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Supporting document 1

Safety assessment – Application A1274

Food derived from disease-resistant banana line QCAV-4

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3.1 Transformation method

To create the QCAV-4 banana line, the Cavendish banana cultivar Grand Nain was transformed using the plasmid pSAN3 (Figure 2). Transformation of Grand Nain was achieved by incubating embryogenic cell suspensions (embryos) derived from immature male flowers with *Agrobacterium tumefaciens* containing the pSAN3 plasmid. The methodology is outlined in flowchart in Appendix 1 and summarised below.

The embryos 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

[3] Part of the banana plant that looks like a trunk.

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 embryos were further maintained on media with the higher kanamycin concentration for 3 months while sub-culturing to fresh medium every month.

The embryos 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[4] 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.

3.2 Detailed description of inserted DNA

Banana line QCAV-4 contains T-DNA from pSAN3 plasmid (Figure 2) and 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.

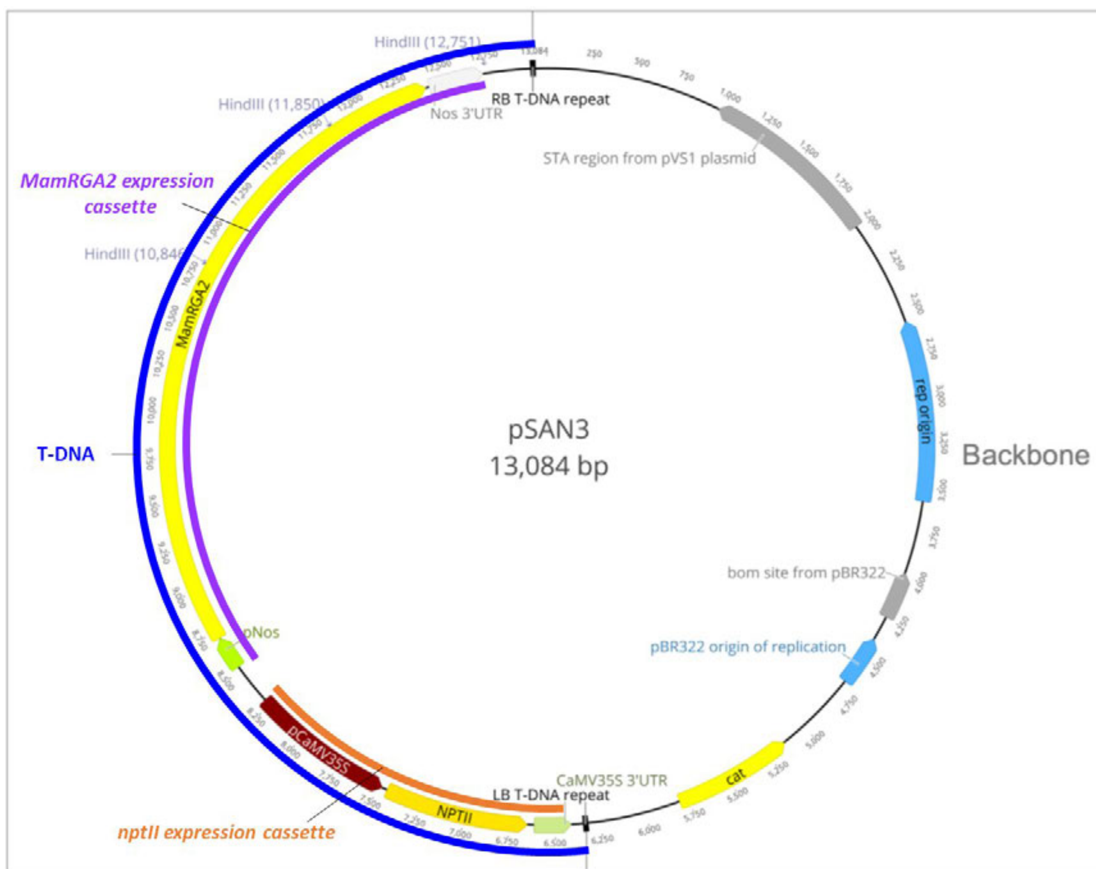


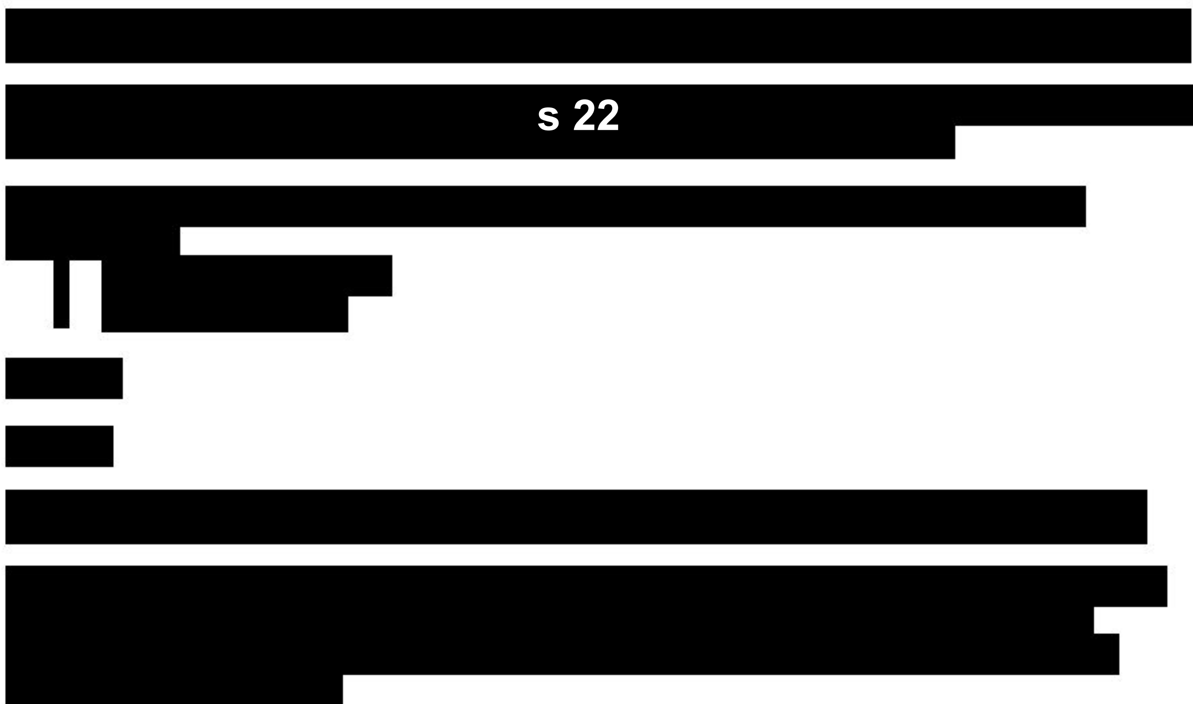
Figure 1: Plasmid map of pSAN3. The T-DNA region comprising the MamRGA2 and nptII expression cassettes is highlighted using the blue bar. The MamRGA2 expression cassette is highlighted using the purple bar. The nptII expression cassette is highlighted using the

[4] 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.

orange bar.

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 from nopaline synthase (NOS) gene from *Agrobacterium tumefaciens*	*MamRGA2* coding sequence from *Musa acuminata ssp. malaccensis*	3'UTR derived from nopaline synthase (*NOS*) gene from *Agrobacterium tumefaciens*
nptII expression cassette	35S Promoter derived from 35S RNA from *Cauliflower Mosaic Virus*	*nptII* coding sequence from Transposon Tn5 of *Escherichia coli* strain K12	3' UTR derived from the 35S RNA of Cauliflower mosaic virus



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 hybridization to three endogenous RGA2 homologs (*MaRGA2*), which were distinguished from *MamRGA2* by their distinctive predicted sizes.

To further confirm the Southern blot results, next-generation sequencing (NGS) was performed on gDNA isolated from the leaves of QCAV-4 (G0) and conventional control. Paired end reads (2x150 bps) 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 depth coverage of 178X.

Comparison of the sequence between QCAV-4 and pSAN3 detected two unique insert-flank junction sites, each comprised of 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 that a single insertion with multiple copies of the intended DNA insert has been integrated into the genome of QCAV-4 (Figure 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 pSAN3 transformation plasmid were aligned. The results of this alignment confirmed there was no integration of pSAN3 backbone sequences, including any antibiotic resistance genes, into 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 Gbps were generated using the PacBio platform with a read length N50[5] 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 was integrated into the host genome (Figure 4). Additionally, the presence of two fragments of rearranged *MamRGA2* expression cassettes between T-DNA 2 and T-DNA 3 were found (Figure 4).

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To examine the T-DNA insertion site, 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 of the conventional control[6] (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 and this corresponded to an intergenic region. The insertion did not disrupt any 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 4). 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.

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[5] 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.

[6] NCBI, Glycine max cultivar Williams 82 v4.0, Assembly Accession GCF_000004515.6.

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