NPTII-AAA85506-Beck  DGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADR
                          250       260
                   ----:----|----:----|----:
NPTII-AAF65391-pSAN3 FLVLYGIAAPDSQRIAFYRLLDEFF
NPTII-pCAMBIA-2200   FLVLYGIAAPDSQRIAFYRLLDEFF
NPTII-AAA85506-Beck  FLVLYGIAAPDSQRIAFYRLLDEFF
```

Figure 15. Protein alignment showing similarities between NPTII. Amino acid sequence comparison of the NPTII protein (accession #AAF65391) encoded by the *npt*II gene in pSAN3, pCAMBIA-2200 and the previously approved original NPTII (accession #AAA85506) sequence from Beck *et al.* (1982).

B.2(b) Bioinformatic amino acid sequence comparison for potential toxicity

B.2(b)(i) MamRGA2

To assess the potential for toxicity of MamRGA2, potential structural similarities shared between the predicted amino acid sequence and sequences in a protein toxin database were evaluated using the Basic Local Alignment Search Tool (BLAST) available within Geneious Prime® version 2022.2.1. An overview of this analysis can be found in section B.1(d)(iii) and study report QUT2023-7.

A blastp search using the BLOSUM45 similarity scoring matrix and the MamRGA2 amino acid sequence as the query sequence did not identify any protein toxins with an E-value lower than 1×10^{-5} but, similarly to ORF 151, identified three protein accessions that contain the keyword "toxin" in UniProt Knowledgebase (Table 6).

B.2(b)(ii) NPTII

A search for potential similarity to protein toxins was also conducted using the NPTII sequence as query and failed to return any match with an E-value lower than 1×10^{-5} (study report QUT2023-7).

B.2(c) Bioinformatic amino acid sequence comparison for potential allergenicity

B.2(c)(i) MamRGA2

To assess the allergenicity potential of MamRGA2 (accession #ACF21694), *in silico* analyses were performed to identify matches between the protein query sequence and known allergens present in the FARRP dataset (study report QUT2023-7). An overview of these analyses can be found in section B.1(d)(ii). These analyses did not identify any known allergens with homology to MamRGA2, indicating that the MamRGA2 protein is unlikely to be an allergen.

B.2(c)(ii) NPTII

Similar analyses were done to assess the allergenicity potential of NPTII (accession #AAF65391) (study report QUT2023-7). These analyses did not identify any known allergens with homology to NPTII, indicating that, as previously demonstrated, it is unlikely to be of allergenicity concern.

B.2(d) MamRGA2 stability to proteolysis in simulated gastric fluid (SGF)

The susceptibility of MamRGA2 to digestion by proteolytic enzymes under acidic conditions was examined using two approaches (study report QUT2023-9). Firstly, a bioinformatic assessment was conducted to identify potential protease cleavage sites using the amino acid sequence and the PeptideCutter tool in the ExPASy Proteomics Site (Gasteiger *et al.,* 2005). This analysis revealed MamRGA2 has multiple cleavage sites for pepsin (308 sites at pH 1.3 and 354 sites at pH >2), trypsin (142 sites), chymotrypsin (71 high-specificity sites, 298 low-specificity sites) and endopeptidases (211 sites). On this basis, MamRGA2 was considered likely to be as susceptible to digestion as most dietary proteins.

The safety of novel proteins inserted into genetically modified plants has routinely included an evaluation of their susceptibility to digestion. To examine this, the susceptibility of semi-purified *E. coli*-expressed MamRGA2 protein to digestion by pepsin in simulated gastric fluid (SGF) was performed. Detailed analysis assessing the suitability and equivalence of the *E. coli*-expressed MamRGA2 protein compared to the plant-expressed MamRGA2 protein is available in study report QUT2023-8. Protein extracts from *E. coli*-expressing MamRGA2 (~200 µg TSP of which MamRGA2 was estimated to be ~13.5 µg by densitometry) were incubated in the presence of SGF pH 1.4 containing pepsin at 37°C for 0, 0.5, 2, 5, 10, 20, 30 and 60 min (Figure 16A-D). Control digestions

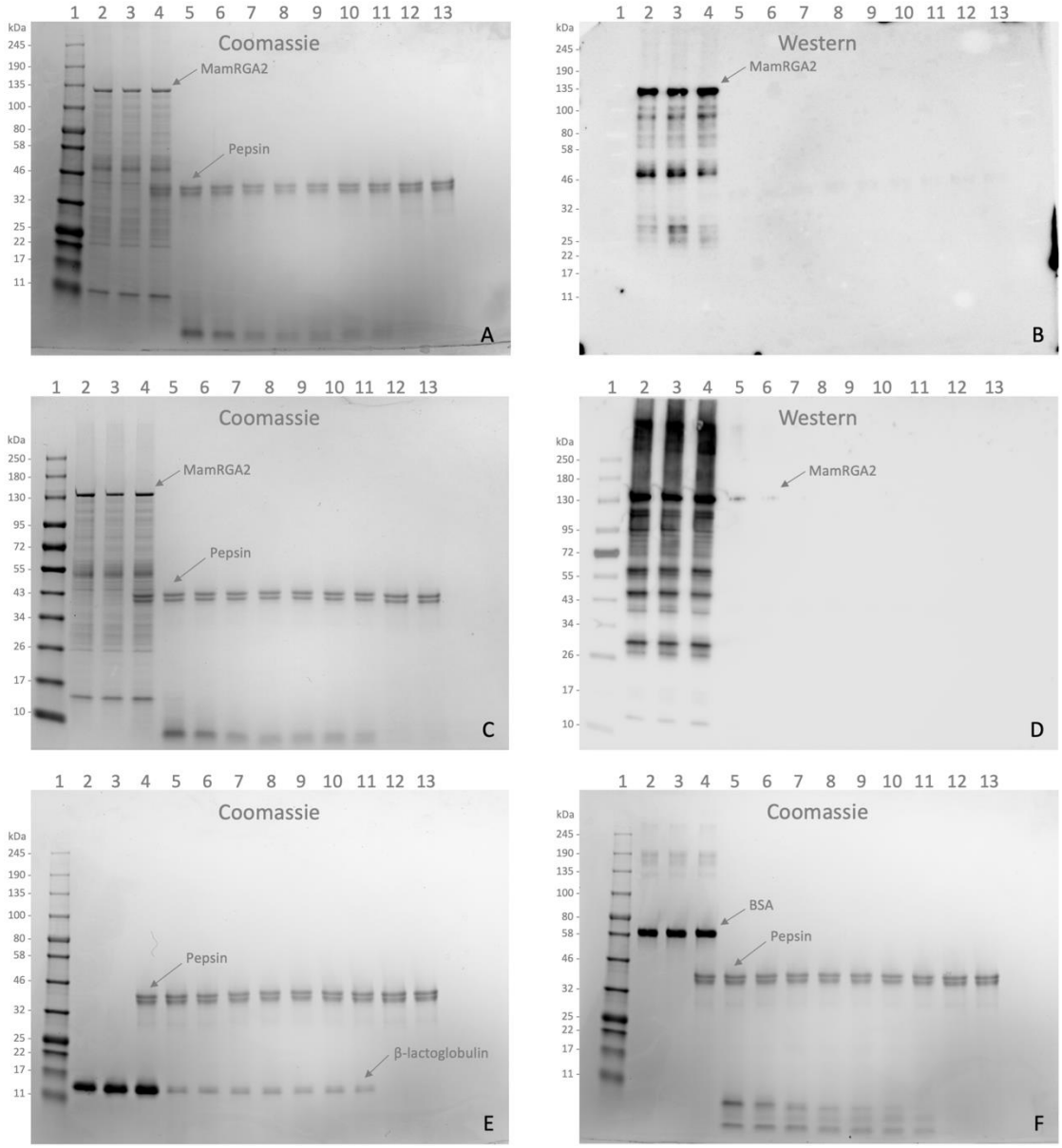


Figure 16. Lability of *E. coli*-expressed MamRGA2 protein in SGF pH 1.4 containing pepsin. Digests of *E. coli*-expressed MamRGA2 (A-D), pepsin-insensitive β-lactoglobulin (β-lac, Cat. #L3908, Sigma) control (E) and pepsin-sensitive bovine serum albumin (BSA, Cat. #P0834, Sigma) control (F), all under denaturing conditions. Panels A, C, E and F: 4-20% Mini-PROTEAN TGX Precast Protein Gel, Coomassie brilliant blue R-250 stain (Cat. #0472-25G, Amresco) and panels B and D: Western immunoblots. Panel B: detection with His_6-tag monoclonal antibody-HRP (Cat. #MA1-135-HRP, ThermoFisher), 1:3,000, chemiluminescent substrate development and exposure time of 20 min. Panel D: detection with primary custom monoclonal mouse anti-MamRGA2 antibody 17F07 (MBS, BBI Solutions), 1:1,000 followed by secondary goat-anti-mouse IgG-HRP (Cat. #62-6520, ThermoFisher), 1:5,000, chemiluminescent substrate development and exposure time of 98 s. Lane 1, molecular weight standards either Colour Pre-stained Protein Standard, Broad Range (11-245 kDa) (Panels A, B, E and F) or (10-250 kDa) (Panels C and D) (Cat. #P7712S and P7719S, New England Biolabs); lane 2-3, *E. coli*-expressed MamRGA2 or control protein incubated in SGF pH 1.4 for 0 and 60 min; lanes 4-11, *E. coli*-expressed MamRGA2 or control protein incubated in SGF pH 1.4 containing pepsin for 0, 0.5, 2, 5, 10, 20, 30 and 60 min; and lanes 12-13, SGF containing pepsin only for 0 and 60 min.

with pepsin-insensitive β-lactoglobulin and pepsin-sensitive bovine serum albumin (BSA) were performed under the same conditions. Samples were removed at stated time points and separated by SDS-PAGE and either stained using Coomassie blue (Figure 16A, C, E and F) or transferred to nitrocellulose membrane for Western blot analysis (Figure 16B and D). Since an N-terminal His_6-tag was added to MamRGA2 for purification purposes, it was also used for detection purposes in Western analysis (Figure 16B) along with the monoclonal mouse anti-MamRGA2 antibody 17F07 (Figure 16D).

The pepsin-insensitive protein, β-lactoglobulin, was still present after 60 minutes of incubation in SGF containing pepsin (Figure 16E), while the pepsin-sensitive protein, BSA, was completely digested within 30 seconds following the addition of pepsin to the assay (Figure 16F). MamRGA2 in SGF was substantially degraded 30 seconds after the addition of pepsin (Figure 16A-D) and was completely undetectable after 2 minutes of digestion (Figure 16D). Faint, low molecular weight degradation products were visible (by Coomassie) in samples subjected to up to 60 minutes of digestion (Figure 16A and C), but these were not detected by Western blot (Figure 16B and D).

The results of this *in vitro* digestion analysis support the conclusion that the MamRGA2 protein, like most conventional dietary proteins, will be readily digested in a typical mammalian gastric environment. Therefore, no increased risk of allergenicity or toxicity would be anticipated from dietary exposure to this protein (Codex, 2003).

B.2(e) Thermal stability of MamRGA2

Although most Cavendish bananas in Australia are consumed as a fresh fruit, some are fried to prepare fritters or banana chips while green (starchy, unripe) bananas have been used to make banana flour for use in baking. As an NLR protein, MamRGA2 is thought to play a role in the activation of the plant defences against Fusarium wilt TR4. As such, it was not possible to design a protein activity assay to assess the functionality of MamRGA2 following exposure to thermal treatments. Therefore, the effect of heat on the structural integrity and immuno-detectability of the MamRGA2 protein was evaluated by SDS-PAGE and Western blot analysis, respectively (study report QUT2023-9). Aliquots of *E. coli*-expressed MamRGA2 (~150 ng) were heated to 60, 75 and 90°C for 15, 30 and 60 minutes, while control samples were kept either at 4°C or 22°C for 60 min before being subjected to SDS-PAGE (Figure 17A). For Western blot analysis, samples were diluted 30x to load the equivalent to 5 ng of MamRGA2 per lane (Figure 17B). The intensity of the ~142 kDa *E. coli*-expressed MamRGA2 protein kept at 4°C was equivalent to that of the one kept at room temperature (22°C). Further, no visible differences in band intensity or degradation of MamRGA2 were observed irrespective of the temperature used or the length of incubation (Figure 17). These results suggest that the MamRGA2 protein is not significantly degraded at temperatures up to 90°C.

B.2(f) Conclusions from assessment of potential toxicity and allergenicity

The weight-of-evidence supporting the lack of identifiable hazards associated with the MamRGA2 protein includes:

1. The history of *Mam*, the source organism of *MamRGA2*, as one of five banana subspecies that are known to have prominent roles in the domestication of bananas, many of which are widely and safely consumed today.
2. The ubiquitous nature of CC-NBS-LRR R proteins such as MamRGA2 in food crops.
3. The known function of these genes in pathogen effector-triggered immunity in plants.
4. The lack of significant amino acid sequence similarity to known or putative protein toxins and allergens.
5. The rapid degradation of MamRGA2 in SGF (pH 1.4) containing pepsin.
6. The absence of detectable levels of MamRGA2 in banana fruit tissue.

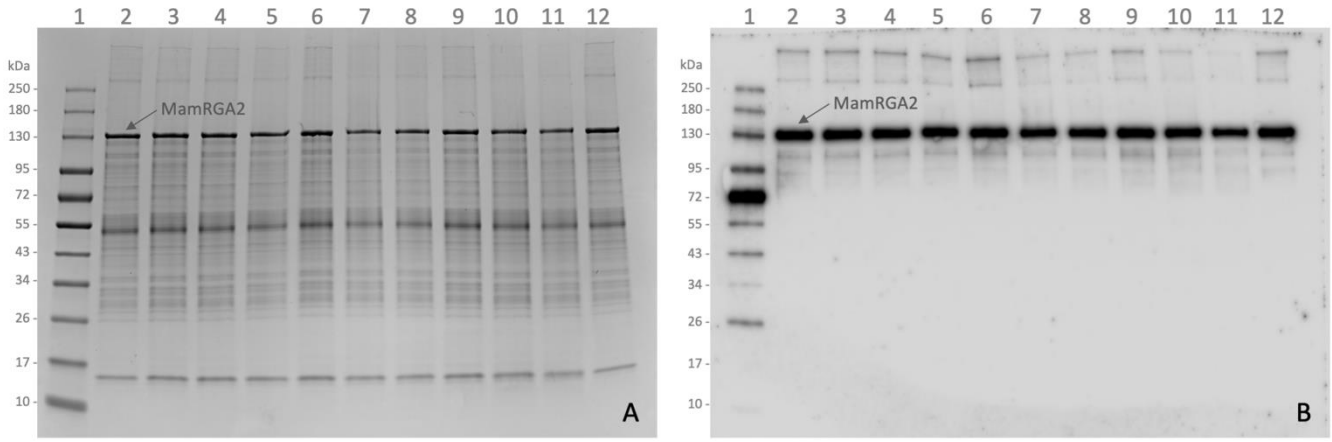


Figure 17. Thermal stability of MamRGA2. A: ~150 ng of heat-treated *E. coli*-expressed MamRGA2 per lane of a 4-20% Mini-PROTEAN TGX Precast Protein Gel, Coomassie brilliant blue R-250 stain (Cat. #0472-25G, Amresco) and B: Western immunoblot of 5 ng of heat-treated *E. coli*-expressed MamRGA2 per lane. Detection with primary custom monoclonal mouse anti-MamRGA2 antibody 17F07 (MBS, BBI Solutions), 1:1,000 followed by secondary goat-anti-mouse IgG-HRP (Cat. #62-6520, ThermoFisher), 1:5,000, chemiluminescent substrate development and auto optimal exposure time of 30 min. Lanes 1, Colour Pre-stained Protein Standard, Broad Range (10-250 kDa) (Cat. #P7719S, New England Biolabs); lanes 2-12, *E. coli*-expressed MamRGA2 treatment as follows: lanes 1, 4°C for 60 min; lanes 2-5, 60°C for 10, 30 and 60 min, lanes 6-8, 75°C for 10, 30 and 60 min, lanes 9-11, 90°C for 10, 30 and 60 min and lane 12, 22°C for 60 min.

In combination, the data described above support the conclusion that the MamRGA2 protein is unlikely to be toxic or allergenic and to represent any hazard to humans or animals. Regarding NPTII, although the sequence present in QCAV-4 is not identical (but 99.6% similar) to the original Beck's sequence (Beck *et al*., 1982) previously approved by various regulatory authorities (including OGTR and FSANZ), the protein retained its full function while bioinformatic searches found no similarity to known or putative protein toxins and allergens. Based on these conclusions, an animal acute toxicity study was not warranted.

B.3. Other (non-protein) new substances

Not applicable

B.4. Novel herbicide metabolites in GM herbicide-tolerant plants

Not applicable.

B.5. Compositional analyses of the food produced using gene technology

The nutritional composition of both banana fruit and peel tissue from event QCAV-4 was compared to fruit and peel tissue derived from its non-GM Grand Nain (GN212-12) counterpart (control). Details on the conditions of these analysis are presented in study report QUT2023-10. Fruit was harvested from the third top hand of full-green (unripe mature) bunches from 10 QCAV-4 plants and 10 non-GM control plants from two generations (generation 5 (ratoon 4) and generation 6 (ratoon 5)) of field grown plants. Fruit was ripened by exposure to ethylene to ripening stage 6 (BAN-C-1, 2001), and pulp sample were removed and stored at -80°C. For six QCAV-4 fruit and two non-GM control fruit from generation 7 (ratoon 6) plants, peel samples were also taken from ethylene-ripened fruit for analysis. Tissue analysis was performed at the National Measurement Institute (https://www.industry.gov.au/national-measurement-institute).

In the absence of a banana-specific OECD consensus document on compositional considerations for new banana

varieties, samples were analysed for the content of (i) proximates (moisture, total fat, total protein, ash, carbohydrates and energy), (ii) three minerals (magnesium, manganese, potassium), and (iii) two vitamins (ascorbic acid (vitamin C) and pyridoxine (vitamin B6)) which represent the highest contributors to the percent daily values from a 2,000 calorie reference diet for adults and children aged four or over (USDA, 2019; https://nutritiondata.self.com/). Further analysis of individual amino acid and fatty acid composition was not deemed a necessary consideration for the safety assessment of event QCAV-4 based upon the following considerations. The recommended daily intake (RDI) of protein for Australian males and females is 64-81 g/day and 46-57 g/day, respectively (NRVANZ, 2006). With a protein concentration of approximately 1 g/100 g (fresh weight, FW) and assuming 100% bioavailability, an average male would need to consume more than 3.2 kg (~25 peeled fruit) of banana per day to ingest 50% of their RDI. Regarding fatty acid intake, the Nutrient Reference Values for Australia and New Zealand (NRVANV, 2006) state that for children, adolescents and adults, the estimated average requirement (EAR), RDI or adequate intake (AI) for total fat has not been set since it is the type of fats consumed that relate to essentiality and to many of the physiological and health outcomes. However, AIs have been set for the most common dietary polyunsaturated fatty acid, linoleic acid (LA), with the female and male AIs established at around 8 and 13 g/day, respectively (NRVANZ, 2006). With a total fat concentration of around 0.2 g/100 g (FW), assuming 100% of banana fat was LA and 100% bioavailability, an average male would need to consume more than 6.5 kg (~50 peeled fruit) of banana per day to ingest the recommended AI for LA. An average Australian male requires a minimum of 23 kg (64 g x 365 days) of protein and 4.7 kg (13 g x 365 days) of linoleic acid per year as extrapolated from the protein RDI and linoleic acid AI for these nutrients (NRVANV, 2006). To meet these requirements for protein and LA, an average Australian male would need to consume a minimum of 2.3 tonnes and 470 kg of banana per year, respectively. Since Australians consume on average only 16 kg of Cavendish banana per year (Hort Innovation, 2022), this would only represent ~0.7% of both their protein (160 g) and fat (32 g assuming 100% LA) yearly requirement. In summary, bananas cannot be considered a significant contributor to the protein and fat intake of the typical Australian diet. Therefore, any changes (even significant) in the amino acid or fatty acid composition of edible parts of event QCAV-4 are inconsequential to the biosafety assessment of this event.

For each analyte tested, the mean (± standard deviation (SD)) and the associated range of the data are provided (Tables 8-10). Any values from the QCAV-4 analyses which extended outside the range of the non-GM controls are highlighted in bold in Tables 8-10. In addition, a combined literature range (CLR) of values for each analyte adapted from the Australian Food Composition Database (FSANZ, 2022) and the FoodData Central database of the U.S. Department of Agriculture (USDA, 2019) is included. Mean values for QCAV-4 analytes which were wholly (value ± SD) outside the range of the non-GM mean values are shaded in green while mean values for QCAV-4 and/or non-GM control analytes wholly outside the CLR ranges are shaded in orange.

The data obtained from the 5th generation of QCAV-4 fruit showed statistical differences with the non-GM counterparts for most analytes except for fat, manganese and pyridoxine (Table 8). Despite these differences, the mean data from all QCAV-4 fruit fell within the range of the non-GM control fruit grown at the same location. Further, for both QCAV-4 and non-GM control fruit, the levels of all analytes fell within the CLR except for manganese, ascorbic acid and pyridoxine which were lower than the CLR. When fruit from the 6th generation was analysed, a statistical difference between the QCAV-4 and the non-GM dataset was only observed for the average fruit manganese concentration (Table 9). Like the analyses from the 5th generation of fruit, all mean QCAV-4 fruit data fell within the reported range of the non-GM control fruit grown at the same location. The levels of all analytes fell within the CLR except for ascorbic acid, which was found to be lower for both QCAV-4 and non-GM control fruit, and manganese, which was only lower than the CLR in fruit from QCAV-4 (Table 9).

Table 8. Generation 5 banana fruit proximates, minerals and vitamins

Analyte	Genotype	n	Mean ± SD	*P* value[1]	Range (min - max)	CLR[2] (min - max)
Moisture (g/100 g)	QCAV-4	10	79.1 ± 1.0	0.0002***	77.6 - **80.4**	71.3 - 80.6
	non-GM	10	76.9 ± 1.1		75.5 - 78.6	
Fat (g/100 g)	QCAV-4	10	0.19 ± 0.00	0.0618	0.19 - 0.19	0.00 - 0.72
	non-GM	10	0.23 ± 0.05		0.19 - 0.30	
Protein (g/100 g)	QCAV-4	10	0.97 ± 0.09	0.0069**	**0.80** - 1.10	0.62 - 1.40
	non-GM	10	1.08 ± 0.06		1.00 - 1.20	
Ash (g/100 g)	QCAV-4	10	0.79 ± 0.11	0.0470*	0.70 - 1.00	0.43 - 1.00
	non-GM	10	0.89 ± 0.10		0.70 - 1.00	
Carbohydrates (g/100 g)	QCAV-4	10	19.1 ± 1.0	0.0018**	**18.0** - 21.0	17.3 - 27.5
	non-GM	10	20.9 ± 1.2		19.0 - 22.0	
Energy (kJ/100 g)	QCAV-4	10	340 ± 17	0.0003***	**320** - 370	287 - 426
	non-GM	10	377 ± 19		350 - 400	
Magnesium (mg/kg)	QCAV-4	10	261 ± 14	0.0004***	**230** - 280	180 - 380
	non-GM	10	294 ± 20		270 - 340	
Manganese (mg/kg)	QCAV-4	10	0.52 ± 0.08	0.1173	0.39 - 0.64	0.93 - 8.29
	non-GM	10	0.65 ± 0.23		0.39 - 0.98	
Potassium (mg/kg)	QCAV-4	10	3,852 ± 178	0.0436*	3,550 - **4,120**	3,000 - 4,260
	non-GM	10	3,652 ± 231		3,360 - 4,030	
Ascorbic Acid (mg/100 g)	QCAV-4	10	1.96 ± 0.18	0.0470*	1.70 - 2.20	4.0 - 15.1
	non-GM	10	1.72 ± 0.31		1.30 - 2.30	
Pyridoxine (mg/100 g)	QCAV-4	10	0.12 ± 0.04	0.6278	0.10 - 0.20	0.19 - 0.42
	non-GM	10	0.13 ± 0.05		0.10 - 0.20	

[1]Independent samples t-Test, significant differences with control asserted at 95%*, 99%**and 99.9%***

[2]Combined literature range (CLR) from the Australian Food Composition Database (F000262: Banana, cavendish, peeled, raw) (FSANZ, 2022) and the FoodData Central database of the U.S. Department of Agriculture, Agricultural Research Service for "Bananas, raw (FDC ID: 173944)", "Bananas, overripe, raw (FDC ID: 1105073)" and "Bananas, ripe and slightly ripe, raw (FDC ID: 1105314)" (USDA, 2019). n = biological replicates; SD = standard deviation.

Table 9. Generation 6 banana fruit proximates, minerals and vitamins

Analyte	Genotype	n	Mean ± SD	*P* value[1]	Range (min - max)	CLR[2] (min - max)
Moisture (g/100 g)	QCAV-4	10	79.2 ± 1.2	0.0913	78.1 - **81.3**	71.3 - 80.6
	non-GM	10	78.1 ± 1.7		75.5 - 80.8	
Fat (g/100 g)	QCAV-4	10	0.19 ± 0.00	1.0000	0.19 - 0.19	0.00 - 0.72
	non-GM	10	0.19 ± 0.00		0.19 - 0.19	
Protein (g/100 g)	QCAV-4	10	1.07 ± 0.29	0.1108	**0.30** - 1.40	0.62 - 1.40
	non-GM	10	1.24 ± 0.13		1.10 - 1.40	
Ash (g/100 g)	QCAV-4	10	1.05 ± 0.41	0.7934	**0.60** - **1.90**	0.43 - 1.00
	non-GM	10	1.10 ± 0.43		0.70 - 1.80	
Carbohydrates (g/100 g)	QCAV-4	10	18.8 ± 1.5	0.3785	**14.0** - 21.0	17.3 - 27.5
	non-GM	10	19.5 ± 1.9		17.0 - 22.0	
Energy (kJ/100 g)	QCAV-4	10	336 ± 25	0.2281	**250** - 360	287 - 426
	non-GM	10	352 ± 32		310 - 400	
Magnesium (mg/kg)	QCAV-4	10	295 ± 20	0.2467	**260** - 330	180 - 380
	non-GM	10	308 ± 28		270 - 370	
Manganese (mg/kg)	QCAV-4	10	0.63 ± 0.14	0.0475*	**0.42** - 0.92	0.93 - 8.29
	non-GM	10	0.88 ± 0.34		0.55 - 1.70	
Potassium (mg/kg)	QCAV-4	10	3,788 ± 204	0.7682	**3,450** - 4,110	3,000 - 4,260
	non-GM	10	3,760 ± 214		3,510 - 4,120	
Ascorbic Acid (mg/100 g)	QCAV-4	10	2.28 ± 0.43	0.1011	1.40 - **3.00**	4.0 - 15.1
	non-GM	10	1.97 ± 0.37		1.30 - 2.60	
Pyridoxine (mg/100 g)	QCAV-4	10	0.33 ± 0.03	0.9436	**0.27** - 0.39	0.19 - 0.42
	non-GM	10	0.34 ± 0.03		0.30 - 0.40	

[1]Independent samples t-Test, significant differences with control asserted at 95%*, 99%**and 99 9%***
[2]Combined literature range (CLR) from the Australian Food Composition Database (F000262: Banana, cavendish, peeled, raw) (FSANZ, 2022) and the FoodData Central database of the U.S. Department of Agriculture, Agricultural Research Service for "Bananas, raw (FDC ID: 173944)", "Bananas, overripe, raw (FDC ID: 1105073)" and "Bananas, ripe and slightly ripe, raw (FDC ID: 1105314)" (USDA, 2019). n = biological replicates; SD = standard deviation.

The concentration of the selected analytes was also measured in peel tissue collected from fruit obtained from six QCAV-4 plants and two non-GM control plants growing in generation 7 (Table 10). A statistical difference between the QCAV-4 and the non-GM dataset was only observed for protein which was found to be significantly higher in the peel of QCAV-4 fruit. Further, the mean values (value ± SD) for protein and magnesium in QCAV-4 peel were found to be outside the range of the non-GM mean values. However, at less than 1% protein content, banana peel is not a large contributor to the protein intake of the human diet making these differences insignificant from a nutritional perspective. Due to the lack of reliable data for banana peel, a CLR could not be accurately determined making the interpretation of our data in the wider context particularly difficult.

In summary, while there were statistical differences in the levels of some of the analytes between QCAV-4 and the non-GM controls, the mean values for proximates, vitamins and minerals from fruit and peel were mostly within the compositional variation reported in the literature. Further, no consistent pattern indicated that expression of the *MamRGA2* and *nptII* transgenes impacted the nutritional composition of QCAV-4. Taken together and considering that bananas are not a major contributor to diets in Australia and that QCAV-4 is not intended to replace 100% of the banana production, it is unlikely that any differences observed would have a nutritional impact on consumers of QCAV-4. Therefore, QCAV-4 should be considered nutritionally equivalent to conventional Grand Nain banana for the levels of all proximates, vitamins and minerals reported.

Table 10. Generation 7 banana peel proximates, minerals and vitamins

Analyte	Genotype	n	Mean ± SD	*P* value[1]	Range (min - max)	CLR[2]
Moisture (g/100 g)	QCAV-4	6	90.6 ± 1.3	0.7670	89.5 - **92.8**	89.8
	non-GM	2	90.3 ± 1.8		89.0 - 91.5	
Fat (g/100 g)	QCAV-4	6	0.47 ± 0.12	0.8641	**0.30** - **0.60**	0.58
	non-GM	2	0.45 ± 0.07		0.40 - 0.50	
Protein (g/100 g)	QCAV-4	6	0.73 ± 0.10	0.0125*	0.60 - **0.90**	0.83
	non-GM	2	0.45 ± 0.07		0.40 - 0.50	
Ash (g/100 g)	QCAV-4	6	2.25 ± 0.79	0.5388	1.70 - 3.80	1.31
	non-GM	2	2.75 ± 1.48		1.70 - 3.80	
Carbohydrates (g/100 g)	QCAV-4	6	5.8 ± 1.5	0.9120	5.0 - 8.0	8.7
	non-GM	2	6.0 ± 2.8		4.0 - 8.0	
Energy (kJ/100 g)	QCAV-4	6	128 ± 26	0.9009	90 - 160	NA
	non-GM	2	125 ± 49		90 - 160	
Magnesium (mg/kg)	QCAV-4	6	125 ± 12	0.0781	**110** - 140	140
	non-GM	2	145 ± 7		140 - 150	
Manganese (mg/kg)	QCAV-4	6	2.55 ± 1.22	0.6446	**1.10** - **4.00**	2.25
	non-GM	2	2.10 ± 0.57		1.70 - 2.50	
Potassium (mg/kg)	QCAV-4	6	8,147 ± 241	0.0721	7,840 - **8,520**	6,479
	non-GM	2	7,645 ± 431		7,340 - 7,950	
Ascorbic Acid (mg/100 g)	QCAV-4	6	0.90 ± 0.00	0.6036	0.90 - 0.90	NA
	non-GM	2	0.90 ± 0.00		0.90 - 0.90	
Pyridoxine (mg/100 g)	QCAV-4	6	0.10 ± 0.02	0.1144	0.08 - **0.12**	NA
	non-GM	2	0.08 ± 0.02		0.06 - 0.09	

[1]Independent samples t-Test, significant differences with control asserted at 95%*, 99%**and 99 9%***
[2]Combined literature range (CLR) difficult to establish due to the lack of quality references for banana peel, however peel data from Cavendish banana cv Grand Nain published by Emaga *et al.* (2007) was converted as a guide. n = biological replicates; SD = standard deviation.

C. Information related to the nutritional impact of the genetically-modified food

Bananas are a major food crop globally and are grown and consumed in more than 100 countries throughout the tropics and sub-tropics. Domesticated bananas are cultivated mainly for their edible fruit and are not known to cause disease or show toxicity in humans or animals and have a long history of safe use as a food. The QCAV-4 banana event described in this submission has been transformed with a gene (*MamRGA2*) derived from a wild banana to confer resistance to the fungal disease Fusarium wilt TR4. The introduction of this gene has no nutritional impact on QCAV-4 based on the following:

(i) Stability of the insert across multiple plant generations
(ii) The presence of *MamRGA2* homologues in Cavendish bananas with a history of safe consumption
(iii) No significant homology to known allergens and toxins
(iv) Compositional analysis of fruit and peel from QCAV-4 showing substantial equivalence to that derived from the non-GM counterpart

In summary, the analysis of event QCAV-4 presented in this submission has not revealed any biologically relevant differences to the non-GM Grand Nain counterpart which would affect its nutritional impact.

D. Other Information

Not applicable.

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