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

**25 September 2023**
**263-23**

## Supporting document 1

Safety assessment – **Application A1274**

Food derived from disease-resistant banana line QCAV-4

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# Executive summary

### Background

Application A1274 seeks approval for the sale and use of food derived from genetically modified (GM) banana line QCAV-4 that has resistance to the fungal disease Fusarium wilt tropical race 4 (TR4), also known as Panama disease.

Disease resistance is conferred by the expression of the novel plant resistance protein MamRGA2, encoded by the *MamRGA2* gene from a wild banana, *Musa acuminata ssp. malaccensis*. The MamRGA2 protein allows the banana plant to detect the presence of the infecting fungus, and triggers the plant's defence response preventing further infection by the fungus. FSANZ has not previously assessed the MamRGA2 protein.

Banana line QCAV-4 also contains a commonly used antibiotic resistance marker gene *nptII* from the ubiquitous gut bacterium *Escherichia coli*. *nptII* encodes the neomycin phosphotranferase type II protein (NPTII) and confers resistance to the antibiotics neomycin and kanamycin. The NPTII protein has been assessed by FSANZ in previous applications.

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

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

### History of use

Banana is one of the most consumed fruit in the world and has a long history of safe use in the food supply. The flesh is commonly consumed raw, however, it sometimes can be processed e.g. dried or frozen, pulped or baked. The peel is less commonly consumed, but can be used in baking and cooking.

### Molecular characterisation

The *MamRGA2* and *nptII* genes were introduced into banana line QCAV-4 via *Agrobacterium*-mediated transformation. Detailed molecular analyses indicate that three copies of the fully functional insert and two incomplete non-functional fragments of the

*MamRGA2* expression cassette are present at a single insertion site / locus in the genome of QCAV-4. There are no extraneous plasmid sequences present in this line.

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

**Characterisation and safety assessment of new substances**

The MamRGA2 protein is present in banana line QCAV-4 at very low levels, particularly in the banana flesh. While the *MamRGA2* gene was derived from a wild banana, there is a history of human exposure to the encoded protein as the source of the gene is one of the main subspecies that has contributed to the genetics of commercial bananas. As a result, homologs of RGA2 of high sequence similarity are present in commercial banana varieties and, like MamRGA2, are also expressed at very low levels.

Bioinformatic studies confirmed a lack of any significant amino acid sequence similarity between MamRGA2 and known protein toxins or allergens. Laboratory studies also demonstrated MamRGA2 is susceptible to the digestive enzyme pepsin and would be thoroughly degraded before it could be absorbed during passage through the gastrointestinal tract. Together with the history of safe exposure to this protein, the evidence supports the conclusion that MamRGA2 is not toxic or allergenic to humans.

The NPTII protein is present at average levels of 4.5 ng/mg fresh weight (fw) in peel and 3.1 ng/mg fw in the flesh. A range of characterisation analyses confirmed the identity of NPTII in QCAV-4. An extensive database demonstrating the safety of NPTII exists. Updated bioinformatic analyses undertaken for this application confirmed that the expressed protein is unlikely to be toxic or allergenic to humans.

**Compositional analyses**

Compositional analyses were undertaken of the banana flesh, as well as some limited analyses of the peel. While some statistically significant differences in mean values were found for some constituents between QCAV-4 and the control, these differences were generally within the range of natural variation for banana. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in QCAV-4 compared to non-GM banana cultivars available on the market.

**Conclusion**

No potential public health and safety concerns have been identified in the assessment of disease-resistant banana line QCAV-4. On the basis of the data provided in the present application and other available information, food derived from QCAV-4 is considered to be as safe for human consumption as food derived from non-GM banana cultivars.

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## List of Abbreviations

| Abbreviation | Definition |
|---|---|
| ATP | Adenosine 5'-triphosphate |
| B-lactoglobulin | Beta-lactoglobulin |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pair |
| COMPARE | COMprehensive Protein Allergen REsource |
| Chr | Chromosome |
| *CyP* | peptidyl-prolyl cis-trans isomerase – banana endogenous reference gene |
| DIG | Digitonin |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme Linked Immunosorbent Assay |
| ER | Endoplasmic reticulum |
| FAO | Food and Agriculture Organization of the United Nations |
| FSANZ | Food Standards Australia New Zealand |
| fw | Fresh weight |
| g | gram |
| Gb | Gigabase |
| gDNA | Genomic DNA |
| GM | Genetically modified |
| kDa | Kilodalton |
| kg | Kilogram |
| LB | Left border |
| LC MS/MS | Liquid chromatography-tandem mass spectrometry |
| LOD | Limit of detection |
| LRR | Leucine-rich repeat |
| *MamRGA2* | Gene encoding resistance protein from wild-seeded banana (*Musa acuminata* ssp. *malaccensis*) |
| MamRGA2 | Protein encoded by the *MamRGA2* |
| MaRGA2 | Resistance protein in Cavendish banana |

| Abbreviation | Definition |
|---|---|
| MT | Million tonnes |
| NB | Nucleotide binding |
| NB-LRR | Nucleotide-binding leucine rich repeat |
| NCBI | National Centre for Biotechnology Information |
| Ng | nanogram |
| NGS | Next Generation Sequencing |
| *nptII* | neomycin phosphotransferase type II (antibiotic-resistance marker gene) |
| NPTII | Protein encoded by *nptII* |
| OECD | Organisation for Economic Cooperation and Development |
| ORF | Open reading frame |
| PCR | Polymerase chain reaction |
| QCAV4-6 | Provisional OECD unique identifier for the banana event selected for commercial approval |
| qRT-PCR | Quantitative reverse-transcription polymerase chain reaction |
| RB | Right border |
| RNA | Ribonucleic acid |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl-sulfate polyacrylamide gel electrophoresis |
| sp | species |
| ssp | subspecies |
| T-DNA | Transfer DNA |
| TR4 | Tropical race 4 |
| µg | microgram |
| VNT1 | Resistance protein homolog from wild potato (*solanum venturi*) |

# 1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Queensland University of Technology to vary Schedule 26 in the Australia New Zealand Food Standards Code (the Code). The variation is to include food from a new genetically modified (GM) banana line QCAV-4, with the provisional OECD Unique Identifier QUT-QCAV4-6. This banana line is resistant to the fungal disease Fusarium wilt tropical race 4 (TR4), also known as Panama disease.

Disease resistance is conferred by the expression of the novel plant resistance protein MamRGA2, encoded by the *MamRGA2* gene from a wild banana, *Musa acuminata ssp. malaccensis*. The MamRGA2 protein allows the banana plant to detect the presence of the infecting fungus, and triggers the plant's defence response preventing further infection by the fungus. FSANZ has not previously assessed the MamRGA2 protein.

The applicant is currently seeking a licence for the commercial cultivation of banana line QCAV-4 from the Gene Technology Regulator (GTR[1]). If approved by the GTR, banana line QCAV-4 may be cultivated in Australia and therefore it is anticipated that fresh fruit and processed food derived from QCAV-4 may be available in Australia and New Zealand.

The applicant has stated that Australia's banana industry mainly serves the domestic market, therefore fresh fruit derived from the GM banana is unlikely to be exported and sold in New Zealand if approved to be cultivated in Australia.

# 2 History of use

## 2.1 Host organism

The host organism is the commercial Cavendish banana and the parental cultivar used for the genetic modification is Grand Nain. The Grand Nain host organism was used as the conventional control for the purposes of comparative assessment with QCAV-4.

Domesticated banana belongs to the Musaceae family and originated in Southeast Asia, where it has been cultivated for human consumption for about 7000 years. It is believed that human migration activities introduced the banana to other parts of the world (Simmonds and Shepherd 1955; Heslop-Harrison and Schwarzacher 2007; Perrier et al. 2011b). Most of the domesticated banana plants are sterile seedless triploids that are derived from inter and intra-specific hybridisations between two wild-seeded diploid ancestors, *Musa acuminata* (genome designation, AA) and *Musa balbisiana* (genome designation, BB) (Simmonds and Shepherd 1955).

The Cavendish banana is a sterile triploid (AAA) subgroup of *Musa acuminata* and is currently the most traded banana variety in the world. There are six cultivars of Cavendish: Williams, Dwarf Cavendish, Dwarf Parfitt, Pisang Masak Hijau, Double and Grand Nain (OGTR 2023). Williams is the most widely grown commercial cultivar, followed by Grand Nain.

Prior to Cavendish, the Gros Michel banana, another subgroup of *Musa acuminata*, was the dominant export banana in the 1950s. Gros Michel was severely impacted by the fungal disease Fusarium wilt tropical race 1 (TR1), which nearly wiped out the banana industry.

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

Resistant cultivars of the Cavendish subgroup were used to replace Gros Michel, but are now succumbing to a new variant of the fungal disease, tropical race 4 (TR4) (Ploetz 2015; Dale et al. 2017).

The banana plant is perennial and contains a false trunk made of leaf sheaths known as a pseudostem[2] (Figure 1A). Banana plants grow from an underground stem known as corm[3] that can produce suckers (Figure 1A) and are used to propagate the plant. The pseudostem produces a single flowerhead, which bears banana fruits with or without fertilisation. The fruit (Figure 1B) is the main product of the banana plant and there are two types of fruits; sweet or dessert banana (referred to as banana) and a less sweet banana (referred to as plantain[4]) (OGTR 2023).

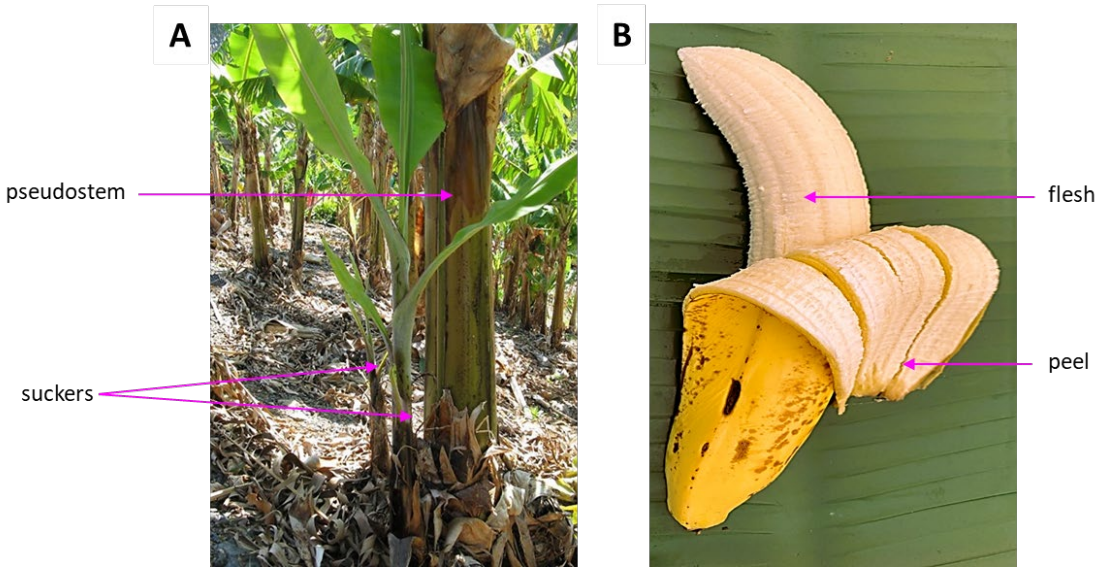



*Figure 1: **A**. Banana plant showing the suckers and pseudostem (Photo adapted from 'The Biology of Musa L. (banana)' (OGTR, 2023). **B**. Banana fruit showing the flesh and peel.*

Globally, banana plant is a major food crop and is grown in approximately 135 countries (FAOSTAT 2023). In 2021, global production of bananas was 137 MT[5] and the top two banana producing countries were India (33.06 MT) and China (23.79 MT) (FAOSTAT 2023).

[2] Part of the banana plant that looks like a trunk.
[3] The corm is the site at which a banana plant produces "suckers", or offshoots of young banana plants that grow in clusters around the "mother" plant.
[4] Often used for cooking.
[5] Million tonnes

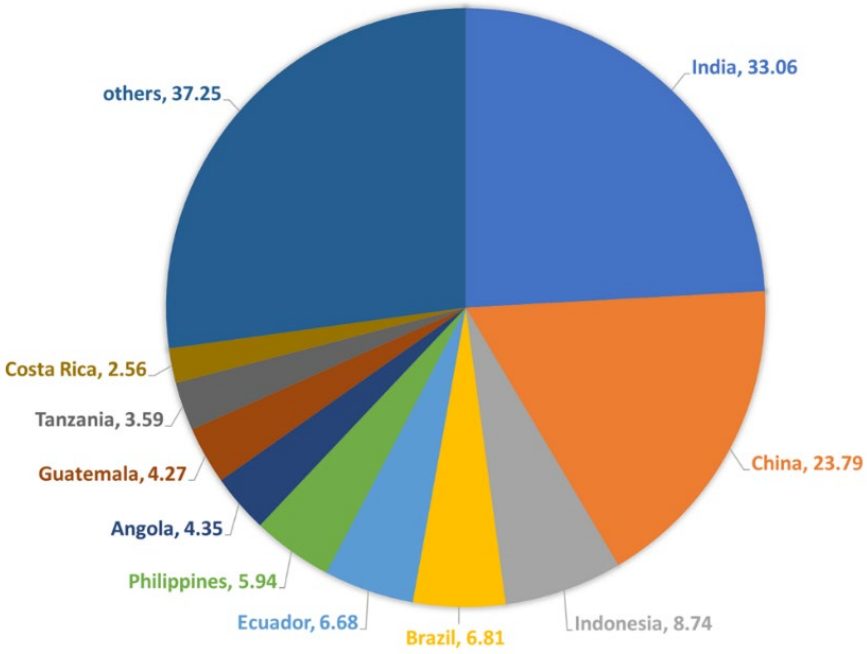



*Figure 2: Major banana producing countries in 2021 (in MT). Data obtained from FAOSTAT (2023)*

Compared to other countries, Australia's banana production is limited, with an estimated 0.35 MT in 2021 (FAOSTAT 2023). Currently, Queensland produces around 94% of Australia's bananas (OGTR 2023). The Australian banana sector mostly serves the domestic market, with little export activity (Hort Innovation 2023).

New Zealand has no commercial banana production (FAOSTAT, 2023). However, there has recently been banana cultivation in various regions of New Zealand's North Island, which currently caters to the local community but seeks to build New Zealand's first banana industry (Rowe 2022). Currently, New Zealand imports bananas from Ecuador, the Philippines, and Mexico to complement its domestic production (TrendEconomy 2023). In 2021, New Zealand imported 0.085 MT of banana (FAOSTAT 2023).

While banana is typically eaten raw as a fresh fruit, it can also be processed and sold in various forms i.e. dried, frozen, or pulped or used in various baked goods. Banana peel is not often consumed raw, however it is used in baking and occasionally in cooking (Hikal et al. 2022). Other parts of the banana plant are also eaten, i.e. the flower and pseudostem are used for cooking in Southeast Asia. Where there is an excess of banana production, all portions of the plant are used as livestock feed (OGTR 2023).

## 2.2 Donor organisms

### 2.2.1 Musa acuminata ssp. malaccensis

The *MamRGA2* DNA sequence encoding the MaMRGA2 protein is derived from *M. acuminata* ssp. *malaccensis*, a wild, seeded diploid banana. *Malaccensis* is one of the main subspecies of *Musa acuminata* to have contributed to the genetics of edible bananas during their domestication (Perrier et al. 2009; Perrier et al. 2011a; Perrier et al. 2011b). The Cavendish banana genome has three endogenous MaRGA2 alleles that are 97% identical to MamRGA2. (Dale et al. 2017)

### 2.2.2 Escherichia coli

The *nptII* gene encodes the NPTII protein and is derived from *Escherichia coli* strain K12. This is a non-pathogenic, facultative anaerobic bacterium commonly found in the gastrointestinal tract of humans and animals. *E. coli* K12 is used globally in the commercial manufacturing of products ranging from amino acids and vitamins for food applications, to recombinant human proteins used in pharmaceutical applications, including protein products used as injectables (JECFA 1991; Huang et al. 2012).

### 2.2.3 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of QCAV-4 (refer to Table 1). These genetic elements are non-coding sequences that are used to regulate the expression of *MamRGA2* and *nptII*.

# 3 Molecular characterisation

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

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

## 3.1 Transformation method

To create the QCAV-4 banana line, the Grand Nain cultivar was transformed using the plasmid pSAN3 (Figure 3). The methodology is outlined in a flowchart in Appendix 1 and summarised below.

Transformation of Grand Nain was achieved by incubating embryogenic cell suspensions (referred to as ECS), derived from immature male flowers, with *Agrobacterium tumefaciens* containing the pSAN3 plasmid. The ECS were then layered on glass fibre filter disks and maintained on media containing antibiotics, kanamycin and timentin, for 3 months. Kanamycin inhibits the growth of untransformed plant cells, while timentin suppresses the growth of excess *Agrobacterium*. The embryos were sub-cultured to a fresh medium every month with increasing concentration of kanamycin to a final concentration of 100mg/L. The ECS were further maintained on media with the higher kanamycin concentration for 3 months while sub-culturing to fresh medium every month.

The ECS were then placed on shoot induction selective medium. The regenerated plantlets were transferred to rooting medium to promote root growth. Up to this point, the plantlets were maintained in selective medium containing kanamycin and timentin.

Rooted plantlets were subsequently multiplied via micropropagation[6] and then transferred to soil, where they were tested for the presence of the *MamRGA2* and *nptII* genes using standard molecular biology techniques. Following the evaluation of insert integrity, gene expression, phenotypic characteristics and agronomic performance, banana line QCAV-4 was selected.

[6] Micropropagation is a method of plant propagation using extremely small pieces of plant tissue taken from a carefully chosen and prepared mother plant and growing these under laboratory conditions to produce genetically identical plantlets.

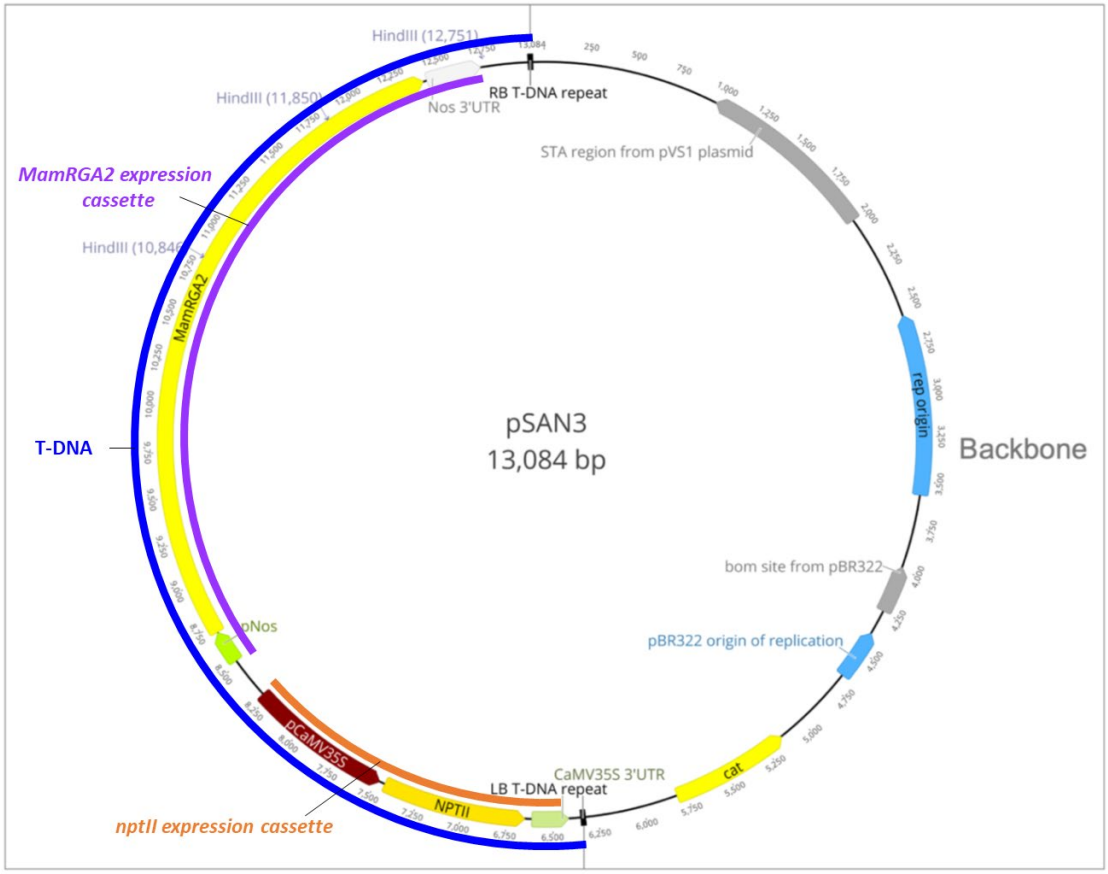



*Figure 3: Plasmid map of pSAN3. The T-DNA region comprising the MamRGA2 and nptII expression cassettes is highlighted using the blue bar. The MamRGA2 expression cassette is highlighted using the purple bar. The nptII expression cassette is highlighted using the orange bar.*

## 3.2 Detailed description of inserted DNA

Banana line QCAV-4 contains transfer DNA (T-DNA) from pSAN3 plasmid (Figure 3). The T-DNA includes the *MamRGA2* and *nptII* expression cassettes. Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. Additional detail, including the plasmid backbone and intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 2.

**Table 1: Expression cassettes contained in the T-DNA of pSAN3**

| Expression cassette | Promoter (Drives expression) | Coding sequence | Terminator (Polyadenylation and termination of transcription) |
|---|---|---|---|
| *MamRGA2* expression cassette | Nos promoter of the nopaline synthase (NOS) gene from *Agrobacterium tumefaciens* | *MamRGA2* coding sequence from *Musa acuminata ssp. malaccensis* | 3'UTR of the nopaline synthase (*NOS*) gene from *Agrobacterium tumefaciens* |
| *nptII* expression cassette | 35S Promoter of the 35S RNA from *Cauliflower Mosaic Virus* | *nptII* coding sequence of the Transposon Tn5 from *Escherichia coli* strain K12 | 3' UTR of the 35S RNA from Cauliflower mosaic virus |

## 3.3 Development of the banana line from the original transformant

After the transformation and selection process, the initial transformant, QCAV-4, was identified following molecular characterisation. New plantlets were generated in tissue culture from QCAV-4 meristems[7]. The newly generated plantlets were maintained in culture and allowed to root. The plantlets with roots were then transferred to soil in greenhouses to acclimatise. The acclimatised plants were used in the 1st field trial to assess their resistance to Fusarium wilt and agronomic performance.

Cultivated bananas are effectively sterile and the use of meristems for multiplication is a characteristic of banana, allowing cultivation through vegetative propagation rather than by sexual reproduction. The progeny arising from this form of asexual reproduction will be genetically identical to the parent plant.

Sucker meristems from promising lines identified in the 1st field trial were used in tissue culture to create new plantlets. Plantlets were allowed to root in culture before being moved to soil to acclimatise. The acclimatised plantlets were subsequently used as the 'plant crop' in a 2nd field trial.

After a fruit bunch on the plant develops and matures, the plant pseudostem dies, and another new pseudostem, known as the first ratoon, arises from another meristem on the basal corm. This process was repeated through multiple ratoons.

Different generations of plants were examined when characterising QCAV-4. Plants generated in tissue culture from the initial multiplication of plantlets are referred to as G0, plants generated from G0 plants are referred to as G1, and so forth (Figure 4). Table 2 summarises the analysis performed and the generations at which QCAV-4 was examined.

[7] The centre of active mitotic cell division where plant growth occurs.

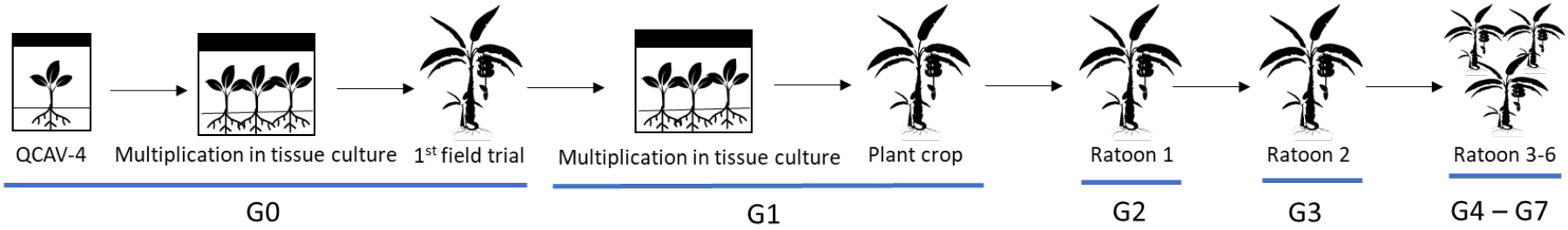



*Figure 4: Propagation path used in the characterisation of QCAV-4*

**Table 2: QCAV-4 generations used for various analyses**

| Analysis | Section | Generation(s) used | Comparators |
|---|---|---|---|
| Number of integration sites | 3.4.1 | G0 | Cavendish cv Grand Nain |
| Absence of backbone and other sequences | 3.4.2 | G0 | Cavendish cv Grand Nain |
| Insert integrity and site of integration | 3.4.3 | G0 | Cavendish cv Grand Nain |
| Genetic stability | 3.4.4.1 | G0, G1, G2, G3 | Cavendish cv Grand Nain |
| Expression of phenotype over several generations | 3.4.4.2 | G1, G2, G3, G4, G5 | Cavendish cv Grand Nain |
| Compositional analysis | 5 | G5, G6, G7 | Cavendish cv Grand Nain |

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

A range of analyses were undertaken to characterise the genetic modification in QCAV-4. These analyses focused on the nature and stability of the insertion and whether any unintended re-arrangements or expression products may have occurred as a consequence of the transformation procedure.

### 3.4.1 Number of integration site(s)

Southern blot analysis was used to analyse the insertion site(s) and determine copy number. Genomic DNA (gDNA), isolated from leaves of the original mother plant (G0), was digested with restriction enzyme *Hin*dIII and hybridised with DIG-labelled probes for *MamRGA2*. gDNA from the conventional control (Cavendish cv Grand Nain) served as a negative control, while plasmid pSAN3 served as a positive control. The results revealed the presence of three copies of T-DNA in the host genome. Additionally, the probe showed hybridisation to three endogenous RGA2 homologs (*MaRGA2*), which were distinguished from *MamRGA2* by their distinctive and predicted sizes.

To further support the Southern blot results, next-generation sequencing (NGS) was performed on gDNA isolated from the leaves of QCAV-4 (G0) and the conventional control. Paired end reads (2x 150 bp) with a total of 106.9 Gb of data was generated using the Illumina platform. Sufficient sequence reads were obtained to cover the inserted T-DNA, with a depth coverage of 178X.

Comparison of the sequence between QCAV-4 and pSAN3 detected two unique insert-flank junction sites, each comprising the inserted T-DNA border sequence joined to a flanking sequence in the banana genome. In addition, multiple T-DNA repeats were detected. This indicates a single insertion site containing multiple copies of the intended DNA insert in the genome of QCAV-4. As expected, no junction sites were detected in the control.

### 3.4.2 Absence of backbone and other sequences

NGS reads from QCAV-4 (G0) and the sequence of the pSAN3 transformation plasmid were aligned. A small number of reads mapped to backbone sequences (Figure 3) however this is likely due to the presence of endogenous bacteria in the original sample used to prepare the gDNA for NGS (Yang et al. 2013; Zastrow-Hayes et al. 2015). Overall, the results of this alignment confirmed the absence of pSAN3 backbone sequences, including any antibiotic resistance genes, in the QCAV-4 genome.

### 3.4.3 Insert integrity and site of integration

To examine the T-DNA insertion site, long-read sequences with a total of 75.9 Gb were generated using the PacBio platform with a read length N50[8] of 17,973 bp and depth coverage of 42X. Comparison of the long-reads with the T-DNA sequence of pSAN3 showed that three copies of the 6702 bp T-DNA from pSAN3, referred to as T-DNA 1, T-DNA 2 and T-DNA 3, were integrated into the host genome (Figure 5).

Additionally, a 6668 bp rearranged fragment of the *MamRGA2* expression cassettes was found between T-DNA 2 and T-DNA 3 (Figure 5). The rearranged fragment consists of a 3042 bp truncated region of *MamRGA2* and a 2672 bp truncated region of *MamRGA2* (with its NOS 3'UTR) recombined in opposite direction and a 142 bp region of the CaMV35S promoter (Figure 5). This sequencing data is consistent with the Southern blotting results

[8] N50 represents the length of the shortest read in the group of longest sequences that together represent (at least) 50% of the nucleotides in the set of sequences.

(see Section 3.4.1).

The *MamRGA2* coding sequences in the 6668 bp rearranged fragment are truncated and therefore non-functional. They are also unlikely to be expressed due to the absence of intact regulatory elements. The presence of these additional DNA sequences does not raise potential safety concerns. The potential for this DNA to lead to protein expression is addressed in Section 3.4.5 .

To determine the location of T-DNA insertion in the host's genome, flanking sequences were obtained by aligning the NGS data of QCAV-4 and pSAN3 plasmid sequence. The identified banana sequences flanking the insertion site were further subjected to homology searches against the reference genome sequence[9] of *Musa acuminata* (Altschul et al. 1990). These searches located the T-DNA insert at a single location in chromosome 6. A 116 bp deletion of the banana genome at the T-DNA integration site was identified which corresponded to an intergenic region. The insertion did not disrupt any endogenous genes or any other known annotated feature in the banana genome.

Several rearrangements were identified at the insert/flank junctions and the T-DNA/T-DNA junctions (Figure 5, orange bars). Such changes are common during *Agrobacterium*-mediated plant transformation due to double-strand break repair mechanisms (Gheysen et al. 1991; Mayerhofer et al. 1991; Gelvin 2021). These changes would not affect the expression of the *MamRGA2* and *nptII* genes.

### 3.4.4 Stability of the genetic changes in QCAV-4

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

#### *3.4.4.1 Genetic stability*

As commercial bananas are vegetatively propagated, standard Mendelian segregation analysis could not be used to determine inheritance. In order to confirm that progenies were genetically identical to the parent and to ensure the stability of the inserted DNA over time, Southern blot analysis was performed on leaf-derived genomic DNA obtained from G0 to G3 plants (Figure 4). gDNA from the conventional control served as a negative control, while plasmid pSAN3 served as a positive control. Each gDNA sample was digested with *Hind*III and hybridised with a *MamRGA2*-specific probe. The results confirmed that the inserted DNA was stably integrated over 3 successive clonal generations and remained stable over this time period for QCAV-

[9] Banana Genome Hub, Musa acuminata Double Haploid-Pahang (version 4), http://banana-genome-hub.southgreen.fr/)

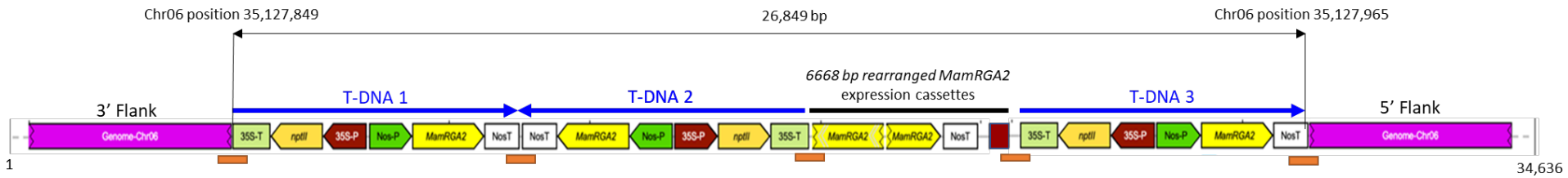



*Figure 5: T-DNA insert present in QVAC-4.The insert contains three fully intact and functional copies of the T-DNA (blue arrows) as well as two fragments of the MamRGA2 expression cassette recombined in opposite direction and inserted between T-DNA 2 and T-DNA 3 (black bar). The insert/flank junctions and T-DNA/T-DNA junction contain various levels of rearrangements and are indicated with orange bars.*

#### *3.4.4.2 Phenotypic stability*

As an intractable protein, the level of MamRGA2 is difficult to quantify (Section 4.1.1). Phenotypic stability was therefore demonstrated by examining the disease resistant trait. The level of disease-resistance was examined in six generations of QCAV-4 (G0, G1, G2, G3, G4 and G5), using plants from two field trials; 1st field trial: 2012 – 2015 and 2nd field trial: 2018 – 2023. The field trials were conducted in a commercial banana farm in the Northern Territory with high Fusarium wilt disease pressure.

The level of disease-resistance was examined by observing the correlation between disease symptoms in infected plants and *MamRGA2* gene expression measured by qRT-PCR[10]. QCAV-4 plants conferred a high level of resistance to Fusarium wilt, with only 2% of plants showing disease symptoms compared to 66% of plants showing disease symptoms in conventional control plants across all generations. The results from the 1st field trial have been published in Dale et al. (2017).

These field trials indicate that the disease-resistance phenotype in QCAV-4 is stable over several generations.

### 3.4.5 Open reading frame (ORF) analysis

A bioinformatic analysis of the QCAV-4 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel open reading frames (ORFs) had been created in QCAV-4 as a result of T-DNA insertion. This analysis also examined whether any putative peptides present in the insert have the potential for allergenicity or toxicity.

The sequence of the entire QCAV-4 insert including sequences spanning the 5' and 3' insert-flank junction of QCAV-4 were translated *in silico* from start-to-stop codon (TGA, TAG, TAA) in all six reading frames in Geneious Prime[11]. A total of 7 ORFs were identified that correspond to putative peptides of eight amino acids or greater in length.

The 7 putative peptides were initially screened using the NCBI protein BLAST search tool[12]. 6 putative peptides did not align significantly (E score $<10^{-5}$) to any protein in this database. Predictably, one had significant homologies with the newly expressed protein MamRGA2.

Putative peptides were used as query sequences in homology searches for known allergens and toxins in established databases. These analyses are theoretical only as there is no reason to expect that any of the identified ORFs would, in fact, be expressed.

#### *3.4.5.1 Bioinformatic analysis for potential allergenicity*

The 7 putative peptides were queried against known allergenic proteins listed in the Allergen Online database[13] (version 21). At the date of the search (27 October 2022), there were 2,233 sequences in the allergen database.

Three types of analyses were performed for this comparison:

(a) full length sequence search – a FASTA36 alignment using a BLOSUM50 scoring matrix. Only matches with E-scores of $< 1\times10^{-4}$ were considered.
(b) 80-mer sliding window search – a FASTA alignment was performed comparing all

[10] Quantitative reverse-transcription polymerase chain reaction.
[11] https://www.geneious.com/
[12] blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch
[13] www.allergenonline.org

contiguous 80 amino acids to the database entries. Only matches of greater than 35% similarity over ≥ 80 amino acids were considered.

(c) 8-mer exact match search – a FASTA alignment was performed comparing all contiguous 8 amino acids to the database entries. Only matches of 100% similarity over 8 amino acids were considered.

The alignment of the 7 putative peptides with the database sequences did not identify any similarities to known allergens in the database. The results of this analysis support the conclusion that there were no matches of significance or concern.

#### *3.4.5.2 Bioinformatic analysis for potential toxicity*

The applicant provided results from *in silico* analysis using a toxin database created in Geneious Prime version 2022.2.1. from a subset of sequences derived from UniProt Knowledgebase[14] that were selected using a keyword search for toxins. At the time of the analysis, 29 August 2022, the collection contained a total of 92,851 sequences.

The 7 putative peptides were examined for the presence of any known toxins found in the toxin database. Significant homology was determined based on a E score of $<10^{-5}$ and a match was identified with a MamRGA2-associated peptide. This peptide is associated with the newly expressed MamRGA2 protein and is considered in detail in Section 4.1.3, where it was concluded the homology is not biologically meaningful and does not raise a safety concern. No other significant homology was found with the putative peptides and known toxins.

### 3.4.6 Conclusion

The data provided by the applicant showed that an integration event has occurred at a single locus in the banana genome. The sequencing data confirmed three fully intact T-DNAs with *MamRGA2* and *nptII* expression cassettes in the genome of QCAV-4. No plasmid backbone sequences, including antibiotic resistance genes, from the transforming pSAN3 plasmid were present. The introduced DNA was shown to be stably inherited from one generation to the next. No new ORFs were created by the insertion that raise potential allergenicity or toxicity concerns.

# 4 Characterisation and safety assessment of novel substances

The main purpose of the characterisation is to describe the nature of any new substances and their phenotypic and biochemical effects on the organism in which they are expressed, particularly in the parts of the organism consumed as food. Typically, the main focus of the characterisation is on newly expressed (or potentially expressed) proteins, but other (non-protein) substances may also be considered.

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

[14] https://www.uniprot.org/help/uniprotkb

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

Two novel substances are expressed in QCAV-4, MamRGA2 and NPTII, and are assessed below.

## 4.1 MamRGA2

The MamRGA2 protein belongs to a group of common plant resistance proteins (R-protein), that contain nucleotide binding (NB) and leucine-rich repeat (LRR) domains. These NB-LRR proteins are used by plants to detect the presence of pathogenic molecules, known as effectors, that are indicative of an infection (Jones and Dangl 2006; McHale et al. 2006).

Once the NB-LRR detects the presence of an effector, the plants' defence mechanisms are activated, leading to the development of an immune response to the pathogen. The pathogen that MamRGA2 recognises and mediates a response to is the fungus, *Fusarium oxysporum* f. sp. cubense Tropical Race 4 (Dale et al. 2017; Wang et al. 2021).

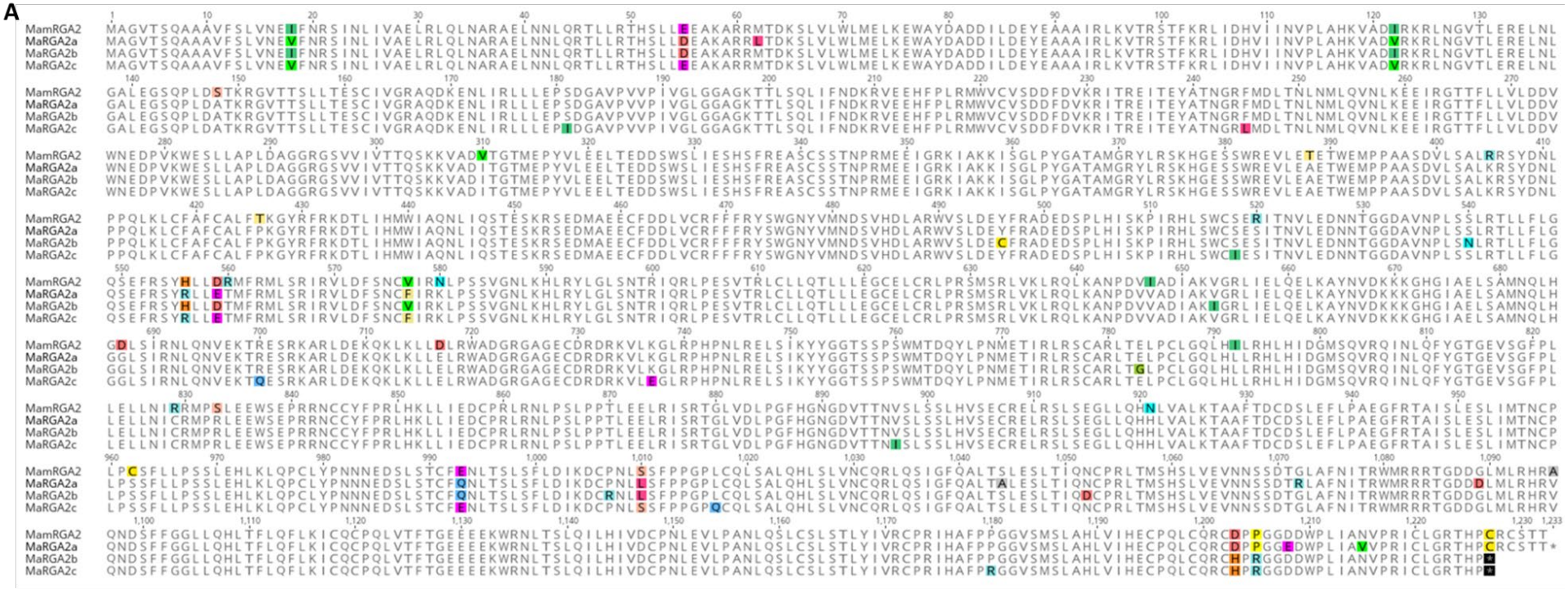
Banana line QCAV-4 expressing MamRGA2 protein shows disease-resistance against Fusarium wilt compared to its conventional control (Dale et al. 2017). The disease-resistance in QCAV-4 is correlated to the expression of *MamRGA2* under high Fusarium wilt disease pressure.

MamRGA2 shares high homology at both the nucleotide and amino acid level to several R-proteins. These proteins are ubiquitous in plants and found in many food crops with a history of safe use, such as tomato, rice, soybean, maize, potato, chickpea, bean, cassava, sorghum and wheat (Baggs et al. 2017; Liu et al. 2021).

*Musa acuminata ssp. malaccensis*, the source of the gene encoding the MamRGA2, is a subspecies of the modern day edible banana. Consequently, the Cavendish banana genome contains three alleles of endogenous RGA2 (MaRGA2) which have a minimum of 97.3% identity to MamRGA2 (Figure 6).

The MamRGA2 gene prepared by the applicant encodes 1232 amino acids, with an expected mass of 139.6 kDa.

**A**




**B**

| 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 6: Amino acid sequence alignment showing differences between MamRGA2 and the three endogenous MaRGA2a, MaRGA2b and MaRGA2c sequences.* ***A.*** *Global alignment with Blosum62 cost matrix generated in Geneious, dissimilar amino acids are highlighted in color.* ***B.*** *percent identity matrix.*

### 4.1.1 Expression of MamRGA2 in QCAV-4 tissue

Western blot analysis was used to examine protein expression in ripe fruit flesh and peel tissue from QCAV-4 and the conventional control. For each tissue analysed, three samples were processed from the 2nd field trial (2018 - 2023) in the Northern Territory. A mouse anti-MamRGA2 monoclonal antibody was generated for detecting MamRGA2 in QCAV-4. The results did not conclusively show the expression of the MamRGA2 protein in QCAV-4. Using a recombinant form of the protein expressed in *E. coli* (Section 4.1.2), the antibody was shown to have a limit of detection (LOD) of ~ 1ng, indicating the amount expressed in QCAV-4 was likely below this amount.

RNA sequencing analysis was performed to identify the tissues in which MamRGA2 was actively transcribed. High-quality RNA was isolated from leaf, root and ripe fruit tissues from QCAV-4 and the conventional control, and sequenced to generate paired end reads (2x 150 bp). The RNA sequencing reads were mapped to known nucleotide sequences of *MamRGA2*, *nptII*, and the three endogenous alleles of *MaRGA2*. The *CyP* gene from banana was used as the endogenous reference gene to normalise the mapped reads.

As expected, transcripts of *MamRGA2* were not detected in the leaf, root or ripe fruit tissue of the conventional control. The expression of the endogenous *MaRGA2* alleles was also negligible in both QCAV-4 and the conventional control. For the introduced *MamRGA2* gene, the highest relative expression was in the root (24X), followed by leaf (20X) with only minimal expression levels in fruit (1X).

These results confirm that *MamRGA2* is being expressed in QCAV-4 albeit at only very low levels.

### 4.1.2 Characterisation of MamRGA2 expressed in QCAV-4 and equivalence to a bacterially-produced form

The equivalence of the QCAV-4- and *E.coli*-derived MamRGA2 proteins must be established before the safety data generated using *E. coli*-derived MamRGA2 can be applied to QCAV-4-derived MamRGA2. Due to low levels of R-proteins and their intractable nature[15], a direct comparison could not be made. However, the applicant provided the results of a series of analytical techniques that characterises the *E. coli*-derived MamRGA2. The results are summarised below.

**Sequence.** Alignment of the translated *E.coli*-derived MamRGA2 sequence is identical to the protein sequence of QCAV-4-derived MamRGA2, translated from the inserted DNA sequence.

**Molecular weight.** Purified *E. coli*-derived MamRGA2 was run on SDS-PAGE then visualised with a Colloidal Blue staining kit. The MamRGA2 band migrates to ~142 kDa, which is equivalent to the expected mass of QCAV-4-derived MamRGA2.

**Immunoreactivity.** Western blot analysis with a mouse monoclonal MamRGA2-specific antibody detected a single MamRGA2 protein in the *E. coli* preparation with a molecular weight of ~142 kDa.

**Peptide mapping.** *E. coli*-derived MamRGA2 was digested with trypsin and chymotrypsin, and analysed by liquid chromatography mass spectrometry (LC-MS/MS). For trypsin digestion 81 unique peptides were identified and mapped, covering 53% of the expected MamRGA2 protein sequence. For chymotrypsin digestion, 129 unique peptides were

[15] Intractable proteins are those that are extremely difficult to isolate and purify. Without the ability to obtain a high amount of purified product, protein characterisation studies cannot be performed

identified and mapped, covering 67% of the expected protein sequence. A total sequence coverage of 82% was achieved when the results from both trypsin- and chymotrypsin-digested MamRGA2 were combined. These results further confirm the expressed protein in *E. coli* is MamRGA2.

The results outlined in this section demonstrated that *E.coli*-derived MamRGA2 is structurally and biochemically equivalent to QCAV-4-derived MamRGA2. The glycosylation analysis for the MamRGA2 sequence would be applicable for both *E.coli*-derived and QCAV-4-derived MamRGA2 (Section 4.1.3). Based on these data, the two proteins are expected to be biochemically and functionally equivalent. It can be concluded that *E. coli*-derived MamRGA2 is a suitable surrogate for QCAV-4-derived MamRGA2 for use in the safety studies described below.

### 4.1.3 Safety of the introduced MamRGA2

**Bioinformatic analyses of MamRGA2**

Bioinformatic analyses, as described in Section 3.4.5.1, were performed to compare the MamRGA2 amino acid sequence to known allergenic proteins in the Allergen Online database (version 21). The search did not identify any known allergens with homology to MamRGA2. No alignments had an E-score of $\leq 1\times10^{-4}$ or met or exceeded the threshold of greater than 35% similarity over ≥ 80 amino acids, and no eight amino acid peptide matches were shared between the MamRGA2 sequence and proteins in the allergen database.

The MamRGA2 amino acid sequence was compared with sequences in the Toxin database, as outlined in Section 3.4.5.2. A blast search identified significant homology to three proteins with an E-value < $1 \times 10^{-5}$. A similar search result was obtained with the MamRGA2-asociated putative peptide identified in Section 3.4.5.2. All of the matched proteins were plant resistance-like proteins that provide protection from pathogenic microorganisms.

A previous blast search using the amino acid sequence of VNT1, a potato R-protein that confers resistance to foliar late blight that was assessed by FSANZ in Application A1139[16], was conducted using the same toxin database. That blast search identified the same three plant-resistance-like proteins identified in the MamRGA2 sequence search.

R-proteins exist in most plants including food crops (McHale et al. 2006) and to date have not been associated with adverse effects in humans or livestock after consumption of food or feed. Significant sequence homology between MamRGA2 and the three R-proteins in the toxin database would be expected based on a similar function and is not itself suggestive of toxicity to humans. The homology therefore does not raise a food safety concern.

[16] https://www.foodstandards.gov.au/code/applications/Pages/A1139.aspx

**Susceptibility of MamRGA2 to digestion**

*E. coli*-produced MamRGA2 was incubated with pepsin (10U enzyme/µg protein) for 0-60 min at 37°C. Reactions occurred under acidic conditions in simulated gastric fluid (Thomas et al., 2004). The positive control was the digestible bovine serum albumin (BSA) and the negative control was the non-digestible β-lactoglobulin incubated with pepsin for 0-60 min. Two additional controls were also used: a no test protein control (pepsin only) and no pepsin control (test protein only). The extent of digestion was visualised by SDS-PAGE with Coomassie Blue staining and Western blotting.

By 0.5 min, visual inspection of the pepsin digestion showed there was no intact MamRGA2 remaining in the reaction mix. The BSA control was rapidly digested by 0.5 min, while the β-lactoglobulin remained present over the course of the reaction. These data indicate that MamRGA2 will be fully degraded by gastric enzymes in the human digestive system.

**Stability of MamRGA2 after exposure to heat**

*E. coli*-produced MamRGA2 was heated for 10, 30 or 60 min at temperatures ranging from 60-90°C. Control samples at 4°C and room temperature were used in the analysis. Control and heated protein samples were run on SDS-PAGE and examined by Western blot to detect the extent of protein degradation, i.e. structural stability. No significant degradation or decrease in signal intensity was observed for MamRGA2 in the control, 60, 75 and 90°C treated samples at 10, 30 and 60 min. These data indicate that MamRGA2 is not significantly degraded at temperatures up to 90°C.

Although MamRGA2 retains intact following heat treatment, this is not directly predictive of allergenicity or toxicity potential (EFSA 2022). The bioinformatic analysis demonstrated the protein does not have any significant amino acid similarity to known allergens or protein toxins of relevance to humans and the digestibility studies suggest that MamRGA2 would be rapidly degraded following ingestion.

**Post-translational modification**

Due to the low expression levels, post-translational modification of MamRGA2 could not be directly evaluated. Instead, the QCAV-4-derived MamRGA2 protein sequence was examined *in silico* using algorithms that detect sequences required for glycosylation. These analyses searched for the signal sequence required for protein transport to the endoplasmic reticulum (ER)[17] and glycosylation-acceptor sites. Although multiple glycosylation-acceptor sites were discovered, the lack of a signal peptide suggests that transit to the ER is unlikely, which would preclude MamRGA2 from being glycosylated *in planta*.

### 4.1.4 Conclusion

The MamRGA2 R-protein is derived from a wild banana and shares homology with R-proteins found in other commonly consumed foods (including commercial banana varieties), indicating a prior history of safe human exposure. Expression studies confirmed very low levels of MamRGA2 in QCAV-4 tissue, similar to native R-proteins. A range of characterisation studies were performed on *E. coli*-produced MamRGA2 confirming its suitability for use in the safety assessment experiments. The MamRGA2 protein is stable to degradation at temperatures of up to 90°C, but is susceptible to pepsin digestion. Bioinformatic analyses showed MamRGA2 had no similarity with known allergens or toxins of relevance to humans. Taken together, this indicates that the MamRGA2 protein is unlikely to

[17] Glycosylation of proteins occurs in the endoplasmic reticulum (ER)

be toxic or allergenic to humans.

## 4.2 NPTII

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

The *nptII* gene is widely used as a selectable marker in the transformation of plants (refer to Section 4.2.3). The gene functions as a dominant selectable marker in the initial, laboratory stages of plant cell selection following transformation (De Block et al. 1984; Horsch et al. 1984). While the *nptII* gene and its encoded protein are present in QCAV-4, it has no function in the commercial line. It is simply a remnant from the initial development stages of the GM banana line. FSANZ have previously assessed and approved 11 events across four commodities which contained NPTII.

### 4.2.1 Expression of NPTII in QCAV-4 tissue

Protein expression in ripe banana fruit was determined by ELISA. An analytical reference standard for NPTII was obtained. In order to determine the sites of accumulation of the protein, samples were collected from QCAV-4 grown in the 2nd field-trial (2018-2023) in the Northern Territory. Flesh and peel tissues were examined from QCAV-4 and conventional control fruits. For each tissue analysed, three samples were processed from the field-trial site.

The results from the protein analysis showed the average levels found in flesh were 3.1 ng/mg of fresh weight (fw) and 4.5 ng/mg fw in peel.

### 4.2.2 Characterisation of NPTII expressed in QCAV-4

The *nptII* gene prepared by the applicant encodes a protein of 265 amino acids. The protein sequence is 99.6% identical to the expected NPTII protein from *E.coli* K12 strain and the sequences used in previous applications assessed and approved by FSANZ. Relative to other sequences, the NPTII protein in QCAV-4 contains an N-terminus deletion of glycine at position 2. This is not expected to affect overall structure, immunoreactivity, enzyme activity or substrate specificity.

Western immunoblot analysis with a commercially available NPTII-specific antibody detected a single NPTII protein with a molecular weight of ~29.1 kDa in QCAV-4 ripe fruit flesh and peel sample preparation. The results confirmed that the NPTII protein found in QCAV-4 is structurally and biochemically equivalent to NPTII found in other plants or from bacteria.

In terms of function, the expression of NPTII protein in QCAV-4 provides the banana with tolerance to kanamycin. This was initially demonstrated during the transformation and selection process (see Section 3.1).

### 4.2.3 Safety of the introduced NPTII

The NPTII protein, encoded by *nptII* gene has now been considered in 11 FSANZ safety

assessments[18]. In previous FSANZ assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans.

The *nptII* gene (and its encoded protein) has a considerable history of use as a selectable marker gene in the development of GM plants (Kumar et al. 2020). Associated with this history of use is a substantial body of evidence to indicate that the presence of NPTII in food derived from GM crops does not pose a significant risk to human health (Flavell et al. 1992; Nap et al. 1992; Fuchs et al. 1993a; Fuchs et al. 1993b).

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

Since the NPTII protein expressed in QCAV-4 has a sequence similarity of 99.6% and is structurally, biochemically and functionally equivalent to the previous NPTII proteins assessed by FSANZ, no further safety evaluation is required other than the examination of updated bioinformatic searches.

**Bioinformatic analyses of NPTII**

The applicant has submitted updated bioinformatic studies for NPTII that looked for amino acid sequence similarity to known protein allergens and toxins (October 2022). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

### 4.2.4 Conclusion

The data presented by the applicant confirms the NPTII expressed in QCAV-4 is identical to previously assessed NPTII proteins, except for a single amino acid deletion at the N-terminus. QCAV-4-derived NPTII is immunoreactive to an NPTII antibody and is functional i.e. provides kanamycin tolerance. The protein is expressed in various plant tissues, including the banana fruit. Updated bioinformatic analyses confirm that NPTII does not have any significant similarity with known allergens or toxins.

# 5 Compositional analysis

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

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

[18] A341, A355, A372, A379, A382, A383, A384, A484, A549, A595, A1029.

potency and level may be significant to health.

## 5.1 Key components

The primary banana food product consumed by humans is the flesh of the fruit. Because there is no OECD Consensus Document on compositional considerations for banana, key components were selected based on the highest contributors to the percent daily values from a 2,000 calorie reference diet for adults and children aged four and up. The key components analysed for the comparison of GM and conventional banana include: proximates, vitamins and minerals.

The compositional analysis did not include fatty acids, amino acids, anti-nutrients or dietary fibre as these are not considered key components for banana.

The Australian Food Composition Database[19] and the FoodData Central database[20] indicate that bananas contain only very low levels of fatty acids and amino acids. which is reflective of the low levels of both fat and protein in the fruit. Bananas also have very low levels of anti-nutrients (Oyeyinka and Afolayan 2019; Ariyo et al. 2021).

In terms of dietary fibre, a banana (Cavendish) contains 2.2 g dietary fibre/100g fw (Australian Food Composition Database). A recent nutrition survey of the Australian population found the average daily dietary fibre consumption ranges from 15 to 25 g/day across all age and gender groups (Fayet-Moore et al. 2018), with bananas contributing only ~ 2.0% of the daily intake, compared to cereals and cereal based products (44%) and vegetables (19%) (Fayet-Moore et al. 2018).

## 5.2 Study design

Bananas from QCAV-4 (G5, G6 and G7 generations) and the conventional control (Cavendish cv Grand Nain) were grown and harvested from a field trial site in the Northern Territory during the 2018 - 2023 growing season. The site was environmentally representative of banana production areas and had a high Fusarium wilt TR4 disease pressure. The field site was established in a randomised complete block design with ten replicates per block. Plants were grown under agronomic field conditions typical for the growing region.

The applicant provided compositional data for:

- Flesh
    - For each generation analysed, 10 samples of QCAV-4 and 10 samples of control were processed.
- Peel
    - 6 samples of QCAV-4 and 2 samples of control were processed.

Ten different analytes were measured in flesh and peel tissues (see Figure 7 for a complete list). Statistical analyses were performed using the SPSS software[21] version 27. Analytes were expressed as either g/100g fresh weight (fw), mg/kg fw or as mg/100g fw, as shown in Figures 10, 11 and 12. For each analyte, 'descriptive statistics' (mean, standard deviation [SD], and range) were generated.

In assessing the significance of any difference between QCAV-4 and the control, a *p*-value of 0.05 was used. Levels for each analyte in QCAV-4 banana were statistically compared to

[19] https://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/foodsearch.aspx
[20] https://fdc.nal.usda.gov/
[21] IBM SPSS Inc

those measured in the control. The maximum and minimum values from the control was calculated to establish the control range i.e. the natural variability of analytes in a plant grown under the same agronomical and environmental conditions.

The magnitude of difference in mean values between QCAV-4 and the control were determined, and this difference was compared to the natural variation of analytes from publically available data. For flesh tissue, the applicant provided a combined range from the Australian Food Composition Database and the FoodData Central database of the United States Department of Agriculture, Agricultural Research Service. FSANZ has further supplemented the ranges from publically available literature (Wall 2006; Hapsari and Lestari 2016; Kookal and Thimmaiah 2018; Fasanya et al. 2019).

For the peel tissue, the applicant provided publically available values for all analytes measured, except ascorbic acid and pyridoxin (Emaga et al. 2007). FSANZ has further supplemented the natural variation ranges from publically available literature (Emaga et al. 2007; Nagarajaiah and Prakash 2011; aboul-Enein et al. 2016; Hassan et al. 2018; Montaño et al. 2019). The natural variation of pyridoxin in peel tissue could not be established due to a lack of reliable reference sources.

These publically available ranges takes into account variability present in non-GM banana cultivars due to a wide range of agronomic and environmental conditions, as well as different genetic backgrounds. These data ranges assist with determining whether any statistically significant differences were likely to be biologically meaningful.

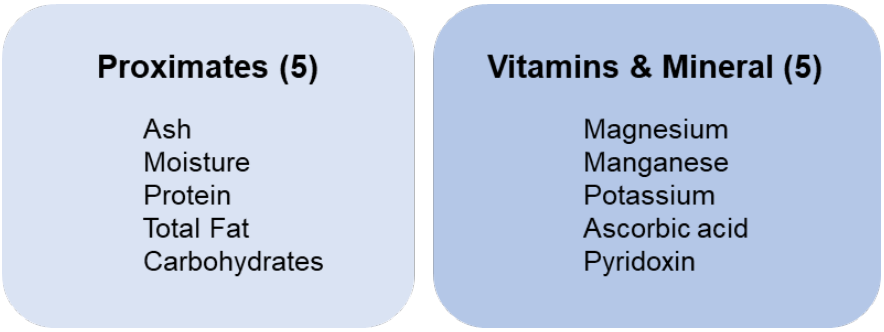



*Figure 7: Analytes measured in flesh and peel tissues*

## 5.3 Analyses of key components in flesh

10 analytes were measured in flesh tissue collected from G5 and G6 banana. A visual overview of all the analytes for G5 and G6 is provided in Figure 8 and Figure 9, respectively.

Of the 10 analytes measured, there were 7 in G5 and one in G6 for which there was a statistically significant difference in mean values between banana line QCAV-4 and the control:

- G5 - moisture, protein, ash, carbohydrate, magnesium, potassium and ascorbic acid;
- G6 - manganese.

For the analytes measured in G5 and G6 where statistically significant differences were found, the QCAV-4 mean values (blue dots) for each of these analytes were within the control range (orange bars) and/or the publically available range (dark grey bars).

Some analyte ranges found in QCAV-4 (blue bars) and control (orange bars) in G5 (manganese and pyridoxin) and G6 (carbohydrate and manganese) were outside the publically available range. However, these differences are either due to differences in

agronomic and environmental conditions and/or due to limited information in the publically available range.

In G6, the minimum range value for protein in QCAV-4 (blue bars) was outside the minimum ranges observed in the control and the publically available data. However, this is due to a single value measured in one of the 10 individual samples analysed for protein (shown in Figure 9 *c*). The difference reported here is most likely an outlier and, as such, this difference is not biologically meaningful.

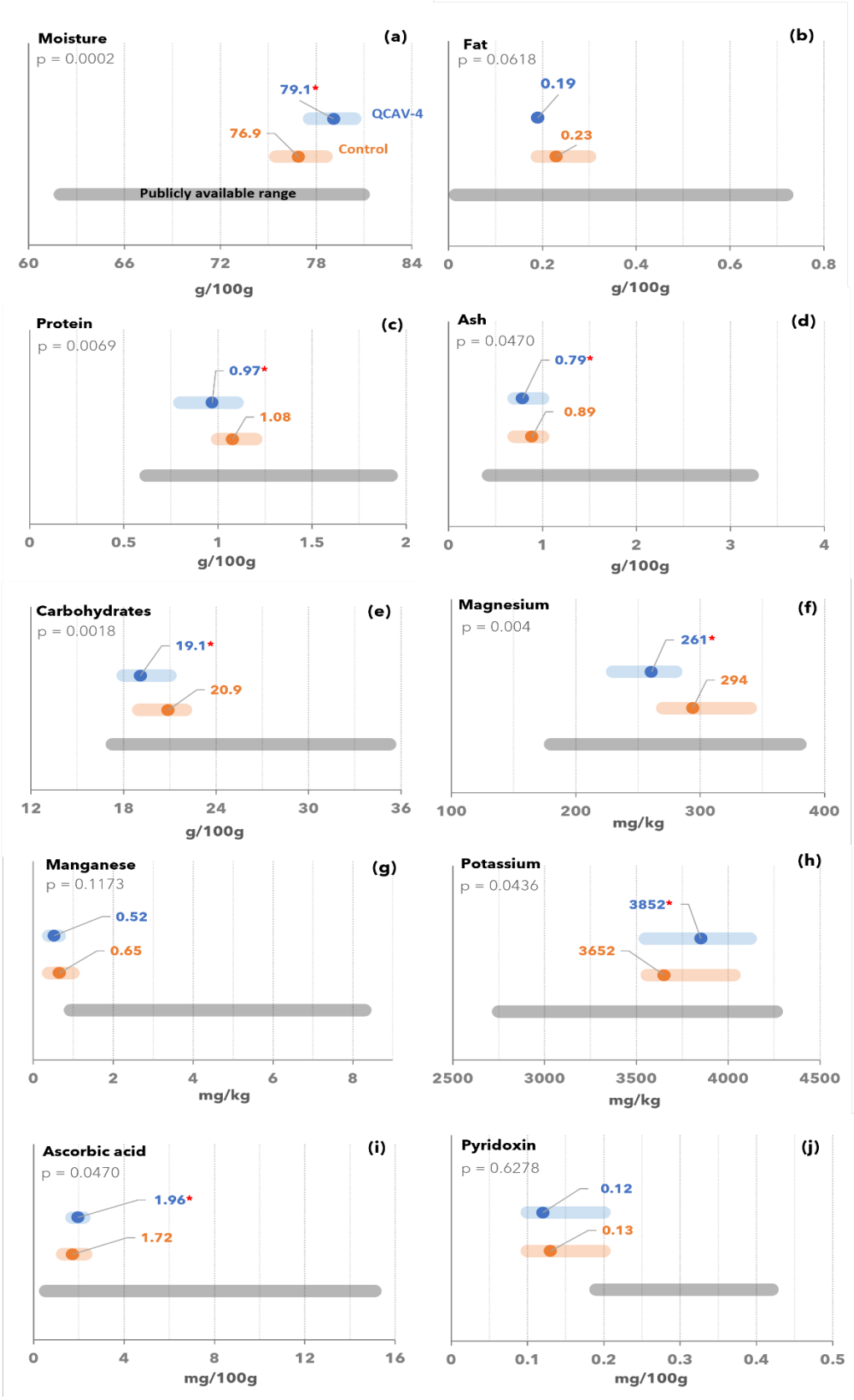



***Figure 8:*** *Visual summary of analytes in flesh tissue from QCAV-4 G5 compared to the conventional control.* ***(a) - (j)*** *Measured means (dots) and ranges for QCAV-4 (blue bars) and the control (orange bars) for the 10 analytes as labelled.* ***(b)*** *The maximum and minimum values for fat in QCAV-4 are the same as the mean value (blue dot).The dark grey bars represent the publically-available ranges for each analyte. Note: the x-axes vary in scale and unit for each analyte. Statistically significant mean values are highlighted in red *.*

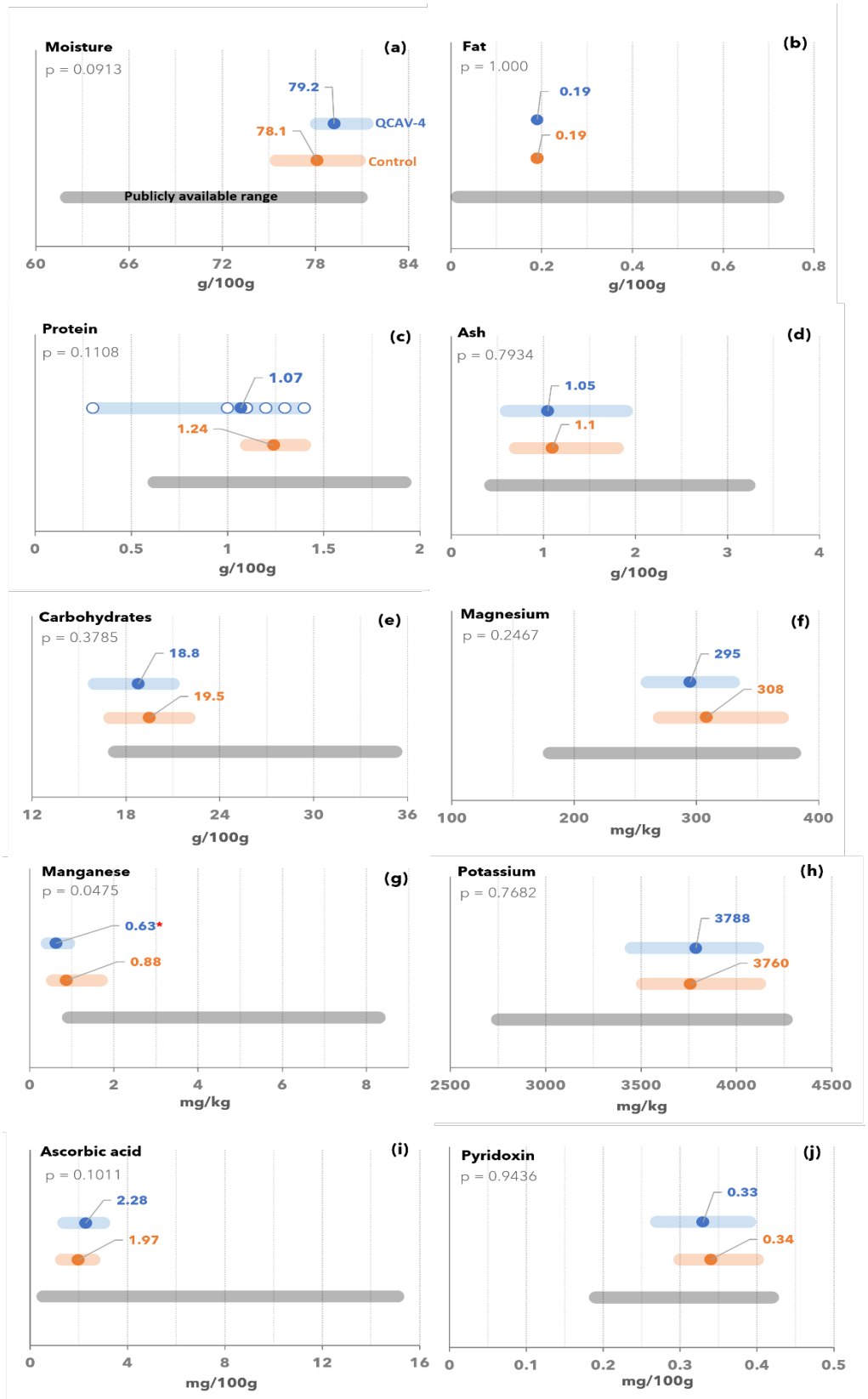



***Figure 9:*** *Visual summary of analytes in flesh tissue from QCAV-4 G6 compared to the conventional control.* ***(a) - (j)*** *Measured means (filled dots) and ranges for QCAV-4 (blue bars) and the control (orange bars) for the 10 analytes as labelled.* ***(b)*** *The maximum and minimum values for fat in QCAV-4 and control are the same as their respective mean values (blue and orange dot).* ***(c)*** *White dots represents individual values of replicates. The dark grey bars represent the publically-available ranges for each analyte. Note: the x-axes vary in scale and unit for each analyte. Statistically significant mean values are highlighted in red *.*

## 5.4 Analyses of key components in peel

10 analytes were also measured in peel tissue collected from G7 banana. A visual overview of all the analytes measured in peel tissue from G7 is provided in Figure 10. In summary, the results are limited but unremarkable.

Due to the severe impact of Fusarium wilt during the field trial, the applicant was only able to collect samples from 2 control plants. This limits the control range. There is also a lack of data in the literature for banana peel to establish a reliable and robust publically available range (Figure 10; data represents five studies). Banana peel analyte ranges in non-GM banana cultivars would undoubtedly be broader.

Acknowledging the limitations of the data, what can be observed in Figure 10 is that the mean values for all the analytes measured in QCAV-4, with the exception of pyridoxin, lie within the in-study non-GM banana control values and/or the publically available range. Due to lack of published literature, the publically available range for pyridoxin could not be determined. The pyridoxin range in non-GM banana cultivar peel is expected to be broader and the measured QCAV-4 mean is expected to fall within this range. Taken together, any significant differences in means between banana line QCAV-4 and control would not necessarily be biologically significant. Banana peel is also not a large contributor to nutrient intake in the human diet.

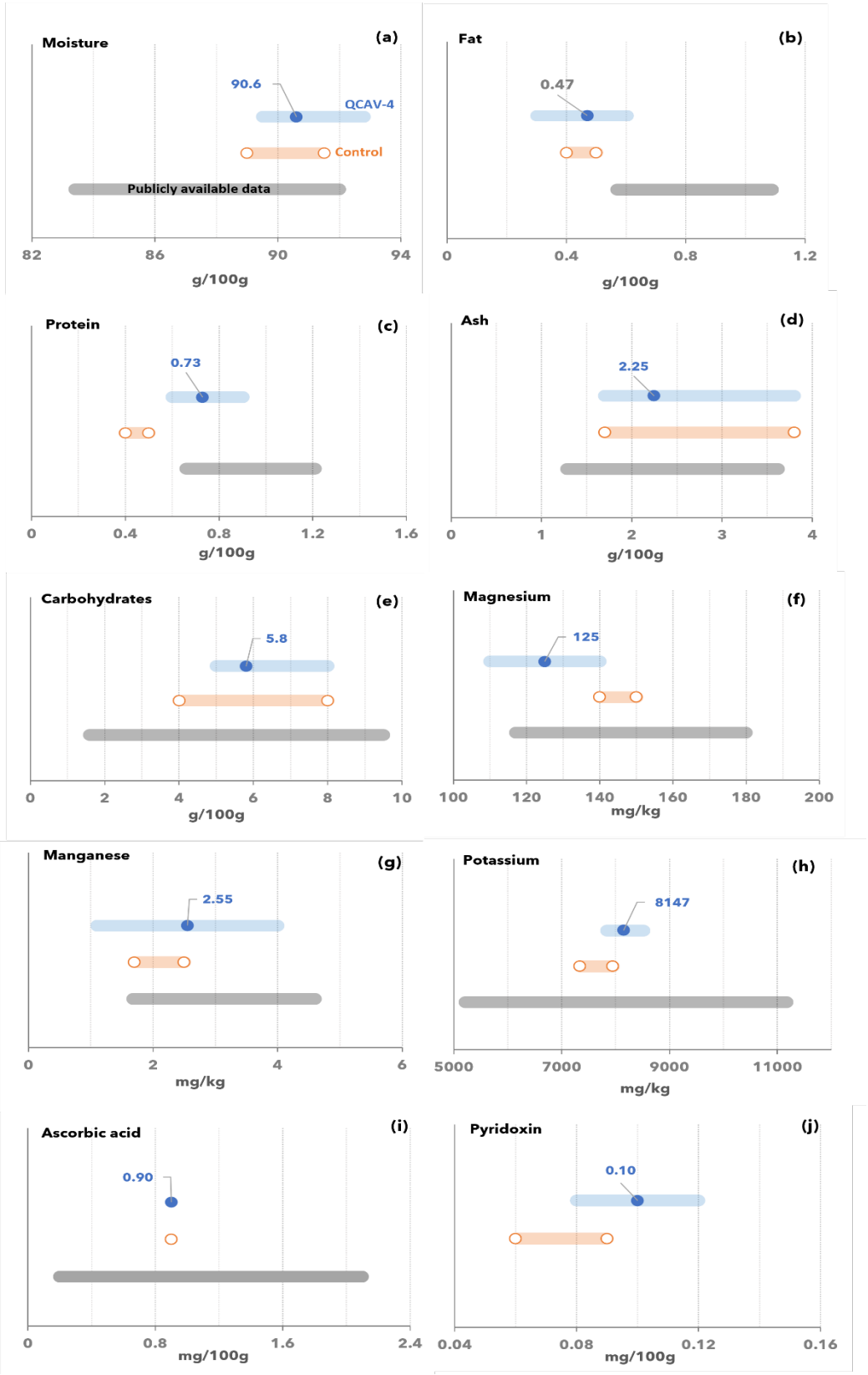



***Figure 10:*** *Visual summary of analytes in peel tissue from QCAV-4 G7 compared to the conventional control.* ***(a) - (j)*** *Measured means (blue dots) and ranges for QCAV-4 (blue bars), individual values for (white dots) connected with orange bars for control (mean was not measured for control due to lack of replicates used. The dark grey bars represent the publically-available range for each analyte.* ***(j)*** *Publically available range could not be established for due to lack of literature. Note that the x-axes vary in scale and unit for each analyte.*

## 5.5 Conclusion

Overall, the compositional data in banana are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in QCAV-4 when compared with conventional non-GM banana cultivars already available in agricultural markets. QCAV-4 banana can therefore be regarded as equivalent in composition to conventional non-GM banana. While the banana peel analysis was limited, the majority of QCAV-4 analytes were within the control values and/or the publically available range.

# 6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with the compositional analysis of the food, such as that presented in Section 5 of this report.

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

QCAV-4 is the result of genetic modifications to confer resistance to the fungal disease, Fusarium wilt TR4, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modifications have not altered the nutrient composition of QCAV-4 banana. The introduction of food derived from QCAV-4 into the food supply is therefore expected to have negligible nutritional impact.

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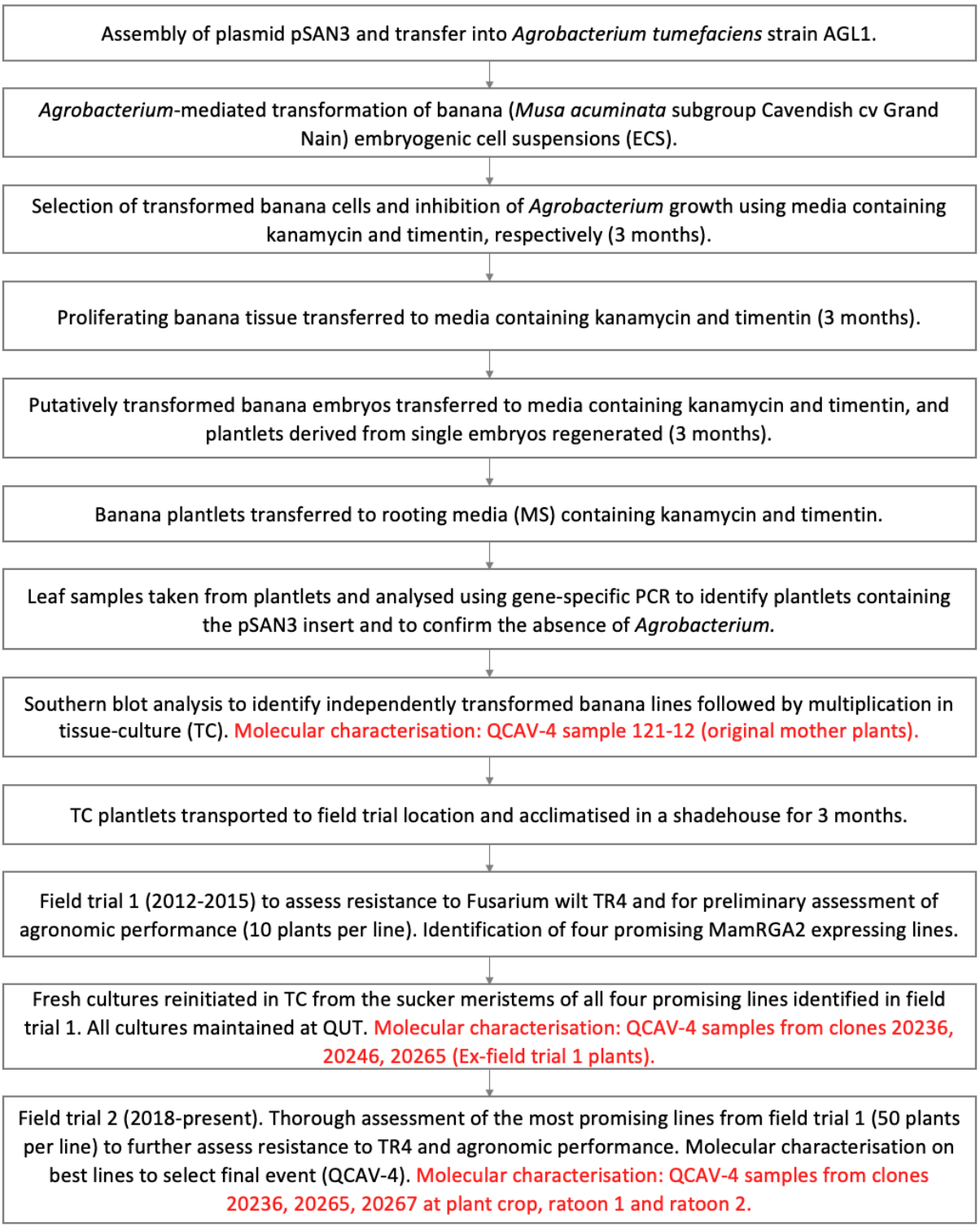
# Appendix 1


Assembly of plasmid pSAN3 and transfer into *Agrobacterium tumefaciens* strain AGL1.
*Agrobacterium*-mediated transformation of banana (*Musa acuminata* subgroup Cavendish cv Grand Nain) embryogenic cell suspensions (ECS).
Selection of transformed banana cells and inhibition of *Agrobacterium* growth using media containing kanamycin and timentin, respectively (3 months).
Proliferating banana tissue transferred to media containing kanamycin and timentin (3 months).
Putatively transformed banana embryos transferred to media containing kanamycin and timentin, and plantlets derived from single embryos regenerated (3 months).
Banana plantlets transferred to rooting media (MS) containing kanamycin and timentin.
Leaf samples taken from plantlets and analysed using gene-specific PCR to identify plantlets containing the pSAN3 insert and to confirm the absence of *Agrobacterium*.
Southern blot analysis to identify independently transformed banana lines followed by multiplication in tissue-culture (TC). Molecular characterisation: QCAV-4 sample 121-12 (original mother plants).
TC plantlets transported to field trial location and acclimatised in a shadehouse for 3 months.
Field trial 1 (2012-2015) to assess resistance to Fusarium wilt TR4 and for preliminary assessment of agronomic performance (10 plants per line). Identification of four promising MamRGA2 expressing lines.
Fresh cultures reinitiated in TC from the sucker meristems of all four promising lines identified in field trial 1. All cultures maintained at QUT. Molecular characterisation: QCAV-4 samples from clones 20236, 20246, 20265 (Ex-field trial 1 plants).
Field trial 2 (2018-present). Thorough assessment of the most promising lines from field trial 1 (50 plants per line) to further assess resistance to TR4 and agronomic performance. Molecular characterisation on best lines to select final event (QCAV-4). Molecular characterisation: QCAV-4 samples from clones 20236, 20265, 20267 at plant crop, ratoon 1 and ratoon 2.

# Appendix 2

## Genetic elements present in the pSAN3 plasmid

| Genetic elements | Relative position | Size (bp) | Source | Description & Function |
|---|---|---|---|---|
| IS[22] | 1-979 | 979 | Binary vector pCanbia-2200 | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| 'STA' region from pVS1 plasmid | 980-1980 | 1001 | | Plasmid stability in culture (Heeb et al. 2000) |
| IS | 1981-2573 | 593 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| Origin of replication | 2574-3574 | 1001 | | Replication protein (Heeb et al. 2000) |
| IS | 3575-3983 | 409 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| Basis of mobility region from plasmid pBR322 | 3984-4244 | 261 | | |
| IS | 4245-4383 | 139 | | |
| Plasmid origin of replication | 4384-4664 | 281 | | High copy number ColE1/pMB1/pBR322/pUC origin of replication. Plasmid origin of replication (Yanisch-Perron et al. 1985) |
| IS | 4665-5140 | 476 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| Chloramphenicol acetyl transferase antibiotic resistance gene | 5141-5800 | 660 | | Confers resistance to chloramphenicol (Prentki et al. 1981) |
| IS | 5801-6320 | 520 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| Left Border (LB) sequence | 6321-6346 | 26 | | Secondary cleavage site releases ssDNA[23] insert from pSAN3 (van Haaren et al. 1989) |
| IS | 6347-6412 | 66 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| *nptII* expression cassette | | | | |
| CaMV35S 3'UTR | 6413-6616 | 204 | *Cauliflower Mosaic Virus* | Poly(A) signal for the termination of *nptII* transcription |
| IS | 6617-6653 | 37 | | |
| *nptII* coding sequence | 6654-7451 | 798 | *Escherichia coli* | Bacterial transposon Tn5, encoding neomycin phosphotransferase (NPTII) confers resistance to kanamycin (Carrer et al. 1993) |
| IS | 7452-7481 | 30 | | |
| 35S Promoter | 7482-8289 | 808 | *Cauliflower Mosaic Virus* | *De novo* expression of the *nptII* gene (Odell et al. 1985; Haq et al. 1995) |
| IS | 8290-8521 | 232 | | Sequence used for cloning (Hajdukiewicz et al. 1994) |
| *MaMRGA2 expression cassette* | | | | |
| *Nos* Promoter | 8522-8705 | 184 | *Agrobacterium tumefaciens* | *De novo* expression of the MamRGA2 gene (Depicker et al. 1982) |
| IS | 8706-8711 | 6 | | |
| *MamRGA2* coding sequence | 8712-12410 | 3699 | *Musa acuminata ssp.* | Generates mRNA that leads to MamRGA2 protein expression providing resistance to *Fusarium oxysporum* f. sp. *cubense* tropical race 4 |

[22] IS – intervening sequence
[23] ssDNA – single-stranded DNA

| Genetic elements | Relative position | Size (bp) | Source | Description & Function |
|---|---|---|---|---|
| | | | *malaccensis* | |
| IS | 12411-12429 | 19 | | |
| *Nos*-ter; poly(A)signal of nopaline synthase gene | 12430-12731 | 302 | *Agrobacterium tumefaciens* | Poly(A) signal for the termination of *MamRGA2* transcription (Depicker et al. 1982) |
| IS | 12732-12996 | 265 | | |
| Right Border (RB) sequence | 12997-13022 | 26 | Binary vector pCanbia-2200 | Primary cleavage site releases ssDNA insert from pSAN3 (van Haaren et al. 1989) |
| IS | 13023-13084 | 62 | Binary vector pCambia-2200 | Sequence used for cloning (Hajdukiewicz et al. 1994) |