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Field selection of elite events of East African highland bananas expressing elevated levels of pro-vitamin A

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Summary

Biofortification of staple crops is a sustainable strategy to deliver essential micronutrients to impoverished populations in developing countries. Banana is a highly valued crop consumed by over 75% of Ugandans. However, the starchy green cooking bananas have very low levels of pro-vitamin A (PVA) and heavy dietary reliance on them has been associated with vitamin A deficiency (VAD). Two banana cultivars, hybrid M9 and Nakitembe, were selected for PVA biofortification. A *phytoene synthase 2a* (*MtPsy2a*) gene was transformed into the selected cultivars under the control of the constitutive maize polyubiquitin1 promoter or the banana fruit-preferred ACC oxidase (ACO) promoter. Plants were regenerated on selective media and putatively transgenic plants confirmed by PCR. A total of 356 and 162 transgenic events for M9 and Nakitembe, respectively, were planted in a confined field trial (CFT). Transgenic plants were assessed against non-transformed controls. Selection was based on phenotype, cycle time, yield, β-carotene equivalents (β-CE) and transgene copy number. There were no significant variations in cycle time, but some phenotypic differences were observed between transgenic and non-transgenic controls. Transgenic fruits had yellow to orange fruit pulps, unlike pulp from non-transgenic controls that were paler. On average, fruit from transgenic M9 and Nakitembe accumulated fourfold and threefold more β-CE than non-transgenic controls, respectively. Five elite lines each of M9 and Nakitembe have been selected for national agronomic performance trials that will aid the selection of lead events to be considered for environmental release.

Introduction

Micronutrient deficiency is the lack of essential vitamins and minerals required by the body in small amounts for proper growth and development (Ritchie and Roser, 2017). The problem is more apparent in the developing world where the majority of the population rely on a few starchy staples that are inherently poor sources of micronutrients. Common micronutrient deficiencies include vitamin A, iodine, iron, zinc and folate. The most affected individuals of the population include children under 5 years of age and women of childbearing age whose physiological status requires nutritious diets.

Vitamin A deficiency (VAD) is the single most important cause of preventable childhood blindness in developing countries, and it contributes significantly, even at subclinical levels, to morbidity and mortality from common childhood infections (Awasthi and Awasthi, 2020; Imdad *et al.*, 2017, 2022). VAD in humans causes adverse health effects termed vitamin A deficiency disorders (VADD) which include drying of the cornea causing night blindness, total blindness, premature deaths (Sommer and Vyas, 2012) and reduced immunity leading to high infant mortality (Bai *et al.*, 2011; Bryce *et al.*, 2008; Herbers, 2003). It is estimated that 250 000 to 500 000 children become blind due to VAD each year, half of whom die within 12 months of losing their sight. In the case of pregnant women, it increases the risk of maternal mortality (WHO, 2009). The primary factor that leads to VAD is a diet low in vitamin A or pro-vitamin A (Awasthi and Awasthi, 2020). VAD is prevalent in Uganda, affecting more than 30% of the vulnerable groups, particularly children under 5 years and women of childbearing age (FANTA-2, 2010).

Different strategies being used to address the problem include dietary supplementation with vitamin A, food fortification, dietary diversification or biofortified crops. Dietary supplementation involves the use of pharmaceutical products such as vitamin tablets and suspensions. This has had successful outcomes in high-risk populations of developed countries (Allen, 2006; Fitzpatrick *et al.*, 2012; Gómez-Galera *et al.*, 2010; Mayo-Wilson *et al.*, 2011), but has sometimes had little impact in particularly hard-to-reach rural communities (Uganda Bureau of Statistics (UBOS) and ORC-Macro, 2001). Food fortification involves adding higher levels of nutrients to food than what the original food provides (Gómez-Galera *et al.*, 2010). It can also refer to the addition of micronutrients to processed foods. Although this strategy has been effective in developed countries, its success can be limited in other settings by financial constraints and a dependence on home-produced food, which is often not diverse enough to meet all nutritional requirements (Chopra and Darnton-Hill, 2007; Darnton-Hill *et al.*, 2002, 2006). Dietary diversification involves increasing the diversity of food consumed to cover the range of all the

essential micronutrients. This requires the implementation of programs that improve the availability and consumption of micronutrient-rich foods. This requires extensive education of communities about essential nutrients in specific foods, which may be resource-intensive (Demment *et al.*, 2003; Fanzo *et al.*, 2013). Uganda has a comprehensive food and nutrition policy that provides for support to these requirements, but due to limited resources, the strategy is severely under-implemented. Development of biofortified crops involves increasing their inherent essential nutritional content and can be achieved through conventional breeding or genetic engineering. Conventional plant breeding involves progressively crossing female and male parents of the same species and selecting elite hybrids for release to farmers, whereas genetic engineering involves the transfer of only one or a few genes for a heritable trait to an already existing cultivar via genetic transformation.

Several crop varieties have been conventionally biofortified and are accepted by consumers (Council for Agricultural Science and Technology (CAST), 2020; Birol *et al.*, 2015). Notable examples include orange-fleshed sweet potato (OFSP) developed by selective breeding at NARO and released in Uganda and other countries to combat VAD (Mwanga and Ssemakula, 2011). Others include maize (Kebede *et al.*, 2021), and iron and zinc dense common beans (Amongi *et al.*, 2021). 'Golden Rice', is perhaps the best publically known example of successful genetic modification for improved PVA content. The first Golden Rice variety, 'Golden Rice 1' involved the introduction of a daffodil *phytoene synthase* (*Psy*) and a bacterial (*Erwinia uredovora*) *phytoene desaturase* (*crtI*) gene under the control of endosperm-specific glutelin and constitutive CaMV-35S promoters, respectively (Ye *et al.*, 2000). The endosperm of transformed rice accumulated up to 1.6 μg/g of mainly β-carotene which nearly met the set target of 2 μg/g at the time. Replacement of the daffodil PSY with a maize PSY gene resulted in a 23-fold increase in total carotenoids in transgenic 'Golden Rice 2', of which 31 μg/g was β-carotene (Paine *et al.*, 2005). The International Rice Research Institute (IRRI) conducted a comprehensive safety testing of 'Golden Rice 2' that resulted it its approval for feed food and processing in Australia, New Zealand, Canada and the United States (International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2022). Biofortification of cassava was successfully accomplished by expressing a bacterial *phytoene synthase* (*crtB*) gene resulting in a 20-fold elevation in total carotenoid content (Sayre *et al.*, 2011). Co-expression of *crtB* with the Arabidopsis *1-deoxy-D-xylulose-5-phosphate synthase* (DXS) gene doubled the carotenoid content further, reaching up to >50 μg/g DW in the cassava tuberous roots (Sayre *et al.*, 2011). Expression of bacterial genes has also been successful in enhancing carotenoid accumulation in maize expressed bacterial CRTB and CRTI genes result in a preferential accumulation of β-carotene in endosperm and a 34-fold increase in total carotenoids in the transgenics compared with WT controls (Aluru *et al.*, 2008).

In Uganda, banana is a key food security crop providing nutrition and income to over 75% of farm households (MAAIF, 2010). The most common types of bananas in Uganda are the East African highland bananas (EAHB, AAA-EA) locally called 'matooke'. The green mature fruits are prepared for consumption using various methods including boiling, steaming, frying or roasting (Akankwasa *et al.*, 2021). However, EAHB bananas contain very low levels of PVA and other critical micronutrients such as iron (Ekesa *et al.*, 2012; Hardisson *et al.*, 2001; Mbabazi *et al.*, 2020). For instance, the preferred cooking cultivars contain only 2 to 15 μg/g dry weight (DW) β-CE which cannot supply sufficient PVA carotenoids to meet the daily requirement of 500 to 800 μg recommended by the World Health Organization (WHO). It is, therefore, no coincidence that there is widespread malnutrition and associated diseases in areas where people over rely on EAHB as a major source of nutrition (Uganda Bureau of Statistics (UBOS) and ICF, 2018).

In Uganda, genetic engineering was preferred over conventional breeding because it enables the introduction of transgenes into already consumer-accepted but sterile EAHB cultivars, thereby preserving key traits while avoiding the need to develop entirely new varieties. There have been two different genetic engineering approaches for enhancing PVA levels in crops, which involve either over-expressing key enzymes or blocking competing branches in the carotenoid biosynthesis pathway. For instance, Golden Rice 2 (GR2) was developed by over-expression of a maize *phytoene synthase* (*Psy*) gene, which resulted in a 37-fold increase in β-carotene compared to non-transgenic controls (Paine *et al.*, 2005). Similarly, over-expression of the maize *Psy* together in combination with a bacterial *phytoene desaturase* gene (*crtI* from *Pantoea ananatis*) resulted in a 34-fold increase in total carotenoid content (Aluru *et al.*, 2008). In potato, tuber-specific silencing of the competing lycopene *ε-cyclase* gene, which directs flux towards α-carotene synthesis, led to a 14-fold increase in β-carotene levels (Diretto *et al.*, 2007). Based on these findings, we adopted the GR2 and maize-based approach as the more effective strategy for biofortification.

A single banana *phytoene synthase* transgene (*MtPsy2a*) under the control of either a constitutive promoter, maize Ubi or a fruit-preferred promoter, banana ACO was previously evaluated using 'Cavendish' banana to evaluate their effect on target PVA levels and agronomic traits. One line contained more than 70 μg/g DW β-CE in the fruit with numerous other lines with greater than 20 μg/g DW β-CE (Paul *et al.*, 2017). This became a springboard to product development in Uganda using preferred banana varieties.

In this study, we report the transformation of two EAHB cultivars with a single banana *phytoene synthase* transgene to biofortify Uganda's most widely consumed staple crop with pro-vitamin A, aiming to enhance vitamin A delivery in hard-to-reach rural communities in Uganda and beyond. Furthermore, we report the assessment of transgenic lines in the field and the selection of elite events to progress through to multi-location field trials in Uganda.

Results

Transformation and primary molecular characterization of transgenic plants

Two banana cultivars, M9 and Nakitembe, were successfully transformed, each with two binary vectors pBMGF-DC-32 and pBMGF-DC-34. A blue staining observed from a GUS assay of a portion of cell suspensions transformed with the genes of interest indicated successful transformation (Figure 1).

Transgenic plants for M9 were generated from cell line M9-498 with a regeneration potential of >60%. The transgene in both M9 and Nakitembe was confirmed by end-point PCR analysis targeting the Ubi-*MtPsy2a* or ACO-*MtPsy2a* promoter-transgene

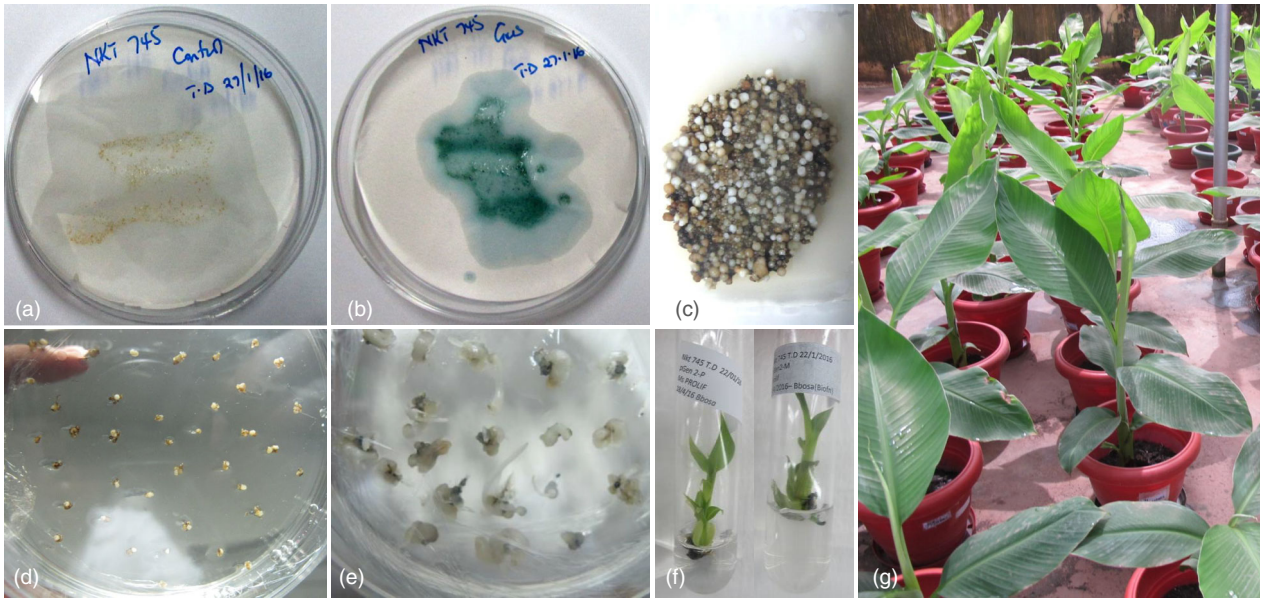

Figure 1 Illustration of transformation of banana embryogenic cell suspensions of cultivar Nakitembe. (a) non-transformed cells from cell line NKT-745; (b) transient GUS expression three days post-transformation; (c) embryos regenerating from transformed cells; (d) segregated embryos on germination media; (e) germinating embryos; (f) regenerated plants and (g) hardened plants in the greenhouse.

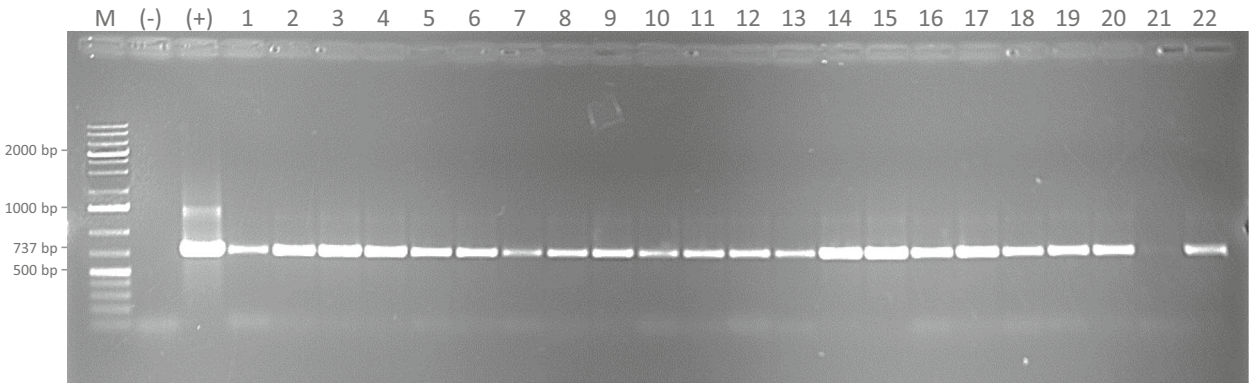

Figure 2 A representative gel showing end-point PCR results with primers targeting the Ubi-*MtPsy2a* junction in M9 transformed plants. M, 100 bp Plus DNA ladder; (−), no template control; (+), plasmid pBMGF-DC-34 control. Lanes 1–22, putatively transgenic plants.

junction (Figure 2; Table 1). When transformed with Ubi-*MtPsy2a* and/or ACO-*MtPsy2a*, out of nearly 500 putatively transgenic M9 plants, 356 were positive for the transgene, giving an overall transformation efficiency of 71.2%. Transformation efficiency varied slightly between the two constructs, with Ubi-*MtPsy2a* positive plants accounting for 57% of total plants generated for M9. Over 95% of all M9 lines confirmed positive did not have latent *Agrobacterium* contamination.

Transgenic plants of AAA-EA cultivar Nakitembe were generated from up to ten cell lines (NKT-660, 731, 732, 737, 739, 742, 743, 745, 746 and 747) with a regeneration potential of 60 to 90%. Out of 1500 putatively transformed plants of Nakitembe generated, only 162 plants were confirmed positive for the presence of the transgene with a transformation efficiency of only 11%. Just like for M9, 57% of the transformed Nakitembe plants were positive for Ubi-*MtPsy2a*. Up to 98.1% of transgenic Nakitembe lines had no latent *Agrobacterium* contamination.

Agronomic and growth characteristics

Cycle time

The first level of selection criterion was trueness to type based on cycle time and phenotypic appearance. Cycle time was measured as the time taken from planting to harvest for the plant crop and from harvest to harvest for the ratoon crop cycles. There were no significant differences in cycle time for control and transgenic plants for either M9 ($P = 0.6342$) or Nakitembe ($P = 0.9855$). The average cycle time for wild-type M9 was 18.1 months for the plant crop (PC) followed by 5.9 and 7.8 months for ratoon 1 (R1) and ratoon 2 (R2) crops. For transgenic M9, the average cycle time was 17.4, 6.3 and 9.1 months for PC, R1 and R2 crops respectively.

Cultivar Nakitembe generally matured faster with controls harvested at 13.3 months after planting, followed by harvesting of R1 and R2 at 5.5 and 5.0 months from the previous harvests,

Table 1 Primers used during the study

Target	Primer name	Sequence (5′ →3′)	Tm (°C)	Amplicon size (bp)
*npt*II	NPTII-F	TGATTGAACAAGATGGATTGCACGC	66	620
	NPTII-R	GATGAATCCAGAAAAGCGGCCAT		
Ubi-*MtPsy2a* junction	UbiInt-F	GATTTTTTTAGCCCTGCCTTC	55	737
	APsy2a-R	TTGTACCTCGATTTCCGCAGGTC		
ACO-*MtPsy2a* junction	ACO-F	GAGAACAAGGCCAAAGGTGGTGG	62	878
	APsy2a-R	TTGTACCTCGATTTCCGCAGGTC		
Banana Actin	Actin-F	CTGGTGATGGTGTGAGCCAC	54	640
	Actin-R	CATGAAATAGCTGCGAAACG		
VirC operon in Ti plasmid pTiBo542	VCF-AGL1	GCCTTAAAATCATTTGTAGCGACTTCG	62	738
	VCR-AGL1	TCATCGCTAGCTCAAACCTGCTTCTG		

Table 2 Agronomic performance of transgenic M9 and Nakitembe banana cultivars versus non-transgenic controls

		M9					Nakitembe				
		Control		Transgenic			Control		Transgenic		
No.	Parameter	*N*	Mean ± SE	*N*	Mean ± SE	*P*-value	*N*	Mean ± SE	*N*	Mean ± SE	*P*-value
1	Plant height (cm)	102	321.1 ± 3.7	702	337.9 ± 1.7	0.0003	45	300.3 ± 9.0	247	292.9 ± 2.9	**0.3472**
2	Plant circumference (cm)	104	75.8 ± 0.8	704	68.6 ± 0.4	0.0000	45	71.6 ± 2.0	247	65.9 ± 0.7	0.0022
3	Number of functional leaves at flowering	107	11.3 ± 0.2	734	10.6 ± 0.1	0.0016	45	9.4 ± 0.2	257	9.4 ± 0.1	**0.9181**
4	Bunch weight (kg)	122	20.3 ± 0.6	890	11.4 ± 0.2	0.0000	45	18.7 ± 1.4	259	11.9 ± 0.6	0.0000
5	Bunch length (cm)	118	86.4 ± 1.4	833	73.8 ± 0.5	0.0000	43	89.6 ± 2.9	245	79.7 ± 1.1	0.0009
6	Bunch circumference (cm)	118	107.6 ± 1.1	833	90.0 ± 0.4	0.0000	43	109.5 ± 2.6	245	100.1 ± 1.0	0.0004
7	Number of hands on a bunch	104	8.5 ± 0.1	762	8.3 ± 0.0	**0.0961**	43	7.4 ± 0.3	254	6.7 ± 0.1	0.0112
8	Number of fingers on top hand	105	18.5 ± 0.5	768	13.2 ± 0.2	0.0000	43	17.8 ± 0.9	254	14.4 ± 0.4	0.0021
9	Number of functional leaves at harvest	118	5.9 ± 0.2	802	4.8 ± 0.1	0.0000	41	4.3 ± 0.2	221	4.5 ± 0.1	**0.5767**
10	Leaf length (cm)	115	277.6 ± 3.5	746	244.6 ± 1.2	0.0000	41	262.8 ± 7.5	221	237.6 ± 3.3	0.0026
11	Leaf radius (cm)	115	74.2 ± 0.9	745	58.9 ± 0.3	0.0000	41	71.4 ± 2.1	221	60.7 ± 0.9	0.0000
12	Fruit length (cm)	118	21.2 ± 0.2	839	19.8 ± 0.1	0.0000	43	20.6 ± 0.5	245	19.1 ± 0.2	0.0038
13	Fruit circumference (cm)	118	13.7 ± 0.1	839	12.2 ± 0.0	0.0000	43	13.6 ± 0.2	245	12.9 ± 0.1	0.0050
14	Petiole length (cm)	114	56.2 ± 0.9	737	57.8 ± 0.4	**0.1093**	41	50.4 ± 1.2	220	51.4 ± 0.6	**0.4070**

Note: Data averages from all assessed plants, regardless of generation. SE, standard error of the mean. Figures in bold indicate non-significant *P*-values. *N*, number of biological replicates.

respectively. The average cycle times for the Nakitembe transgenic plants were 13.6, 5.5 and 5.4 months for the PC, R1 and R2 crops, respectively.

Similarly, the cycle time of plants transformed with promoters Ubi or ACO did not significantly differ from their wild-type controls. These observations confirm that the transgene for enhancement of PVA in banana did not affect the trueness to type of M9 ($P = 0.21243$) and cultivar Nakitembe ($P = 0.7232$).

Phenotypic characteristics were assessed through direct observation of plant morphology. It was impossible to differentiate WT controls from transgenics of both M9 and Nakitembe based on the external phenotypic appearance of bunches or fruits (Table 2, Figure 3). M9 controls and transgenics had more significant differences in parameters assessed than Nakitembe (Table 2). Distinct differences between control and transgenic fruits were observed only after longitudinal sectioning. The fruit pulp of transgenic M9 appeared yellow to orange in contrast to the usual cream coloured pulp of controls. Similarly, the fruit pulp of Nakitembe transgenics was deep yellow to light orange in colour compared to the yellow colour of controls (Figure 3).

Yield

In order to select for suitable transgenic lines, yield was considered the second-level criterion and set at a lower limit of not more than 20% of the yield for the wild-type control. M9 controls performed better than transgenic lines with an overall average of 20.3 kg per bunch compared to 11.4 kg for transgenic lines. The average yield for transgenic M9 was 12.9, 9.8 and 11.5 kg in the PC, ratoon 1 and ratoon 2 crops, respectively. Similarly, non-transgenic Nakitembe control lines had bigger bunches with an average of 18.7 kg compared to 11.9 kg for transgenic lines. The average yield measured as bunch weight of transgenic Nakitembe was 9.6, 11.7 and 19.4 kg for the PC, ratoon 1 and ratoon 2 crops, respectively. Consequently, the lower 20% yield limit was set at 15.5 kg for M9 and 14.5 kg for Nakitembe based on respective overall control yields.

In general, yields were reduced in transgenic plants compared to the controls irrespective of the promoter used. Yield reductions were more significant in M9 than in Nakitembe ($P < 0.001$). On

Figure 3 Representative photographs of bunches and fruit from control (wild-type) versus transgenic M9 and Nakitembe (NKT) lines.

average, M9 WT controls weighed 20.3 kg compared to 11.5 and 11.4 kg for transgenic lines harbouring ACO-*MtPys2a* and Ubi-*MtPys2a*, respectively. In Nakitembe, bunch weight reduced from 18.7 kg in controls to 11.6 and 12.1 kg in transgenic lines containing the ACO-*MtPys2a* and Ubi-*MtPys2a* constructs, respectively (Figure 4).

β-Carotene equivalents (β-CE)

All the lines that met the required criteria for trueness to type and yield (bunch weight) were subjected to the third level of selection criteria, being the PVA content at a minimum of 20 µg/g DW β-CE.

The β-CE levels increased significantly in both M9 and Nakitembe transgenics compared to their respective WT controls ($P < 0.001$). M9 controls had an overall average β-CE of 5.6 µg/g DW compared to 21.6 µg/g DW in transgenic lines. There was no significant difference in β-CE content between PC and R1 crops. However, the R2 crop had significantly lower β-CE levels compared to the first two generations.

Nakitembe controls, on the other hand, had an overall average β-CE of 13.9 µg/g DW (range 0.9 to 33.6 µg/g DW), whereas transgenics had 35.7 µg/g DW (range 2.2 to 149.6 µg/g DW). No significant variations were observed in the overall β-CE content between generations, although increases were noted from generation to generation in transgenics. Ubi-*MtPsy2a* lines generally had higher β-CE levels (overall average of 37.8 µg/g DW) compared to ACO-*MtPsy2* lines (overall average of 30.8 µg/g DW).

Higher β-CE levels in M9 WT controls were significantly related to smaller bunches ($P = 0.01$). Similarly, high β-CE levels in transgenic M9 lines were significantly associated with smaller bunches ($P < 0.0001$). No significant relationship between β-CE and yield was observed in Nakitembe controls or transgenics (Figure 5).

Selection of elite lines

Field selections were primarily based on yield and β-CE. Any lines that satisfied the two criteria at any of the three generations studied were considered candidate lines. Any phenotypic abnormalities (dwarfness) resulted in inferior bunches that were automatically rejected based on yield. A total of 62 M9 and 45 Nakitembe lines met these criteria (Tables 3 and 4). Out of the 62

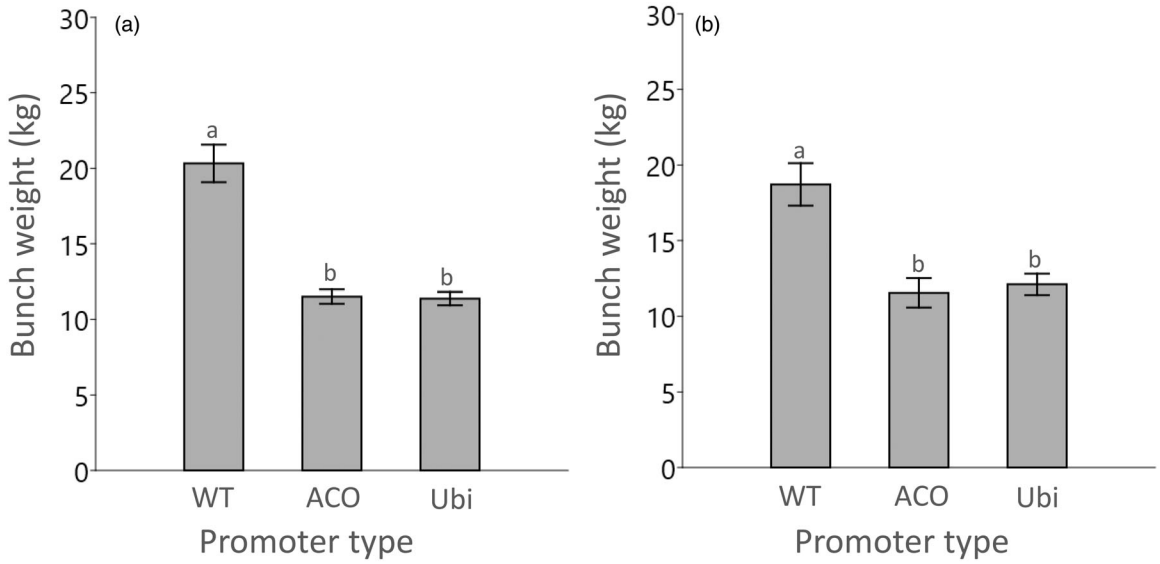

Figure 4 Influence of promoter activity on bunch weight of wild-type control and transgenic M9 and Nakitembe plants. (a) M9 plants; (b) Nakitembe plants. Data represent the mean ± SE. Statistical analysis was performed using one-way ANOVA followed by Mann–Whitney pairwise comparisons. Different letters indicate significant differences ($P < 0.001$).

selected M9 lines, 52.5% had been transformed with construct ACO-*MtPsy2a* and 47.5% with Ubi-*MtPsy2a* (Table 3). In contrast, Nakitembe had a smaller fraction (28.9%) of selected lines containing ACO-*MtPsy2a* while the majority (71.1%) were of Ubi-*MtPsy2a* (Table 4).

The selection for M9 elite lines was mainly based on the crop performance across generations where the majority of selected lines met both yield and β-CE criteria. Line M9-12141 was included in the selection because of an acceptable copy number and a clean T-DNA insertion revealed by sequence analysis.

Selected M9 elite lines had a significant yield difference between PC and R1 ($P < 0.001$) and R2 crops ($P < 0.001$). Ratoon 1 and R2 had no significant yield variations ($P = 0.1095$). Bunch weight ranged from an average of 7.3 kg (Ubi-*MtPsy2a*, 3 copies) to 18.5 kg (ACO-*MtPsy2a*, 1 copy). No overall significant difference was observed in β-CE levels among M9 elite lines at all generations. β-CE levels ranged from 7.2 μg (ACO-*MtPsy2a*, 6 copies) to 45.9 μg/g DW (ACO-*MtPsy2a*, 5 copies) in M9 selected lines (Table 3).

On the other hand, significant yield variations were observed between all generations of Nakitembe elite lines ($P < 0.001$). Yield ranged from 11.2 kg (ACO-*MtPsy2a*, 2 copies) to 35.0 kg (Ubi-*MtPsy2a*, 4 copies). Like observed in M9, β-CE levels in elite Nakitembe lines did not vary significantly between generations ($P > 0.05$). β-CE levels ranged from 19.1 μg (ACO-*MtPsy2a*, 1 copy) to 76.1 μg/g DW (Ubi-*MtPys2a*, 7 copies) in selected Nakitembe lines (Table 4).

Yield and β-CE variations were not unique to only transgenic lines. Similar variations were also observed in WT controls. On average, yields of M9 control ranged from 18.3 to 23.6 kg and β-CE ranged from 4.0 to 6.9 μg/g DW (Table 3). In Nakitembe, yields of control ranged from 15.0 to 29.1 kg and β-CE ranged from 11.2 to 15.4 μg/g DW (Table 4).

Copy number

A fourth criterion based on the number of copies of the gene inserted in the genome of transgenic lines was used to select lines with the least possibility of T-DNA disrupting existing open reading frames (ORFs) or creating new ones. The priority was to select lines containing only 1–2 copies of the *MtPsy2a* gene. Interestingly, most M9 and Nakitembe lines that met yield and β-CE levels criteria were found to contain 1–2 copies of the gene.

Up to 29% of the selected M9 lines contained one copy of the gene while 31% had two copies, with the remaining lines containing 3 to 13 copies (Table 3). Nakitembe had the highest proportion of lines containing a single copy of the gene (53.3%), while another 31% contained two copies of the gene. Seven lines of Nakitembe contained undesirable copy numbers ranging from 3 to 7 (Table 4). Only those lines with 1 to 2 copies of the transgene integrated were selected as potential elite events.

Discussion

Despite intensive efforts to reduce micronutrient malnutrition, more than 40% of the world's population still suffer from at least one of the major micronutrient deficiencies, that is, iron, zinc, vitamin A or iodine (Ritchie and Roser, 2017) and their prevalence is rising, especially in developing countries. The situation not different in Uganda, where a section of the population that is hard-to-reach, especially children below 5 years and young women, continue to grapple with the 'hidden hunger' (Uganda Bureau of Statistics (UBOS) and ICF, 2018; Uganda Bureau of Statistics (UBOS) and ICF International, 2012; Uganda Bureau of Statistics (UBOS) and Macro International, 2007; Uganda Bureau of Statistics (UBOS) and ORC-Macro, 2001).

There has been substantial progress in the micronutrient biofortification of crop plants as a sustainable health intervention (Malik and Maqbool, 2020). Typical examples of pro-vitamin A carotenoid (PVAC) biofortified crops include Golden Rice (Paine *et al.*, 2005), maize (Aluru *et al.*, 2008; Naqvi *et al.*, 2009; Zhu *et al.*, 2008), wheat (Cong *et al.*, 2009; Wang *et al.*, 2014) potatoes (Diretto *et al.*, 2007), cassava (Beyene *et al.*, 2018) and banana (Paul *et al.*, 2017). The results reported here represent progress so far with the biofortification of EAHB.

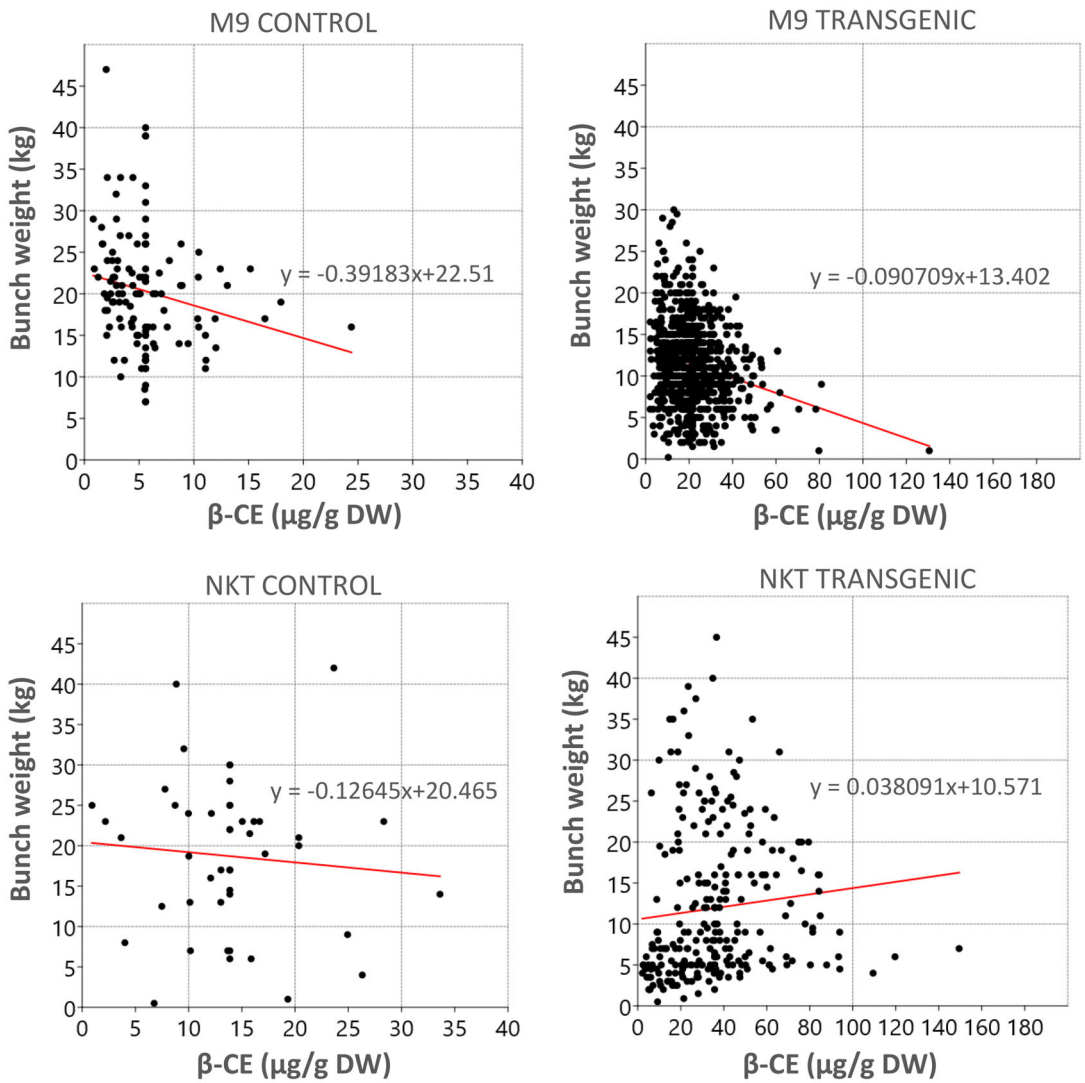

Figure 5 Relationship between β-CE levels and bunch weight in WT controls and transgenic lines of M9 and Nakitembe (NKT).

The purpose of this study was to elevate levels of PVA in EAHB by transforming them with PVA-enhancing genes. Plants with elevated levels of PVAC and without yield penalty or undesirable phenotypic effect would then be selected for multi-locational evaluation. Bananas with enhanced PVAC offer a more sustainable delivery system to mitigate vitamin A deficiency (VAD) in impoverished populations that depend on it as a major source of nutrition.

We report the successful transformation of both M9 and Nakitembe cooking bananas with the banana *MtPsy2a* transgene under the control of a maize ubiquitin promoter and a banana-derived ACO promoter. We set out with an ambitious target to generate 200 independent transgenic lines for each of the two vectors for the two cultivars. However, a total of 356 M9 lines (153 lines were transgenic for ACO-*MtPsy2a* and 203 with Ubi-*MtPsy2a*) and 162 Nakitembe lines (69 lines consisting of ACO-*MtPsy2a* and 93 Ubi-*MtPsy2a*) independent transgenic lines were generated in this study. Transformation and regeneration efficiencies have been observed to vary between different banana varieties (Tripathi *et al.*, 2015) and several factors likely contributed to this outcome. Genotype-specific responses to *Agrobacterium* infection, differential competence for somatic embryogenesis and variation in tissue culture responsiveness all play a role in determining transformation success. Nakitembe, in particular, is known to be more recalcitrant under *in vitro* conditions, which may account for the lower number of lines recovered. Additionally, the physiological state of explants, susceptibility to oxidative browning and callus proliferation capacity can further influence regeneration efficiency, especially during large-scale transformation efforts such as this one.

Our initial molecular characterization after acclimatizing putatively transgenic plantlets in the greenhouse was based on the simple PCR detection of the transgene and the absence of *Agrobacterium* contamination. At that stage, the number of plants advanced to CFT could have been drastically reduced if molecular characterization had included copy number determination and vector backbone detection.

Nakitembe was more recalcitrant to transformation; however, those lines that were successfully transformed had a higher proportion of T-DNA single-copy insertions (53.3%) compared to M9 lines (29%). The apparent ease of transformation in M9 led to a greater frequency of multiple T-DNA insertions, which

Table 3 Salient features of M9 transgenic and control lines based on yield, β-CE and transgene copy number

			Plant crop		Ratoon 1 crop		Ratoon 2 crop	
Construct	T-DNA copy #	*N*	Bunch weight (kg)	β-CE (μg/g DW)	Bunch weight (kg)	β-CE (μg/g DW)	Bunch weight (kg)	β-CE (μg/g DW)
ACO-*MtPsy2a*	1	9	**18.5 ± 1.0**	22.1 ± 3.2	10.9 ± 1.6	21.6 ± 3.6	10.8 ± 1.5	13.6 ± 4.0
	2	10	16.6 ± 1.1	21.1 ± 2.2	10.2 ± 1.4	27.0 ± 3.0	11.9 ± 1.4	24.4 ± 5.7
	3	4	17.6 ± 0.9	28.3 ± 2.4	8.0 ± 1.1	25.2 ± 2.9	11.6 ± 1.9	28.3 ± 5.4
	4	1	18.0 ± 0.0	31.3 ± 0.0	**16.0 ± 0.0**	20.9 ± 0.0	16.0 ± 0.0	23.2 ± 0.0
	5	1	18.0 ± 0.0	22.2 ± 0.0	ND	ND	13.0 ± 0.0	**45.9 ± 0.0**
	6	2	16.0 ± 0.0	34.8 ± 4.3	9.0 ± 1.0	30.8 ± 7.5	9.8 ± 2.8	**7.2 ± 0.0**
	7	1	17.0 ± 0.0	27.2 ± 0.0	10.0 ± 0.0	27.3 ± 0.0	8.0 ± 0.0	ND
	13	1	16.0 ± 0.0	25.7 ± 0.0	9.0 ± 0.0	**31.7 ± 0.0**	12.0 ± 0.0	20.5 ± 0.0
	X	3	14.2 ± 2.2	19.9 ± 2.7	10.2 ± 2.7	20.7 ± 6.7	**17.0 ± 1.7**	21.8 ± 6.8
Ubi-*MtPsy2a*	1	9	16.8 ± 0.3	22.7 ± 0.6	**7.5 ± 1.2**	22.7 ± 3.2	11.5 ± 1.9	15.0 ± 2.3
	2	9	16.8 ± 0.3	22.7 ± 0.6	**7.5 ± 1.2**	22.7 ± 3.2	11.1 ± 1.9	15.0 ± 2.3
	3	2	17.0 ± 1.0	23.0 ± 1.4	11.0 ± 2.0	19.8 ± 7.0	**7.3 ± 2.8**	25.0 ± 13.6
	4	4	15.6 ± 1.1	**41.5 ± 2.7**	10.5 ± 1.6	31.2 ± 2.4	13.1 ± 2.7	37.6 ± 4.4
	X	6	**12.5 ± 3.4**	**18.5 ± 2.6**	11.8 ± 3.2	**16.0 ± 4.5**	16.7 ± 1.8	28.4 ± 4.5
Wild-type	-	44	18.3 ± 0.8	6.9 ± 0.8	19.2 ± 0.7	5.3 ± 0.7	23.6 ± 1.5	4.0 ± 0.5

Note: Data represent the mean ± SE, β-CE, β-carotene equivalents; DW, dry weight; ND, no data; *N*, number of biological replicates; X, copy number not determined. Figures in bold represent the highest and lowest bunch weights and β-CE levels obtained from transgenic lines in the three crop cycles.

Table 4 Salient features of selected Nakitembe transgenic lines and WT controls based on yield, β-CE and transgene copy number

			Plant crop		Ratoon 1 crop		Ratoon 2 crop	
Construct	T-DNA copy #	*N*	Bunch weight (kg)	β-CE (μg/g DW)	Bunch weight (kg)	β-CE (μg/g DW)	Bunch weight (kg)	β-CE (μg/g DW)
ACO-*MtPsy2a*	1	7	14.7 ± 1.8	44.7 ± 6.0	21.9 ± 2.3	36.8 ± 5.4	33.0 ± 1.2	**19.1 ± 2.5**
	2	5	**11.2 ± 0.7**	47.4 ± 9.2	19.8 ± 2.1	**26.2 ± 7.5**	**26.7 ± 2.3**	47.4 ± 8.2
	7	1	16.5 ± 0.0	**76.1 ± 0.0**	22.5 ± 0.0	32.9 ± 0.0	29.0 ± 0.0	26.9 ± 0.0
Ubi-*MtPsy2a*	1	17	17.4 ± 1.2	35.7 ± 5.1	19.7 ± 1.4	35.5 ± 3.6	34.4 ± 3.0	34.5 ± 6.3
	2	9	15.2 ± 0.6	47.2 ± 5.8	22.6 ± 1.9	37.2 ± 5.8	32.0 ± 1.5	43.2 ± 14.4
	3	3	19.0 ± 3.0	53.9 ± 15.7	19.3 ± 0.3	**62.5 ± 9.4**	ND	ND
	4	2	19.3 ± 0.7	46.0 ± 33.4	**18.3 ± 3.8**	43.1 ± 17.0	**35.0 ± 0.0**	**53.4 ± 0.0**
	6	1	**21.0 ± 0.0**	**31.6 ± 0.0**	**23.0 ± 0.0**	35.0 ± 0.0	ND	ND
Wild-type	-	20	15.0 ± 1.5	15.1 ± 1.8	18.0 ± 1.8	11.2 ± 2.1	29.1 ± 3.2	15.4 ± 3.0

Note: Data represent the mean ± SE, β-CE, β-carotene equivalents; DW, dry weight; ND, no data. *N*, number of biological replicates. Figures in bold represent the highest and lowest bunch weights and β-CE levels obtained from transgenic lines in the three crop cycles.

subsequently resulted in more M9 lines being rejected during selection based on copy number. Although banana is clonally propagated and does not face issues of transgene segregation, lines with one or two T-DNA insertions are generally preferred. Multiple insertions increase the risk of disrupting endogenous genes or regulatory elements, potentially causing unintended phenotypic effects and complicating biosafety evaluations. Regulatory agencies also favour events with simple, well-characterized insertion profiles, as these reduce uncertainty during molecular characterization and approval. From a biological perspective, higher transgene copy number does not necessarily enhance trait expression and may instead disrupt metabolic balance, particularly in tightly regulated pathways such as carotenoid biosynthesis (Tang *et al*., 2007). Thus, selecting for low-copy events remains a critical consideration, even in clonally propagated crops.

The levels of β-CE increased by fourfold and threefold on average in transgenic M9 and Nakitembe, respectively. The choice of promoter had no significant difference in elevating β-CE levels in M9; however, the use of the Ubi promoter generally resulted in Nakitembe lines with considerably higher β-CE levels than those transformed using ACO. While it may be expected that the fruit-specific and ripening-inducible ACO promoter would outperform Ubi in fruit, particularly during ripening, the observed trend is consistent with the fact that fruit in this study were harvested at physiological maturity, prior to the onset of ripening. Under these conditions, the ethylene-responsive ACO promoter is minimally active, whereas Ubi drives constitutive expression throughout fruit development (Paul *et al*., 2017). The significant effect of Ubi in Nakitembe may also be influenced by the smaller and unbalanced sample set in this genotype, combined with its known phenotypic variability.

Based on our target of 20 μg/g DW (or 5 μg/g FW), any of the 62 lines of M9 or 72 lines of Nakitembe developed and selected in this study would meet 50% of the vitamin A RDA for preschool children (400 μg retinol activity equivalents) if only 645 g of

matooke is consumed. This is much easier than having to consume 4.6 kg or 1.7 kg of non-transformed M9 or Nakitembe, respectively, to meet the same RDA. This takes into account 25% losses of PVA during cooking (Mbabazi *et al*., 2020), and a conservative 12:1 β-carotene to retinol conversion ratio (Van Loo-Bouwman *et al*., 2014). Since most of the generated and selected lines contain well over 20 μg/g DW, consumption of much less amounts of cooked matooke can result in attaining the RDA levels.

The overall variation observed in β-CE levels from generation to generation was not significant in Nakitembe controls as well as transgenics. However, R2 of M9 had significantly lower β-CE levels in both controls and transgenic. Since M9 β-CE trends were similar in both controls and transgenics, trait expression could be reminiscent of prevailing environmental cues. Similarly, within the lines, we did not find any single M9 line with a consistent level of β-CE from one generation to another, except FT-12220 that was discarded because it had four copies of the transgene integrated (Supplementary information). Variations in PVAC content have also been reported in 'Golden Rice' and in transgenic maize (Aluru *et al*., 2008; Paine *et al*., 2005). Seasonal variation resulting from environmental impact has been observed to play a role in PVAC variations (Mbabazi *et al*., 2020; Paul *et al*., 2017). Of the two cultivars used therefore, Nakitembe exhibited more resilience to environmental influence and expressed the β-CE trait more consistently from generation to generation.

In this study, wild-type controls outperformed transgenics in yield, with reductions of 44% in M9 and 36% in Nakitembe. Carotenoid biosynthesis follows a complex pathway involving several intermediates and branches. The first committed and rate-limiting step in carotenoid biosynthesis is catalysed by PSY, which converts GGPP into phytoene, thereby controlling flux into the carotenoid pathway that ultimately leads to the production of lycopene, α-carotene and β-carotene. Over-expression of *MtPsy2a* may deplete the shared GGPP pool, reducing the availability of this key precursor for other isoprenoid-derived pathways, including those involved in the biosynthesis of plant hormones such as cytokinins and gibberellins, which are essential for growth and development (Cazzonelli and Pogson, 2010; Ruiz-Sola and Rodríguez-Concepción, 2012). This metabolic flux effect was more pronounced in the wild-type M9, which has a substantially lower inherent β-CE content (5.4 μg/g dry weight – DW) compared to Nakitembe (13.9 μg/g DW) (Table 2). This may reflect an upstream bottleneck limiting the supply of precursors to the carotenoid pathway, or alternatively, a stronger feedback inhibition mechanism in M9 that constrains carotenoid accumulation even when *MtPsy2a* is over-expressed. Interestingly, the choice of promoter did not significantly affect yield in either cultivar, indicating that both are suitable for PVA biofortification in EAHBs. As expected, control plants produced larger bunches but had lower β-CE levels. These findings suggest that increasing β-CE content using the current strategy may impact yield. However, firm conclusions should await multi-location agronomic trials with appropriate replication across all lines.

The number of M9 lines (62 lines) that satisfied primary selection criteria (fruit β-CE levels and yield) was higher than those of Nakitembe (45), largely due to a higher number of M9 events planted. Selected elite M9 lines represented 17.1% of the weaned and planted events, while those of Nakitembe represented 27.7% of planted lines, indicating a more precise insertion of the transgene in Nakitembe.

Most of the selected elite events had only one or two copies of the transgene integrated, as determined by a probe-based droplet digital PCR (ddPCR) assay targeting the *nptII* selectable marker gene. This marker, present in all constructs used, enabled accurate copy number estimation across all events while avoiding potential interference from endogenous *psy* sequences. Although ddPCR provides high specificity and sensitivity, it does not detect complex T-DNA arrangements such as tandem repeats, truncated insertions or rearrangements. To address these limitations, we confirmed ddPCR estimates through whole genome sequencing of all elite lines, which allowed comprehensive assessment of true T-DNA structure, chromosomal insertion sites, and the absence of vector backbone sequences. This multi-tiered approach strengthens confidence in the structural characterization of the transgenic events and mitigates the known limitations of PCR-based methods (Pucker *et al*., 2021). The majority of events met our target criterion of having no more than two insertions, a key selection filter to minimize risks of disrupting or silencing endogenous open reading frames or generating novel ORFs at junction sites (Umesha *et al*., 2024; Wilson *et al*., 2006). While copy number remains an important parameter for regulatory assessment, its practical relevance for clonally propagated crops such as banana, where seed formation is not a concern, remains an open question.

Conclusion

Our study demonstrates that the expression of the *MtPsy2a* transgene under the control of either the ACO or Ubi promoter effectively increases PVAC levels in banana fruit to concentrations sufficient to contribute to the alleviation of VAD in populations that rely heavily on banana as a staple food. Our findings show that both promoters are effective in driving *MtPsy2a* expression in either cultivar, offering flexibility in promoter choice for pro-vitamin A biofortification strategies. However, the observed yield reductions in transgenic lines warrant further investigation. Future studies should examine the expression of key genes and changes in metabolite profiles within the carotenoid and related metabolic pathways to understand the underlying causes. Additionally, these preliminary yield observations must be validated through rigorously designed and replicated agronomic trials across multiple environments to assess their consistency and any potential trade-offs associated with the biofortification trait.

To that effect, ten elite events were selected in this study and have been advanced to multi-location confined field trials at four sites in Uganda. These events were chosen based on their carotenoid content, transgene copy number and agronomic performance. The upcoming trials will be critical for evaluating their performance across diverse environments and for determining their suitability for future deployment as biofortified cultivars. These trials will generate agronomic, food safety and environmental safety data for submission to the National Biosafety Committee as part of the biosafety clearance process. In parallel, the Variety Release Committee conducted independent evaluations while the enactment of the Genetic Engineering Regulatory Bill is awaited.

The Banana21 project began in 2005 with strong support from the Gates Foundation and the promise of a technology that could transform nutrition and save thousands of lives. Two decades later, that promise remains undiminished. While neighbouring countries across Africa and Southeast Asia (including Nigeria, Kenya and the Philippines) have embraced genetically modified crops to address food security and public health challenges, Uganda continues to stall in the absence of a functional biosafety

law. As a result, we are now in the process of initiating a trial to evaluate and release the pro-vitamin A banana in Kenya, where regulatory frameworks permit the approval and deployment of genetically engineered crops. Despite the scientific progress and proven potential of these technologies, regulatory inertia in Uganda threatens to keep life-saving innovations out of reach for those who need them most.

Experimental procedures

Plant materials

Two popular cultivars grown throughout Uganda (M9 and Nakitembe) were chosen for this study. M9 is a conventionally bred EABH with the following major traits: (1) resistance to black Sigatoka; (2) tolerance to weevils and nematodes; (3) tolerance to drought; and (4) high yielding (Kephas *et al*., 2015). This cultivar is expected to become more important in the lowland and mid-altitude areas ranging from 1150 to 1300 m above sea level (masl) where pest and disease pressure as well as drought effects are high. Nakitembe, on the other hand, is a local EAHB and was chosen because of its excellent food quality attributes and good presence in the banana market. This cultivar would become recommended for the highland areas (>1300 masl) where disease pressure is low.

Generation of transgenic bananas

Transgenic bananas were generated via *Agrobacterium-mediated* transformation of embryogenic cell suspensions using *A. tumefaciens* strain AGL1 as described by Khanna *et al*. (2004). Cell suspensions of M9 and Nakitembe cultivars were generated, and each was transformed with two binary vectors pBMGF-DC-32 and pBMGF-DC-34 (Figure 6). Binary vector pBMGF-DC-32 consisted of *Musa acuminata* × *troglodytarum* cv. Asupina *phytoene synthase 2a* (*MtPsy2a*; GenBank: JX195659) gene under the transcriptional control of a banana fruit-preferred aminocyclopropane-1-carboxylate oxidase (ACO; GenBank: AF221107) promoter. Binary vector pBMGF-DC-34 consisted of *MtPsy2a* under the control of a constitutive maize polyubiquitin1 (Ubi1) promoter (Dugdale *et al*., 2000). Transformation success for the cell suspensions was ascertained using a β-glucuronidase (GUS) reporter gene *uidA* containing a catalase intron from *Escherichia coli* under the control of the Ubi promoter (Khanna *et al*., 2004). Selection of putatively transgenic plants was mediated by the *neomycin phosphotransferase* II (*npt*II) gene (Beck *et al*., 1982) (Figure 6).

The banana embryogenic cell suspension and recombinant *Agrobacterium* were co-cultured for 4–5 days before transfer to selection media supplemented with Kanamycin at 100 mg/L for 2–3 months until mature embryos formed. Embryos were transferred to embryo germination media and subsequent plantlets cultured onto plant regeneration media. Regenerated plants were transferred to sterile soil in weaning cups and maintained in a humid chamber for 2 weeks in the greenhouse. Successfully hardened plants were transferred to freshly sterilized soil in pots of 200 mm diameter and acclimatized for 2 months prior to planting in the field.

Histochemical GUS assay

Histochemical staining of transformed cells was based on the procedure described by Jefferson *et al*. (1987). Transformed cells

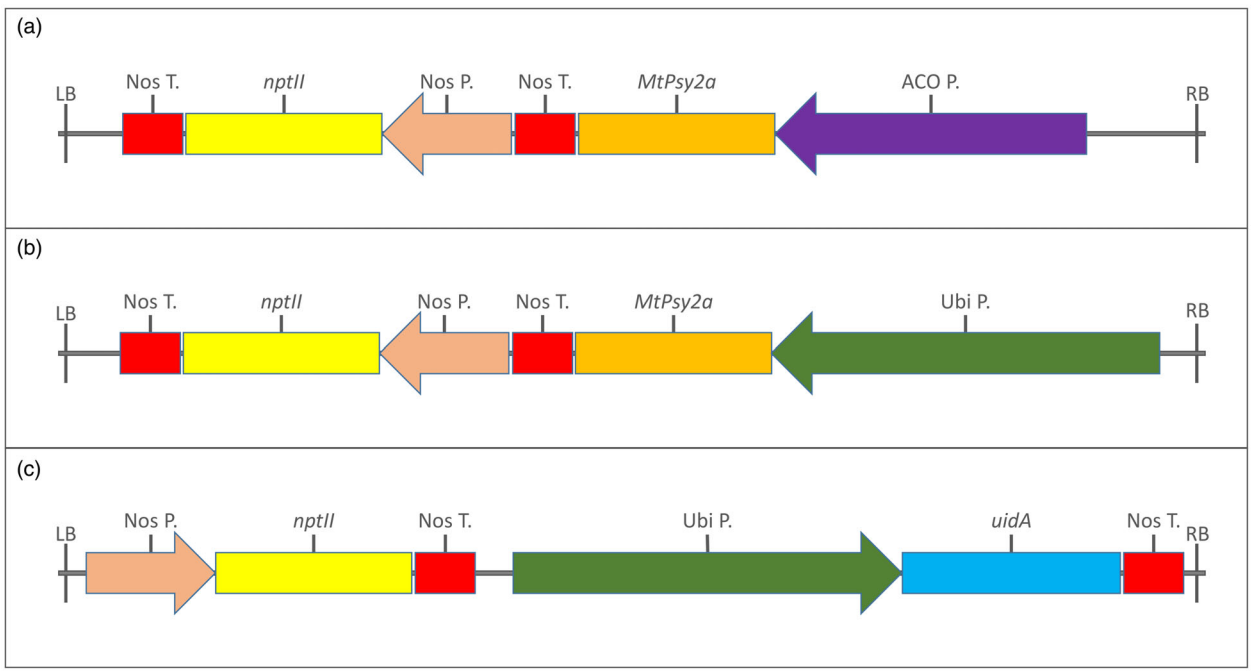

Figure 6 Schematic representation of T-DNA regions of the constructs used to modify EAHBs for increased levels of PVA. (a) T-DNA section showing ACO promoter driving the expression of *MtPsy2a* in a binary vector named pBMGF-DC-32; (b) T-DNA section showing Ubi promoter driving the expression of *MtPsy2a* in a binary vector named pBMGF-DC-34 and (c) T-DNA section showing Ubi promoter driving the expression of *uidA* in a binary vector named pBIN-Ubi-Gus. LB, left border; Nos T., nopaline synthase terminator; Nos P., nopaline synthase promoter; *npt*II, neomycin phosphotransferase selectable marker gene; *MtPsy2a*, *phytoene synthase 2a* gene from *Musa acuminata* × *troglodytarum* cv. 'Asupina'; *uidA*, β-glucuronidase (GUS) reporter gene containing a catalase intron from *Escherichia coli*; ACO P., banana aminocyclopropane-1-carboxylate oxidase promoter; Ubi P., maize polyubiquitin 1 promoter; RB, right border.

were incubated at 37 °C overnight in X-Gluc solution composed of 0.1% (w/v) 5-bromo-4-chloro-3-indoyl-β-glucuronic acid, 100 μM sodium phosphate (pH 7), 0.5 μM potassium ferrocyanide, 0.5 μM potassium ferricyanide and 10 μM ethylenediaminetetraacetic acid (EDTA). Cell suspensions were scored as GUS positive if any deep indigo blue colour was present.

Detection of transgenes in the transformed banana lines

Genomic DNA for PCR was isolated from leaf samples collected from hardened and potted plants using the CTAB protocol adapted from Stewart Jr. and Via (1993). Leaf tissue (1 g) was ground in liquid nitrogen and incubated in 700 μL of CTAB extraction buffer at 65 °C for 30 min. The total genomic DNA (gDNA) was extracted using 700 μL of chloroform-isoamylalcohol (24:1) v/v and precipitated using an equal volume of isopropanol. The pellet was then washed with 1 mL of 70% cold ethanol, the gDNA was treated with RNase A, re-extracted and re-precipitated. The final precipitate was washed, dried and re-suspended in sterile nuclease-free water. The concentration and purity of DNA were determined using a NanoDrop™ UV–Vis spectrophotometer (Thermo Fisher Scientific) according to Desjardins and Conklin (2010). DNA quality was confirmed by PCR using primers designed to amplify the housekeeping gene *Actin*.

To detect the presence of the transgene, end-point PCR reactions with ACO-*MtPsy2a* and Ubi-*MtPsy2a* primer pairs were performed (Table 1). Latent *Agrobacterium* contamination analysis was performed on transgene-positive lines using *virC1* and *virC2* region-specific primers for AGL1. Transgene-negative plants were confirmed using *npt*II gene-specific primers. PCR reactions contained 50 ng of plant gDNA, 1.2 mM $MgCl_2$, 0.4 μM of each of the primer pairs, 1× PCR buffer, 0.24 mM dNTPs and 0.02 U Taq per reaction of 20 μL. The reaction mixture was subjected to an initial denaturation step of 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at the optimized annealing temperature (Tm) for each primer pair and extension at 72 °C for 2 min followed by a final extension step of 72 °C for 5 min. The PCR products and a 100 bp Plus DNA ladder (Bioneer) were separated on 1.5% agarose gels in 1× TAE buffer at 150 V for 40 min. The gels were stained with ethidium bromide (0.5 μg/mL), and images were captured using the GBOX Syngene gel documentation system (SYNGENE, UK) fitted with a digital camera.

Tracking lines

The Banana Tracker™ database system was used as a central data storage facility for all information ranging from gene constructs, banana cell line development and transformation to field plant data collection. All positive transgenic lines registered in the Banana Tracker™ database were assigned unique field trial (FT) numbers. Subsequently, details about any line such as cell line, vector, transformation date and laboratory book reference could be easily tracked. This guaranteed the traceability and quality of the selection process and outcomes.

Establishment of transgenic lines in confined field trials

Transgenic lines that were confirmed positive for the transgene of interest and had no latent *Agrobacterium* contamination were planted in a phased manner in a confined field trial (CFT) approved by the National Biosafety Committee of the Uganda National Council for Science and Technology (UNCST) under permit number NBC/GM/CFT/001/09. The CFT, located at the National Agricultural Research Laboratories – Kawanda (N00°24.900, E032°32.053, N00°24.778, E032°32.051, N00°24.778, E032°32.102 and N00°24.902, E032°32.101), was planted between August 2014 and September 2017. In order to increase the number of events to select for PVA content, a larger number of single independently transformed lines for each cultivar were planted in the CFT. The trial comprised 356 transgenic lines of M9, where 153 lines were transgenic for ACO-*MtPsy2a* and 203 with Ubi-*MtPsy2a*. Up to 162 transgenic Nakitembe lines were planted, with 69 lines consisting of ACO-*MtPsy2a* and 93 Ubi-*MtPsy2a*.

Each transgenic line was planted at a spacing of 3 × 3 m between plants and rows. For every ten transgenic lines planted, a non-transgenic cell line control plant was included randomly. Standard agronomic practices for planting banana were implemented, including the application of farm yard manure of approximately 15 kg/planting hole at planting, desuckering to maintain only the PC (mother plant), ratoon 1 (daughter) and ratoon 2 (granddaughter). Male buds were cut off after the formation of the last hand of the bunch. These were disposed of in incineration pits as a biosafety precaution to prevent the persistence of pollen in the environment.

Collection of agronomic data

Data were collected on the following key agronomy variables: plant height, plant girth, number of functional leaves at flowering, bunch weight, bunch length, bunch circumference, number of hands on a bunch, number of fingers on top hand, number of fingers on middle hand, fruit pulp colour, number of functional leaves at harvest, leaf length, leaf radius, fruit length and fruit circumference.

Dates of planting and harvesting were recorded. Cycle time was calculated as the time (in months) from planting to harvest of the PC and from harvest to harvest for ratoon crops. Mature bunches were harvested by cutting the peduncle 10 cm above the first ridge above the proximal hand. Bunches were weighed, including the rachis, using weighing scales (kg).

Collection and processing of fruit samples

At least three mature green fruits collected from the top, middle and bottom parts of the bunch were delivered to the laboratory, approx. 200 m away. All fruit samples were processed within 48 h from harvesting. The fruits were peeled under dim light conditions and pulp chopped into approx. 1 cm^3 pieces. Processed fruits were frozen in Petri dishes at −80 °C for at least 12 h and freeze-dried at −50 °C under a vacuum of 0.1 mBar for 72 h. Equal proportions of freeze-dried tissues were ground into a homogeneous composite powder using ceramic mortars and pestles.

Analysis of carotenoids using HPLC

Carotenoids were extracted from 200 mg of freeze-dried fruit samples and analysed by HPLC as previously described by Buah *et al*. (2016). Total carotenoids and β-CE were expressed in microgram per gram of dry weight (μg/g DW).

Criteria for selection of elite events

A set of criteria was defined to guide a stepwise selection of elite events from the single plant evaluation trial for progression to a replicated national performance trial. The selection criteria included a 4-step progressive assessment:

- Step 1: Trueness to type based on plant cycle time and phenotypic appearance.
- Step 2: Yield value not less than 20% of the bunch weight of cell line controls.
- Step 3: PVA content at a minimum of 20 μg/g dry DW β-CE, based on (a) bioconversion ratio of 6:1 of α- and β-carotene to retinol in humans, (b) meeting at least 50% Estimated Average Requirement (EAR), (c) daily consumption of cooked bananas of 500 g of freshly cooked matooke and (d) dry matter and processing losses taking into account 75% moisture content and 25% losses during steaming and boiling; and finally.
- Step 4: Transgene copy number targeting lines with one or two copies of T-DNA inserts in the genome.

Determination of transgene copy number

Transgenic lines that met the selection criteria steps 1–3 were further analysed for the number of copies of integrated T-DNA using a duplex probe-based droplet digital PCR (ddPCR) assay. The digital PCR measured the number of copies of the selectable marker *npt*II using the banana Minichromosome Maintenance Complex Component 5 (*mcm5*) gene (GenBank: XM_009383287.2) as an internal single-copy reference gene. Total gDNA was extracted from 20 mg of lyophilized leaf tissue using a GenElute™ Plant Genomic DNA Miniprep Kit (SIGMA) and 1 μg digested with *Hind*III was used as a template in the ddPCR copy number detection assay. After optimization, all selected M9 and Nakitembe lines were assessed.

Statistical analysis

All statistical analysis and graphical presentation were performed using Paleontological statistics software package for education and data analysis version 4.03 (Hammer *et al*., 2001). The range, means and standard errors were calculated and displayed as bar graphs. A one-way ANOVA was conducted to compare means of transgenic and non-transgenic controls followed by the Mann–Whitney pairwise comparisons. A generalized linear model was constructed to establish the relationships between yield and β-CE content.

Author contributions

SB – Conducted experiments, collected, analysed and interpreted data and wrote the first draft of the paper. JT – Conducted experiments and collected data. PN – Generated cell suspensions used for transformations and managed tracking system. JK – Supervised SB, JT and GA and provided critical review of the manuscript. GA – Conducted experiments (initial transformations). JY – Designed and tested constructs, interpreted data, reviewed and edited the manuscript. RH – Provided overall supervision of JY. JD – Conceptualized and designed the study and mobilized funds. WT – Conceptualized and designed the study and mobilized funds. All co-authors read and made significant contributions to all sections of the paper.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Summary of field selection of M9 transgenic lines based on yield, β-CE and transgene copy number.
Table S2 Summary of field selection of Nakitembe transgenic lines based on yield, β-CE and transgene copy number.
Table S3 Field performance of M9 control lines based on yield and β-CE levels.
Table S4 Field performance of Nakitembe control lines based on yield and β-CE levels.