

Precision bred organism marketing notice (reference: PBM/26/MUAC/001)

Information provided to the Secretary of State alongside a notice of intention to market a precision bred plant under schedule 2 of the Genetic Technology (Precision Breeding) Regulations 2025.

1. Name and address of the person with overall responsibility for marketing the precision bred plant

Tropic Biosciences UK Ltd.
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Colney Lane,
Colney, Norwich, England, NR4 7GJ,
United Kingdom

2. General description of the precision bred plant

a) Genus and species

Musa acuminata

b) Intended alterations to characteristics of the plant

Damage of banana fruits results in enzymatic browning which is a major cause of banana fruit loss. Specifically, enzymatic browning has been reported to cause around 20% of banana yield losses (information gathered from the banana sector by Tropic) and is believed to be responsible for greater than 50% of yield losses across susceptible fruit (Iqbal et al., 2019; Shrestha et al., 2020). Polyphenol oxidase (PPO) enzymes catalyse the enzymatic browning reaction. Under resting conditions, the nuclear-encoded PPO enzymes are largely localised to plastids whilst their phenolic substrates are primarily found in the vacuole. With mechanical damage of banana fruits, including peeling, bruising and slicing, PPO enzymes and their phenolic substrates are released and come into contact with oxygen. As a result, PPO enzymes can catalyse

the oxidation of their substrates promoting the formation of brown melanin pigments. The process of enzymatic browning can affect fruit appearance, taste and texture and can result in significant losses of banana fruit yields. Reducing PPO gene expression and PPO enzymatic activity in the banana fruit was therefore targeted as a strategy to produce a reduced browning banana with limited yield losses to enzymatic browning.

c) Types of genetic changes introduced to cause these alterations

Targeted PPO edits were introduced with gene editing to produce loss of function insertion or deletion mutations and reduce the production of functional PPO proteins in the banana fruit. Characterisation of the reduced browning banana phenotype in line TRB011002 demonstrated a reduction in browning in the flesh tissue of the banana fruits during post-harvest browning assays.

d) Techniques of modern biotechnology used to make these genetic changes

A CRISPR/Cas9 DNA editing approach was utilised to produce the reduced browning banana. This editing technology sees Cas9 endonuclease protein directed by guide RNA (gRNA) molecules to specific target DNA sequences. Cas9 then functions to precisely cleave target DNA and introduce a double-stranded break (DSB). Such a DSB can subsequently be repaired via error-prone mechanisms, such as the non homologous end joining (NHEJ) pathway or the microhomology-mediated end joining (MMEJ) pathway, which can result in DNA edit formation. DNA edits in target genes often disrupt gene function, for example, by introducing frameshifts and/or premature stop codons within a coding sequence. The CRISPR/Cas9 editing was performed in *M. acuminata* through the transient expression of the editing machinery in embryogenic cells following treatment with *Agrobacterium tumefaciens*. The *A. tumefaciens* used carried a plasmid encoding Cas9 and specific gRNAs within a T-DNA region also encoding a selectable marker gene. The banana cells synthesised the Cas9 protein and the gRNAs which targeted Cas9 to specific sequences resulting in the formation of targeted DSBs. Then, DSB repair introduced gene edits.

3. Intended use of the precision bred plant

The banana fruit harvested from the reduced browning banana variety is intended for import, retail, and consumption purposes akin to conventional banana fruit.

4. Intended genetic changes made

(a) the details of genetic changes made;

CRISPR/Cas9 was used to introduce edits in the coding sequence of all three *M. acuminata* PPO1 alleles. For this, two gRNAs were used to specifically target Cas9 to the first exon of PPO1. Targeting this region with two specific gRNAs increased the likelihood of Cas9-mediated disruption in the 5' region of the PPO1 gene across all three alleles. Molecular characterisation analyses of genomic DNA extracted from leaves of the reduced browning banana confirmed the presence of one single base pair insertion in one allele (allele 1), one single base pair deletion in two alleles (alleles 2 and 3) and a seven base pair deletion in one allele (allele 2), across the two gRNA target sites in the PPO1 gene. The targeted edits introduced in the reduced browning banana are summarised in Figure 1. In all three alleles of PPO1, the introduced edit or edits cause a frameshift and the formation of a premature stop codon which are expected to significantly disrupt the protein expression.

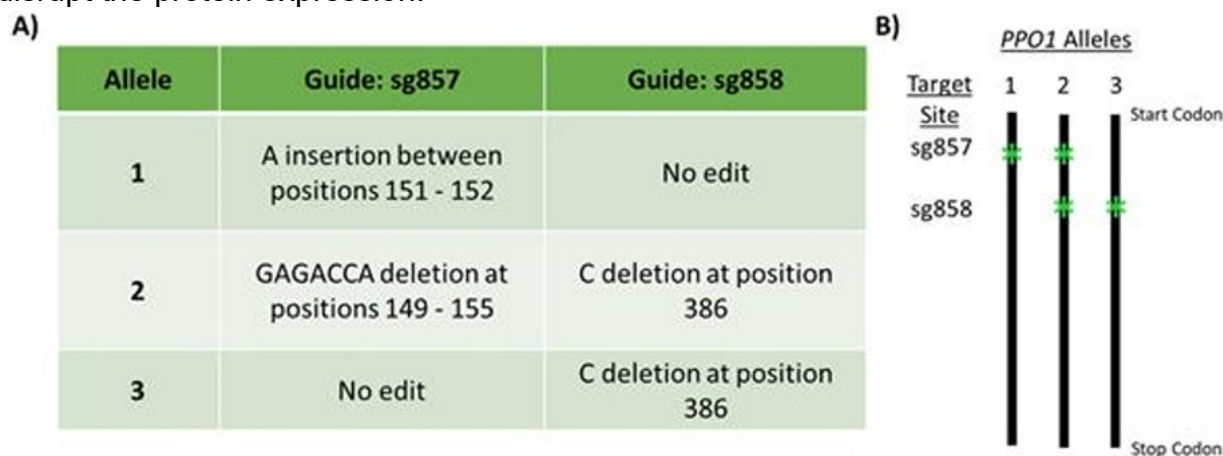


Figure 1. Edits in the reduced browning banana line TRB011002. **(A)** Table outlining the nature of the targeted edits. Edits are described by their nucleotide position in the wild-type consensus relative to the relevant first coding nucleotide (nucleotide position 1). **(B)** Graphical representation of the edits, where the '*' symbols represent edits at target sites.

(b) the location of genetic changes;

The targeted edits introduced in the reduced browning banana are located in PPO1 gene sequences in chromosome 6 of the banana nuclear genome. The locations of the targeted edits introduced in the reduced browning banana are summarised in Figure 1.

The sequences referred to in this application derive from Tropic's in-house reference genome which is highly accurate for Tropic's Cavendish banana varieties and allowed differentiation of all three PPO1 alleles. This genome is not assembled to chromosome level. As such, the absolute attribution of PPO1 alleles to a specific copy of chromosome 6 is not possible with this genome. Therefore, the allele numbers shown for PPO1 in this application were arbitrarily assigned to the allele sequences and do not relate to a determined chromosome.

The gene ID for PPO1 is Ma06_g31080. The code derives from the genome published by Martin et al. (2016), which is known as DH-Pahang v2.0. This code has been applied to our in-house reference genome. Information on Ma06_g31080 can be found on the EnsemblPlants genomic database, which is publicly accessible via the following link: https://plants.ensembl.org/Musa_acuminata/Search/New?db=core (accessed March 2026). On the Banana Genome Hub, information can be found using transcript ID information [Ma06_t31080.1, as follows: https://banana-genome.hub.southgreen.fr/features/Ma06_t31080.1 (accessed March 2026)

(c) the stability of genetic changes;

Asexual reproduction by vegetative propagation is possible for all banana varieties, wild and cultivated, and is the only naturally occurring viable means of reproduction for triploid varieties such as those of the *M. acuminata* Cavendish subgroup. Fruit in the triploid *M. acuminata* varieties develops through parthenocarpy and does not require pollination, with the female ovules shrivelling within the fleshy, seedless and sterile banana fruit. After the fruiting cycle, the main pseudostem dies and multiple suckers emerging from the corm will begin the plant cycle once more. In vitro micropropagation is also used for vegetative propagation of banana plants. Due to the asexual reproduction of bananas, the reduced browning banana line TRB011002 is stable, and its progenies are expected to retain the targeted DNA edits during propagation. Additionally, stability of the DNA edits was confirmed by molecular characterisation of multiple plant generations

d) when a genetic change involves the insertion of any genetic material, a description of all the genetic elements inserted and the organism from which they originated;

Comprehensive molecular characterisation was used to identify the reduced browning banana line TRB011002 as non-transgenic and edited, confirming no plasmid genetic material was inserted in the genome.

e) the purpose of the genetic changes described above in sub-paragraphs (a) to (d), including how they alter the characteristics of the precision bred plant

Gene editing of PPO1 introduced insertion or deletion edits into all three PPO1 alleles of the reduced browning banana line TRB011002. These edits resulted in frameshifts in the protein coding sequences and the introduction of premature stop codons disrupting PPO1 protein coding. Such mutations are expected to prevent functional PPO1 protein production and significantly reduce PPO levels in the banana fruit resulting in reduced enzymatic browning and a reduced browning

banana variety. Post-harvest assessment of browning in banana flesh tissue demonstrated that reduced browning banana line TRB011002 displays a statistically significant reduction in flesh tissue browning compared to a conventional control banana line.

5. Unintended genetic changes made

(a) the details of genetic changes made;

No unintended genetic changes are present in the reduced browning banana line TRB011002 resulting from the application of modern biotechnology.

(b) the location of genetic changes;

Not applicable.

(c) the stability of genetic changes;

Not applicable.

6. How the intended genetic changes were introduced

(a) which techniques of modern biotechnology were used;

A CRISPR/Cas9 DNA editing approach was utilised to produce the reduced browning banana line TRB011002. This editing technology sees Cas9 endonuclease protein directed by guide RNA (gRNA) molecules to specific target DNA sequences. Cas9 then functions to precisely cleave target DNA and introduce a double-stranded break (DSB). Such a DSB can subsequently be repaired via error-prone mechanisms, such as the non-homologous end joining (NHEJ) pathway or the microhomology-mediated end joining (MMEJ) pathway, which can result in DNA edit formation. DNA edits in target genes often disrupt gene function, for example, by introducing frameshifts and/or premature stop codons within a coding sequence.

The CRISPR/Cas9 approach targeted endogenous PPO1 alleles to create the reduced browning banana. This method involved *A. tumefaciens*-mediated expression of the CRISPR/Cas9 machinery from plasmid T-DNA (Figure 2). To generate non transgenic and edited lines, banana embryogenic cell suspensions (ECS) were treated with *A. tumefaciens* carrying the plasmid. To enrich for cells transiently expressing the T-DNA genes in free from, i.e. not integrated into the genome, a short period of selection was performed with the aminoglycoside antibiotic G418 (Geneticin). A longer period of selection pressure would instead bias towards stable T-DNA integration. The banana

cells synthesised the Cas9 protein and gRNA molecules which targeted Cas9 to specific genomic sites to introduce DSBs. The banana endogenous repair mechanisms could then introduce DNA edits in the target sequences.

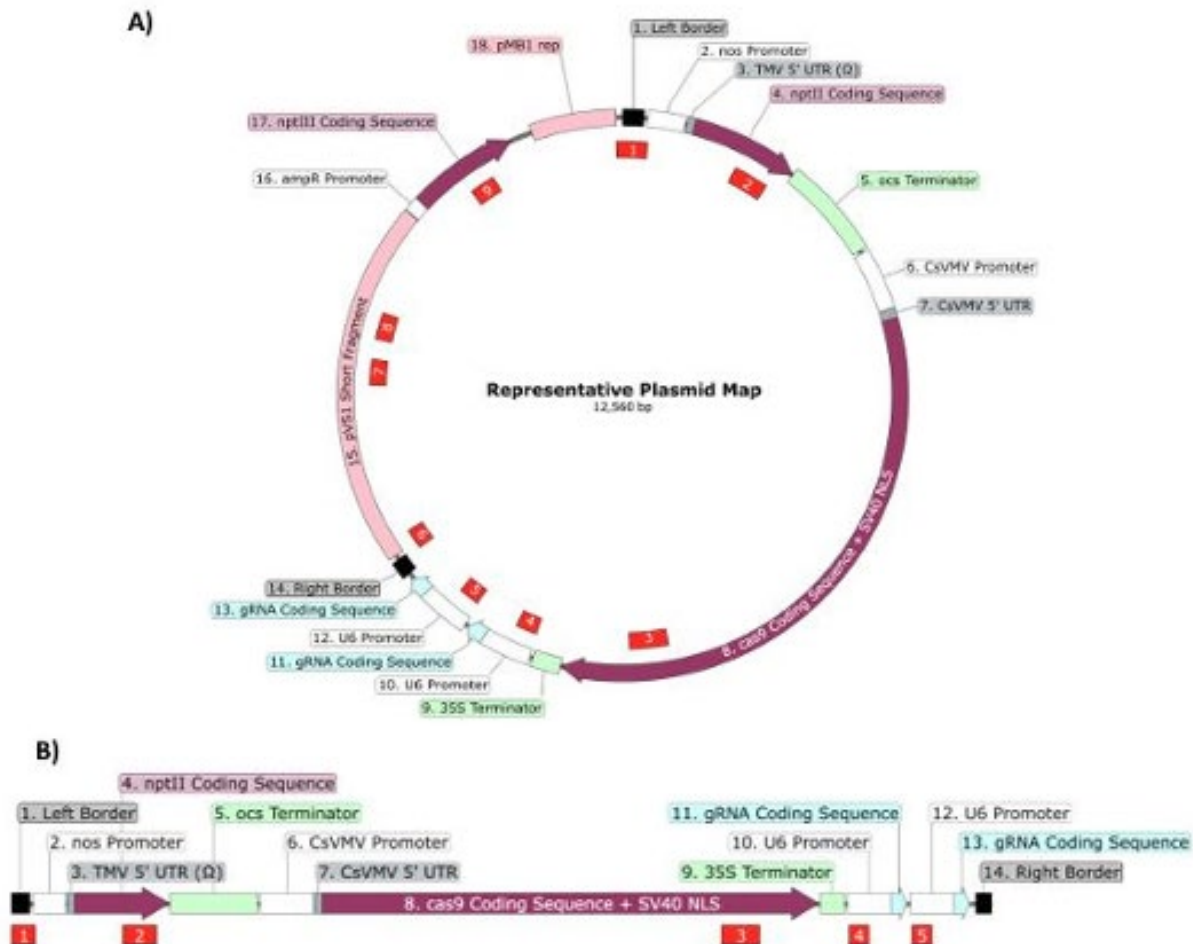


Figure 2. Map of the plasmid used in the genetic manipulation of the reduced browning banana line TRB011002. Map of the **(A)** plasmid and **(B)** T-DNA used in the genetic manipulation of the reduced browning banana line TRB011002. The T-DNA region is enclosed within the Left and Right Borders. Numbers in red boxes correspond to the amplicons from the quantitative polymerase chain reaction (qPCR) analyses used to confirm the absence of plasmid DNA in the genome of the reduced browning banana line TRB011002. In the initial qPCR screen, a primer pair targeting regions 4 and 5 was used. In the subsequent full molecular characterisation qPCR analyses, 8 primer pairs targeting regions 1 – 9 were used.

Following the *A. tumefaciens* treatment, the banana embryogenic cells were regenerated into individual plants *in vitro*. These individual plants were then maintained *in vitro* with vegetative micropropagation used to produce multiple, genetically identical individuals. During this process, lines were selected using three phases of molecular

characterisation on genomic DNA (gDNA) samples taken from the individual plants which confirmed the targeted edits and absence of foreign plasmid DNA integration in the reduced browning banana line TRB011002.

(b) information about any transgenic intermediates used;

No transgenic intermediates were used in generating the reduced browning banana line TRB011002.

7. Analysis and procedures used

(a) description of the analysis and procedures used to confirm the plant only contains genetic sequences that could arise by traditional processes.

Three phases of molecular characterisation, termed 'initial screen', full molecular characterisation' and 'whole genome sequencing (WGS)', were performed to identify and characterise the reduced browning banana line TRB011002 (Figure 3). The first two stages of molecular characterisation were performed on the initial plant of the reduced browning banana line TRB011002 which was then micropropagated in vitro to produce multiple, clonal individuals before WGS. For this WGS, multiple leaf samples were taken from the reduced browning banana line TRB011002 plants and pooled. Through these multiple molecular characterisation phases, the reduced browning banana line TRB011002 was confirmed as edited as targeted without unintended genetic changes and non-transgenic (confirmed absence of integration of plasmid DNA).

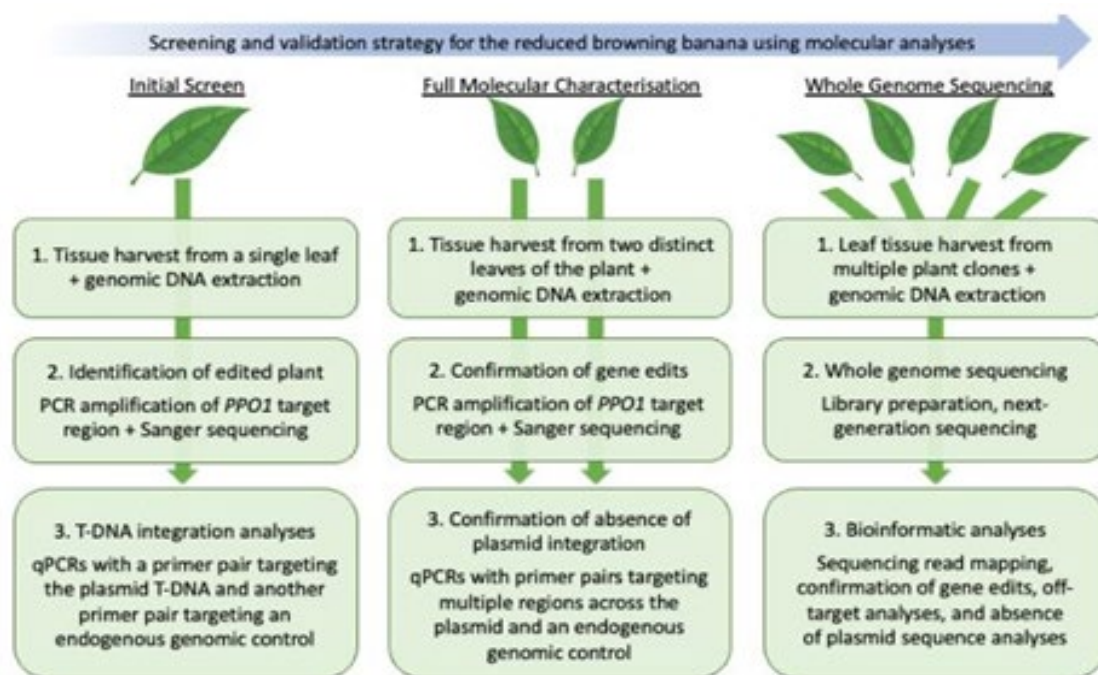


Figure 3. Flow chart highlighting the molecular characterisation undertaken on the reduced browning banana line TRB011002.

Absence of foreign plasmid DNA integration:

The method used to edit the reduced browning banana line TRB011002 was designed to produce edited plants without plasmid DNA integration. Nonetheless, three phases of molecular characterisation incorporating qPCR and WGS analyses, were used to assess for and confirm the absence of plasmid DNA in the genome of the reduced browning banana line TRB011002. In the initial screen, genomic DNA (gDNA) was extracted from a single leaf sample of the initial reduced browning banana plant which was regenerated from the *A. tumefaciens*-treated embryogenic cells. Absence of the T DNA in this sample was assessed using qPCR analyses with one primer pair designed to amplify from elements in the T-DNA and another primer pair targeted to an endogenous banana gene, phytoene desaturase (MaPDS). The sample from the initial plant of the reduced browning banana line TRB011002 gave specific amplification with the primer pair targeted to the endogenous banana gene but not the T-DNA-targeted primer pair indicating that the T-DNA sequence was unlikely to have integrated into the plant genome. The same result was detected with a wild-type control banana gDNA sample whilst a transgenic control banana gDNA sample gave specific amplification with both primer pairs, confirming the technical success of the assay.

In the full molecular characterisation phase, the initial plant of the reduced browning banana line TRB011002 was resampled for further molecular characterisation. Two gDNA samples were extracted from distinct leaves of the initial plant and subjected to

further qPCR analyses to test for the absence of plasmid DNA integration. These qPCR investigations were performed using 8 primer pairs targeting 9 regions of the T DNA and plasmid backbone. Additionally, for each sample, a control qPCR reaction was performed with a primer pair targeting the endogenous banana gene. The plasmid specific primers failed to specifically amplify from gDNA extracted from the reduced browning banana plant. This was also the case for gDNA extracted from a negative control wild-type banana plant. In contrast, these primers successfully amplified from a positive control plasmid DNA sample carrying each primer pair targeted DNA sequence. The control qPCR reactions using a primer pair targeted to the endogenous banana gene successfully amplified from all banana gDNA samples but not the plasmid DNA sample. These analyses therefore indicated that plasmid sequences are absent from the genome of the reduced browning banana line TRB011002.

Finally, in the third phase of molecular characterisation following micropropagation gDNA for WGS was isolated from a pooled leaf sample taken from multiple clonal plants of the reduced browning banana line TRB011002. The WGS data for the reduced browning banana line TRB011002 was assessed to further confirm the absence of foreign DNA integration from the plasmid. For this, bioinformatic analyses were used to map the WGS reads to the reference banana genome and the plasmid map. The resulting coverage was assessed and visualised with the Integrative Genomics View (IGV) software (Robinson et al., 2011). The WGS demonstrated the absence of plasmid DNA integration into the genome of the reduced browning banana line TRB011002.

From the qPCR and WGS analyses, the absence of foreign plasmid DNA integration into the genome of the reduced browning banana line TRB011002 was validated across multiple clonal individuals and phases of vegetative propagation.

Addendum: Description of characterisation of the genetic changes made

The three phases of molecular characterisation were also used to identify and confirm the targeted edits introduced in the reduced browning banana line TRB011002. In the initial molecular screen, a single-leaf gDNA sample was extracted from each regenerated plant and subjected to polymerase chain reaction (PCR) and Sanger sequencing analyses to identify individuals with targeted DNA edits. This analysis identified the initial plant of the reduced browning banana line TRB011002 as containing targeted edits.

Next, the initial plant of the reduced browning banana line TRB011002 was resampled for a 'full molecular characterisation' with two gDNA samples extracted from distinct leaves of the plant. Target PCR amplifications and Sanger sequencing were used to fully characterise the targeted DNA edits. The initial plant of the reduced browning banana line TRB011002 was then micropropagated in vitro to produce multiple, clonal individuals.

Finally, WGS was conducted on gDNA extracted from a pooled sample of leaf tissue taken from multiple plant clones of the reduced browning banana line TRB011002. The

WGS data from the reduced browning banana line TRB011002 was used to further validate the targeted edits. To do so, the WGS reads aligning to target sites were visualised with the IGV software (Robinson et al., 2011). This confirmed the edits previously identified by PCR and sequencing. These analyses confirmed the presence of the genetic changes made in reduced browning banana line TRB011002.

8. Other precision bred plants covered by this marketing notice

(a) Total number of precision bred plants being notified

The notification is for a single line, reduced browning banana line TRB011002 as described.

(b) Confirmation of precision breeding criteria

Not applicable – only one plant being notified

(c) Variations in intended genetic changes introduced

Not applicable – only one plant being notified

(d) Variations in unintended genetic changes introduced

Not applicable – only one plant being notified

References

Iqbal A, Murtaza A, Huw W, Ahmad I, Ahmed A and Xu X (2019). Activation and inactivation mechanisms of polyphenol oxidase during thermal and non-thermal methods of food processing. *Food and Bioproducts Processing*, 117, 170-182. doi:10.1016/j.fbp.2019.07.006

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