

Golden bananas in the field: elevated fruit pro-vitamin A from the expression of a single banana transgene

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Summary

Vitamin A deficiency remains one of the world's major public health problems despite food fortification and supplements strategies. Biofortification of staple crops with enhanced levels of pro-vitamin A (PVA) offers a sustainable alternative strategy to both food fortification and supplementation. As a proof of concept, PVA-biofortified transgenic Cavendish bananas were generated and field trialed in Australia with the aim of achieving a target level of 20 µg/g of dry weight (dw) β-carotene equivalent (β-CE) in the fruit. Expression of a Fe'i banana-derived phytoene synthase 2a (*MtPsy2a*) gene resulted in the generation of lines with PVA levels exceeding the target level with one line reaching 55 µg/g dw β-CE. Expression of the maize phytoene synthase 1 (*ZmPsy1*) gene, used to develop 'Golden Rice 2', also resulted in increased fruit PVA levels although many lines displayed undesirable phenotypes. Constitutive expression of either transgene with the maize polyubiquitin promoter increased PVA accumulation from the earliest stage of fruit development. In contrast, PVA accumulation was restricted to the late stages of fruit development when either the banana 1-aminocyclopropane-1-carboxylate oxidase or the expansin 1 promoters were used to drive the same transgenes. Wild-type plants with the longest fruit development time had also the highest fruit PVA concentrations. The results from this study suggest that early activation of the rate-limiting enzyme in the carotenoid biosynthetic pathway and extended fruit maturation time are essential factors to achieve optimal PVA concentrations in banana fruit.

Introduction

Micronutrient deficiency, often referred to as hidden hunger, occurs when intake and absorption of vitamins and minerals are too low to sustain good health and development. The World Health Organization (WHO) estimates that 190 million pre-school children are deficient in one of the major micronutrients, vitamin A. Vitamin A deficiency (VAD) alone is responsible for almost 6% of child deaths under the age of 60 months in Africa and 8% in South-East Asia (WHO, 2011). For the vast majority of these children, VAD is almost exclusively the result of inadequate intake of dietary vitamin A or pro-vitamin A (PVA) although exacerbated by other health conditions. Similar levels of VAD are also evident in women of childbearing age in these same regions (WHO, 2011). These levels of VAD continue despite the implementation over many years of extensive alleviating strategies such as supplements and food fortification. These strategies have been demonstrably successful, but there remain persistently high and unacceptable levels of VAD particularly in sub-Saharan Africa and south Asia (Stevens *et al.*, 2015).

In an effort to significantly reduce VAD in these regions, strategies aimed at increasing the dietary intake of particularly α- and β-carotene together as PVA are being developed or implemented. These include programmes to encourage growing and

consuming staple foods with high levels of PVA. In some instances, such foods or crops with the desired agronomic and consumer traits are already available and can therefore be easily deployed (HarvestPlus, 2012). However, the majority of accepted cultivars and landraces of staple crops are low in micronutrients such as PVA and iron, and therefore, it is necessary to develop new varieties with enhanced levels of these micronutrients. This can be achieved either through conventional breeding or by genetic modification where the traits are not available within the accessible germplasm or cannot be easily introgressed into acceptable cultivars. These two approaches are known as biofortification.

The best-known example of biofortification by genetic modification, and the most advanced in terms of development, is 'Golden Rice'. In Ye *et al.*, 2000 and colleagues reported the generation of transgenic rice expressing the daffodil phytoene synthase (*Psy*) gene under the control of an endosperm-specific rice glutelin promoter together with the bacterial (*Pantoea ananatis* formerly known as *Erwinia uredovora*) phytoene desaturase (*CrtI*) gene under the control of the constitutive CaMV 35S promoter. The endosperm of selected lines was yellow, and one heterozygous line contained 1.6 µg/g dry weight (dw) total carotenoids. Paine *et al.* (2005) subsequently reported the development of the second generation of 'Golden Rice', which

was engineered with a maize (*Zea mays*) *Psy* gene and the *CrtI* gene under the control of the glutelin promoter. One Golden Rice 2 elite event had a 23-fold increase in total carotenoids over the original Golden Rice with a total carotenoid level of up to 37 $\mu\text{g/g}$ dw in the endosperm of which 31 $\mu\text{g/g}$ was β -carotene. A number of other important food crops have been or are being developed to enhance the level of PVA through genetic modification.

Bananas are the world's most important fruit crop and one of the top 10 crops by production. They are widely grown in the wet tropics and subtropics forming an important dietary component both raw as a dessert fruit and cooked often as the major source of carbohydrate. In a number of countries, bananas are the principal staple food including Uganda where consumption levels average 0.5 kg per person per day rising to around 1 kg per person per day in some regions (Komarek, 2010; Smale and Tushemereirwe, 2007). In East Africa, the staple cultivar is East African highland banana (EAHB) (*M. acuminata* AAA-EA) prepared primarily by steaming or boiling whereas, in West Africa, plantains are dominant and are usually fried or roasted (Fungo and Pillay, 2011). In both regions, the level of VAD is high. In Uganda, it varies from 15% to 33% in children under 60 months with similar levels in women of childbearing age (UDHS - Uganda Demographic and Health Survey, 2006). Unlike rice endosperm, banana fruit contains PVA and, in some instances, very high levels particularly Fe'i bananas of Micronesia and Papua New Guinea (Englberger *et al.*, 2003). Bananas with β -carotene equivalent (β -CE) levels of 340 $\mu\text{g/g}$ dw have been reported whereas the dominant dessert banana cv 'Cavendish' has between 1 and 4 $\mu\text{g/g}$ dw β -CE and the EAHB clone, 'Nakitembe', has approximately 10 $\mu\text{g/g}$ β -CE dw (Englberger *et al.*, 2006a; Mbabazi, 2015). Unfortunately, domesticated bananas have very low male and female fertility rendering conventional breeding extremely difficult. Thus, the introgression of the high PVA traits of Fe'i bananas for instance into farmer preferred EAHB selections would be practically impossible. However, genetic modification of bananas is well established.

Here, we report the 'proof-of-concept' technology required towards the generation of PVA-biofortified EAHB varieties in Uganda. The 'Cavendish' dessert banana was genetically modified, and greatly enhanced PVA levels were demonstrated in the fruit of plants grown in the field in Australia.

Results

The target

At the outset, it was important to identify a target fruit level of β -carotene equivalents necessary to help alleviate VAD in Uganda. The target was set at delivering 50% of the estimated average requirement (EAR) of vitamin A in vulnerable populations which for children under 60 months is 120 $\mu\text{g/day}$ and for females ranging from 235 $\mu\text{g/day}$ up to 445 $\mu\text{g/day}$ for lactating mothers. An estimated bioconversion 6:1 ratio of β -carotene equivalents (β -CE) to vitamin A from cooked banana pulp was used (Bresnahan *et al.*, 2012) with an estimated consumption of cooked bananas of 300 g/day for children and 500 g/day for women. The α - and β -carotene retention after steaming or boiling was also estimated at 70% (Mbabazi, 2015). Using these parameters, banana fruit needed to contain β -CE levels of at least 20 $\mu\text{g/g}$ dw to achieve 50% of the EAR.

There were three major technical constraints at the commencement of this project that influenced the research strategy:

(i) very little information was available regarding the expression of transgenes in bananas generally and more specifically in banana fruit, (ii) the time from transformation to harvestable fruit ranges from 2 to 2½ years, and (iii) it was clearly impractical to take large numbers of transgenic bananas through to fruit in the greenhouse. Therefore, a large number of independent transgenic lines were generated to enable the testing of a wide range of promoter and transgene combinations. Initially, a single plant per transgenic line was planted in the field with, in most instances, between 10 and 30 transgenic lines per construct. For more information, refer to the 'History of the project' section of the Supplementary information document.

Promoter characterization

Three promoters were selected as possible candidates for expressing PVA-related transgenes in banana fruit, and these were characterized for levels and patterns of expression in transgenic bananas. The promoters included the constitutive maize polyubiquitin promoter (Ubi) and two promoters isolated from banana, the expansin 1 promoter (Exp1) and the ACC oxidase promoter (ACO) which were predicted to be fruit specific. These promoters were fused to the β -glucuronidase reporter gene (*uidA*), the cassettes transformed into bananas and the transgenic plants established in the field. β -glucuronidase (GUS) protein levels were measured in the fruit pulp using ELISA; however, this approach could not be used for leaf or peel material because of very high background levels. Therefore, for leaf and peel samples, MUG fluorometric assays were used to estimate enzyme activity rather than protein levels.

GUS activity was measured in the leaves of six independent transgenic lines for each promoter. As expected, there were high but variable levels of GUS activity in the leaves of all six plants where *uidA* was under the control of the Ubi promoter (Figure 1a). In the leaves of the wild-type control plant and plants where *uidA* was under the control of either the Exp1 or ACO promoter, there was undetectable to negligible GUS activity (Figure 1b and c).

Pulp samples from the fruit of the same lines described above were collected at 3, 6, 9 and 12 weeks post-bunch emergence (S3, S6, S9 and S12) and also at 'full green' (FG), when the bunches were harvested, and 'full ripe' (FR). The FG stage is equivalent to the stage when cooking bananas are harvested in Uganda. GUS protein was not detected in the wild-type at any fruit development stage. In contrast, appreciable but varying levels of GUS protein were detected in the pulp of banana fruit from S3 through to FR in the six Ubi-*uidA* lines (Figure 1d) confirming the constitutive nature of the Ubi promoter. No reproducible trend in GUS protein levels across the six lines was observed as fruit matured from S3 to FR except that average protein accumulation was lowest at the earliest stage, S3. In the six Exp1-*uidA* lines examined, no appreciable GUS expression was detected in fruit pulp from S3 through to FG with the exception of FR fruit from lines FT258 and FT263 (Figure 1e). This indicated that the Exp1 promoter is activated very late during fruit development. Very low levels of GUS expression were detected in the fruit pulp of the ACO-*uidA* lines from S3 to S9 (Figure 1f). With the exception of line FT736 which peaked fourfold higher than any other line, the overall trend was that GUS expression slowly increased from S9 through to FG and plateaued at FR. These results indicated that the ACO promoter was activated earlier than Exp1 during fruit

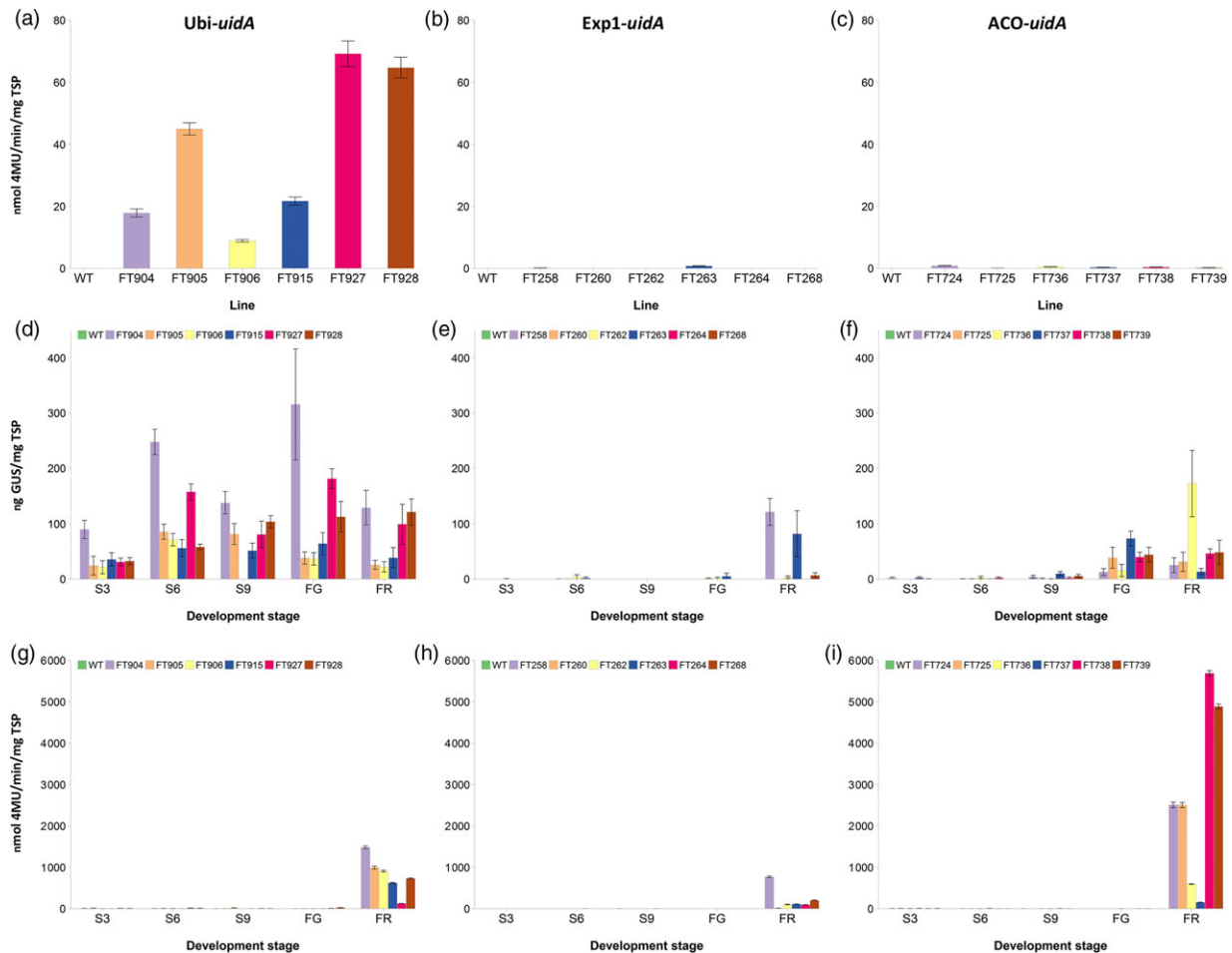


Figure 1 Analysis of promoter activity in wild-type and transgenic Cavendish banana lines. GUS activity was measured in leaf (a, b, c), and peel (g, h, i), while GUS protein concentration was measured in pulp tissue (d, e, f). Ubi promoter (a, d, g); Exp1 promoter (b, e, h); and ACO promoter (c, f, i). WT, wild-type FT432. S3, S6 and S9 represent 3, 6 and 9 weeks post-bunch emergence, respectively. FG, full green and FR, full ripe. Error bars: \pm SD.

development and more consistently at the FG and FR stages (Figure 1e and f).

The pattern of Ubi driven GUS expression in fruit peel was unexpectedly different from fruit pulp with low expression from S3 to FG followed by a substantial increase at FR (Figure 1g). In the peel of Exp1-*uidA* lines, the pattern of GUS expression was similar to that observed in the fruit pulp (Figure 1h). ACO-*uidA* lines had very little GUS expression in the peel up to FG; however, there was a dramatic increase to levels higher than either the Ubi or Exp1 lines at FR (Figure 1i).

PVA analysis: plant and ratoon crops

Domesticated bananas grow as a perennial crop. The initial plant, the plant crop, develops a corm and a pseudostem from which the original bunch is produced. After the bunch is harvested, the pseudostem dies and is replaced by a second pseudostem producing the first ratoon crop which develops from a sucker originating from the corm. Similarly, a second ratoon crop is produced and so on.

The same three promoters used to assess GUS expression were used to drive the expression of three carotenoid biosynthesis transgenes. These transgenes were the phytoene synthase 1 gene from maize (*ZmPsy1*) used in Golden Rice 2, a phytoene synthase

2a gene isolated from the Fe'i banana cultivar Asupina (*MtPsy2a*) (Mlalazi *et al.*, 2012) and the bacterial phytoene desaturase gene *PaCrtI* also used in Golden Rice 2. *ZmPsy1* and *MtPsy2a* were transformed into Cavendish banana singly and in combination with *PaCrtI*.

A total of 244 transgenic lines were confirmed to contain the respective PVA transgene(s) by PCR. Southern blot analyses showed that the transgene copy number varied from one to more than 10 copies (Figure S1 and S2). For each line, a single plant was established in the field together with 50 non-transgenic control plants. This initial randomized trial was designated Field Trial 1 (FT-1), and the plant crop was assessed over a period of 16 months. Forty-eight transgenic lines either died or were stunted and did not produce fruit. Therefore, fruit was harvested from a total of 196 transgenic lines of which 153 samples were selected for the initial plant crop fruit analysis (Table 1). Fruit was harvested at FG, ripened to FR and sampled at both stages. The sample used for the initial PVA level screen consisted of a single fruit taken from the middle of the bunch from each of 153 transgenic lines together with the equivalent sample from the fruit of 15 non-transgenic control plants. Following lyophilization and total carotenoids extraction, each sample was analyzed by HPLC and β -CE levels calculated. The most important outcomes

Table 1 Data summary from the transgenic banana field trial

Promoter-transgene	Number of plants in the field	Number of plants harvested	Number of plants analyzed (first cut)*	β-CE in FG fruit (μg/g dw)		β-CE in FR fruit (μg/g dw)	
				Range	Average	Range	Average
Wild-type	50	50	15	0.6–3.8	1.5	0.8–5.8	3.1
Exp1- <i>MtPsy2a</i>	33	28	28	1.2–8.6	3.3	0.8–10.0	2.8
Exp1- <i>MtPsy2a</i> + Ubi- <i>PaCrtl</i>	13	8	5	0.2–2.3	1.0	1.3–3.4	2.1
Exp1- <i>ZmPsy1</i>	32	26	26	1.2–4.6	2.6	1.3–15.2	4.5
Exp1- <i>ZmPsy1</i> + Ubi- <i>PaCrtl</i>	18	11	8	0.2–1.8	1.0	1.5–3.6	2.4
Exp1- <i>ZmPsy1</i> + Exp1- <i>PaCrtl</i>	7	7	2	–	–	2.1–11.0	6.5
Exp1- <i>PaCrtl</i>	29	26	3	–	–	1.7–2.6	2
ACO- <i>MtPsy2a</i>	30	29	28	11.2–15.3	13.3	3.4–13.4	8.3
ACO- <i>ZmPsy1</i>	30	18	18	5.4–10.9	7.6	2.5–24.6	9.0
ACO- <i>ZmPsy1</i> + Exp1- <i>PaCrtl</i>	6	4	3	10.6–25.7	17.1	7.8–16.7	13.2
Ubi- <i>MtPsy2a</i>	9	7	7	1.4–19.1	6.8	3.5–16.1	7.4
Ubi- <i>ZmPsy1</i>	10	5	5	0.3–13.6	6.1	1.0–16.1	7.9
Ubi- <i>PaCrtl</i>	27	27	20	–	–	1.0–3.7	1.8
Total	294	246	168	–	–	–	–

*First cut relates to an initial screening of the fruit of transgenic banana lines done by HPLC and using only a single fruit collected from the middle position of the bunch. FG, full green and FR, full ripe.

of this initial screening included: (i) there was obvious variation between individual control plants and also variation within lines with the same promoter-transgene combination, (ii) the highest expressing transgenic line for each of the different transgenes or combinations of transgenes contained higher levels of β-CE than the highest control plants except those lines containing Exp1-*PaCrtl* or Ubi-*PaCrtl* alone or where Ubi-*PaCrtl* was combined with Exp1-*MtPsy2a* or Exp1-*ZmPsy1*, (iii) in all transgenic lines and controls, the fruit contained higher levels of α-carotene than β-carotene (Figure S3), and (iv) *PaCrtl* alone had little effect on fruit PVA levels when driven by either the Exp1 promoter or the Ubi promoter; as such, none of the single transgene *PaCrtl* lines were progressed through for further analysis.

From this initial plant crop screen, 63 transgenic lines were selected for more comprehensive analysis. The selection included lines representing high, average and low PVA accumulation. As preliminary data revealed considerable variation in PVA accumulation across the bunch (data not shown), carotenoids were extracted from a composite sample including equal amounts of fruit taken from the top, middle and bottom of the bunch. Further, although fruit samples from the selected lines were to be analyzed across three generations (plant crop followed by first and second ratoon crops), the trial was hit by a severe cyclone in February 2011. As a consequence, all lines were blown over resulting in fruit from some lines in either the first or the second ratoon crops not being available for analysis.

For this project, the PVA levels at FG were considered more important as cooking bananas are harvested at this stage in Uganda. However, FR data were also collected as cooking bananas are usually consumed over a number of days post-harvest. The β-CE levels in FG and FR fruit from each of the selected 63 transgenic lines are presented in Table 2. Where expression of *MtPsy2a* or *ZmPsy1* was controlled by the Exp1

promoter, the level of β-CE in the plant crop and first ratoon increased from FG to FR in 43 of 53 samples analyzed (81%) (Table 2). A similar increase was seen in 82% (14 of 17 samples) of the analyzed samples where the Ubi promoter was used to drive the expression of the same transgenes. However, when ACO was used as a promoter, this number reduced to 50% (13 of 26 samples).

In the plant crop, there were no lines that met the target level of 20 μg/g dw β-CE where the composite sample of three fruit per bunch was analyzed. The highest β-CE level at FG was 18.7 μg/g dw in Ubi-*MtPsy2a* line FT328 that subsequently died. In addition, only eight lines had PVA levels greater than 10.0 μg/g dw β-CE at FG. These were two Ubi-*ZmPsy1* lines (FT287 and FT309 with 13.4 and 11.9 μg/g dw β-CE, respectively), two Ubi-*MtPsy2a* lines (FT328 and FT324 with 18.7 and 11.7 μg/g dw β-CE, respectively), two ACO-*MtPsy2a* lines (FT504 and FT518 with 16.6 and 15.9 μg/g dw β-CE, respectively) and two ACO-*ZmPsy1* + Exp1-*PaCrtl* lines (FT584 and FT587 with 17.1 and 11.5 μg/g dw β-CE, respectively). To investigate whether a correlation existed between transgene expression levels and accumulation of carotenoids, the expression of the *ZmPsy1* and *MtPsy2a* transgenes was determined in the FG fruit of a selection of lines using RT-PCR (Figure S4 and S5) and qRT-PCR (Figure 2). *MtPsy2a* line FT246 had the highest relative expression of the transgene followed by line FT324, FT518 and FT295 (Figure 2a). Expression was considerably lower in the other three lines tested. Expression of *ZmPsy1* was highest in line FT584 followed by FT309 while similar, but lower expression was seen in lines FT287, FT467, FT475, FT479 and FT585 (Figure 2b). Lines FT187 and FT192 had low expression.

When FG fruit from the next generation (first ratoon crop) were analyzed, 68% (23 of 34) of samples showed an increase in β-CE from the plant crop (Table 2). Interestingly, every line

Table 2 PVA carotenoid concentration in the fruit pulp of selected wild-type and transgenic Cavendish banana lines across four generations

Promoter-transgene	Line #	Plant crop		1st ratoon crop		2nd ratoon crop		Sucker crop	
		FG	FR	FG	FR	FG	FR	FG	FR
		β-carotene equivalents (μg/g dw)							
Wild-type	Average (n _z = 6)	2.6	3.1	2.2	2.2	1.7	2.3	6.0	7.4
Exp1-MtPsy2a	FT246	7.3	9.3	8.3	8.5	NA	NA	18.2	19.6
	FT544	3.4	4.6	NA	NA	NA	NA	NA	NA
	FT545	3.4	4.7	NA	NA	NA	NA	NA	NA
	FT342	2.8	4.2	7.4	4.6	NA	NA	10.6	9.0
	FT335	2.5	4.0	2.4	3.3	NA	NA	NA	NA
	FT343	2.3	3.8	2.9	3.4	NA	NA	NA	NA
	FT242	2.2	3.7	2.0	2.0	NA	NA	8.6	8.0
	FT233	2.0	3.3	1.9	2.6	NA	NA	NA	NA
	FT341	1.4	3.1	1.8	2.0	NA	NA	9.3	8.1
Exp1-MtPsy2a + Ubi-PaCrtI	FT244	1.5	2.8	2.6	1.9	NA	NA	NA	NA
	FT245	0.9	1.8	1.8	4.6	NA	NA	NA	NA
	FT220	0.7	1.4	1.7	1.9	NA	NA	NA	NA
	FT232	0.2	1.9	1.8	1.5	NA	NA	NA	NA
Exp1-ZmPsy1	FT534	9.3	11.9	NA	NA	NA	NA	NA	NA
	FT536	9.1	8.9	NA	NA	NA	NA	NA	NA
	FT317	6.6	7.9	NA	NA	NA	NA	11.4	15.6
	FT192	4.3	6.4	NA	NA	NA	NA	20.3	31.2
	FT538	3.8	9.4	NA	NA	NA	NA	10.9	14.8
	FT187	3.5	5.3	1.8	1.8	9.5	9.9	7.0	11.0
	FT311	2.9	6.1	3.1	2.5	NA	NA	NA	NA
	FT201	2.3	3.2	1.9	3.2	NA	NA	9.1	15.0
	FT319	1.9	3.2	3.5	2.6	NA	NA	NA	NA
	FT318	1.5	2.7	1.9	2.4	NA	NA	NA	NA
	FT210	1.3	2.8	1.2	2.5	NA	NA	NA	NA
	Exp1-ZmPsy1 + Ubi-PaCrtI	FT217	1.8	2.4	1.5	2.4	NA	NA	NA
FT196		1.6	4.2	2.9	2.5	NA	NA	NA	NA
FT207		1.5	3.5	2.4	2.7	NA	NA	NA	NA
FT195		1.0	2.6	NA	NA	NA	NA	NA	NA
FT208		0.8	2.7	2.1	2.1	NA	NA	NA	NA
FT205		0.7	2.6	1.0	7.4	NA	NA	NA	NA
FT213		0.4	1.8	NA	NA	NA	NA	NA	NA
ACO-MtPsy2a		FT504	16.6	12.0	12.1	11.7	NA	NA	20.0
FT518	15.9	10.7	NA	NA	NA	NA	23.1	35.9	
FT511	9.4	7.3	5.3	6.0	NA	NA	14.4	13.6	
FT508	9.2	7.1	4.2	3.8	NA	NA	15.6	19.0	
FT498	8.9	9.9	8.6	8.4	NA	NA	NA	NA	
FT506	6.0	5.9	NA	NA	NA	NA	NA	NA	
FT516	4.2	7.4	NA	NA	NA	NA	NA	NA	
FT497	4.1	10.5	NA	NA	NA	NA	13.4	12.9	
ACO-ZmPsy1	FT492	9.4	8.5	NA	NA	NA	NA	NA	NA
	FT467	7.3	10.4	NA	NA	NA	NA	10.5	13.4
	FT468	NA	NA	7.3	6.6	NA	NA	NA	NA
	FT475	4.3	10.5	NA	NA	NA	NA	20.7	22.0
	FT493	4.3	18.7	7.0	6.0	NA	NA	NA	NA
	FT476	2.9	5.4	4.5	4.7	NA	NA	NA	NA
	FT487	2.7	15.1	NA	NA	NA	NA	NA	NA
	FT479	2.7	13.6	NA	NA	NA	NA	20.5	16.2
	FT483	1.7	9.1	NA	NA	NA	NA	18.8	16.9
	ACO-ZmPsy1 + Exp1-PaCrtI	FT584	17.1	11.2	NA	NA	NA	NA	27.0
FT587		11.5	NA	NA	NA	NA	NA	21.5	22.2
FT588		7.7	10.1	NA	NA	NA	NA	NA	NA
FT585		7.3	5.6	NA	NA	NA	NA	12.2	11.5
Ubi-MtPsy2a	FT328	18.7	18.9	NA	NA	NA	NA	NA	NA
	FT324	11.7	16.1	NA	NA	26.6	33.4	55.0	50.1
	FT294	6.6	9.7	13.5	9.4	13.5	12.0	29.0	25.2
	FT295	5.4	6.1	6.6	6.6	4.8	4.5	10.5	12.0
	FT296	4.2	6.0	5.6	4.1	12.5	13.1	NA	NA
	FT327	3.0	4.9	NA	NA	NA	NA	NA	NA
	FT330	2.5	4.9	3.6	4.0	NA	NA	11.9	15.1
	Ubi-ZmPsy1	FT287	13.4	15.8	NA	NA	24.3	21.8	39.7
FT309		11.9	14.7	NA	NA	40.4	39.3	46.9	18.5
FT298		0.7	2.5	1.1	1.3	NA	NA	NA	NA
FT302		0.6	1.5	1.4	2.0	NA	NA	NA	NA

FG, full green and FR, full ripe.

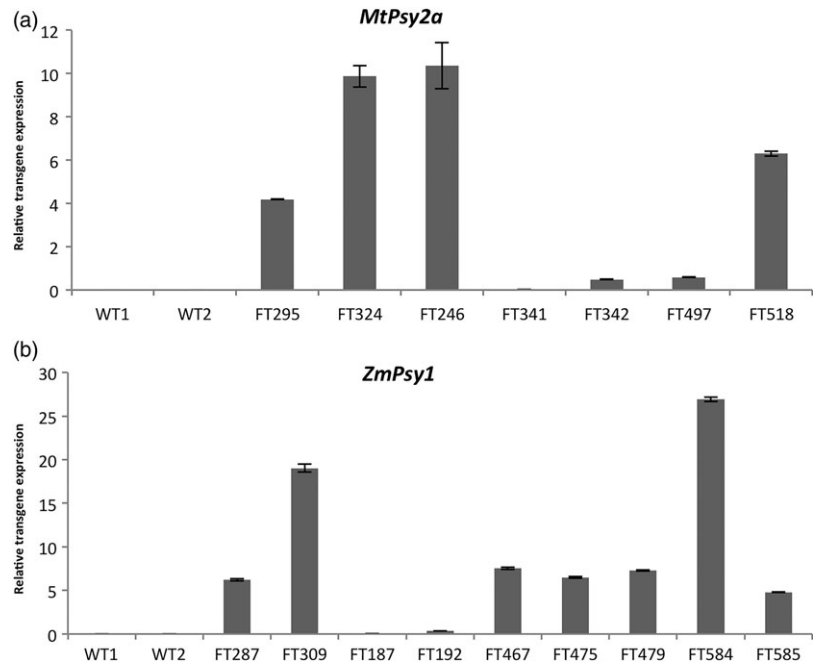


Figure 2 Analysis of mRNA expression levels in the FG fruit pulp of selected transgenic Cavendish banana lines by qRT-PCR. (a) *MtPsy2a* lines and (b) *ZmPsy1* lines. FG, full green. WT, wild-type with WT1 = FT167 and WT2 = FT430. Values are normalized expression levels \pm SEM.

analyzed that contained either Ubi-*ZmPsy1* or Ubi-*MtPsy2a* showed an increased accumulation in β -CE from the plant crop. However, again, no line accumulated over the 20 $\mu\text{g/g dw}$ β -CE target level. FG fruit from two lines had PVA levels above 10 $\mu\text{g/g dw}$ β -CE in the ratoon crop: ACO-*MtPsy2a* line FT504 with 12.1 $\mu\text{g/g dw}$ β -CE down from 16.6 $\mu\text{g/g dw}$ β -CE in the plant crop and Ubi-*MtPsy2a* line FT294 with 13.5 $\mu\text{g/g dw}$ β -CE up from 6.6 $\mu\text{g/g dw}$ β -CE in the plant crop. Due to the impact of the 2011 cyclone, only seven lines could be assessed in the second ratoon. FG fruit from three of these lines accumulated above target levels of PVA. Ubi-*ZmPsy1* line FT309 had FG fruit with 40.4 $\mu\text{g/g dw}$ β -CE, more than double the target, while Ubi-*MtPsy2a* line FT324 and Ubi-*ZmPsy1* FT287 accumulated 26.6 and 24.3 $\mu\text{g/g dw}$ β -CE, respectively (Table 2).

Carotenoid accumulation throughout fruit development was also monitored in selected lines from each of the single promoter-*Psy* combinations in the second ratoon crop at 3, 6 and 9 weeks post-bunch emergence as well as FG and FR (Figure 3). For the four lines where the transgene was under the control of the Ubi promoter, PVA levels were elevated (above 15 $\mu\text{g/g dw}$ β -CE) from the earliest fruit collection time point (S3) irrespective of whether the transgene was *ZmPsy1* or *MtPsy2a* (Figure 3a and d). For the two lines with the highest PVA levels, the general trend was increasing PVA during fruit development to a maximum at FG for Ubi-*ZmPsy1* line FT309 or at FR for Ubi-*MtPsy2a* line FT324. In contrast, accumulated PVA levels in the fruit of Exp1-*ZmPsy1* and Exp1-*MtPsy2a* lines remained below 5 $\mu\text{g/g dw}$ β -CE from the emergence of the bunch all the way through to S9 (with the exception of line FT342 which accumulated 6.6 $\mu\text{g/g dw}$ β -CE at S9) followed by an increase towards maturity with a maximum of 9.9 $\mu\text{g/g dw}$ β -CE at FR in Exp1-*ZmPsy1* line FT187 (Figure 3b and e). During fruit development of lines containing ACO-*ZmPsy1*, PVA levels were lowest at S3 and S6 for two of the lines but moderately higher than the Exp1-*ZmPsy1* lines at those stages (Figure 3c). PVA levels peaked for those same two lines at either S9 or FG. In contrast, the highest PVA level in line FT476 was at

S3. For the ACO-*MtPsy2a* lines, again one line, FT511, had maximum PVA accumulation at S3, while the other two lines had maximums at S9 or FG (Figure 3f). Overall, the PVA accumulation pattern during fruit development reflected the expression profiles previously observed in transgenic lines where the same three promoters were used to drive the expression of *uidA* (Figure 1). The constitutive Ubi promoter provided consistent stronger expression throughout fruit development followed by the ACO promoter and finally Exp1.

During the plant and ratoon crops, the phenotype of each plant was recorded at regular intervals from planting to bunch harvest. None of the 50 wild-type control plants showed altered phenotypes and fruit developed normally (Figure 4a–c). The presence of the *PaCrt1* transgene did not appear to affect phenotype. However, three categories of altered phenotypes were observed in the transgenic lines: stunting, 'golden leaf' and 'golden bunch'. For the 'golden leaf' phenotype, the youngest leaf would consistently unfurl with a bright yellow colour ('golden') and progressively turn to green as it matured (Figure 4g). Fruit on the 'golden bunch' emerged bright orange instead of green (Figure 4d). As the fruit matured and filled, it progressively turned greener to a mixture of green and orange at harvest (Figure 4e and f). Fruits with increase PVA levels displayed a pulp ranging from deep yellow to bright orange (Figure 4h and i). Of the original 244 transgenic lines planted in the trial, 65 had the 'golden leaf' phenotype of which 29 were also stunted; 29 had the 'golden bunch' phenotype. The 'golden leaf' and 'golden bunch' phenotypes were highly transgene dependent where lines with those phenotypes invariably contained the *ZmPsy1* transgene in contrast to lines containing the *MtPsy2a* transgene. The 'golden leaf' phenotype was observed in 27 of 57 (50%) lines observed carrying Exp1-*ZmPsy1* alone or together with either Exp1-*PaCrt1* or Ubi-*PaCrt1*. More importantly, the 'golden bunch' phenotype which only occurred in ACO-*ZmPsy1* or ACO-*ZmPsy1* + Exp1-*PaCrt1* lines was recorded in 94% (29 of the 31) of the lines assessed. In contrast, across all 85 lines containing

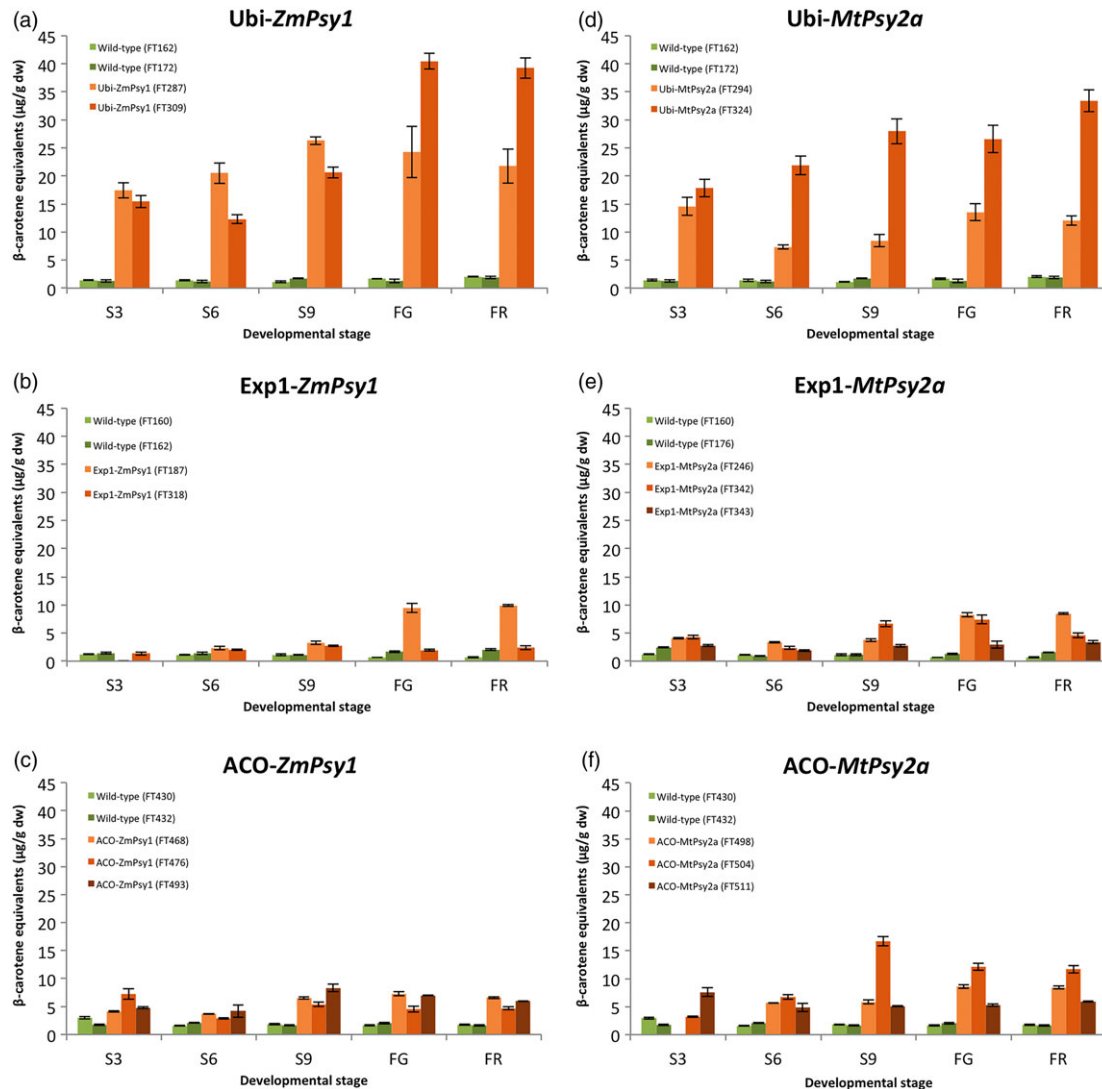


Figure 3 PVA carotenoid accumulation in the pulp of wild-type and selected transgenic Cavendish banana lines during fruit development. S3, S6 and S9 represent 3, 6 and 9 weeks post-bunch emergence, respectively. FG, full green and FR, full ripe. Error bars = \pm SD.

MtPsy2a, only 7 (8%) had the ‘golden leaf’ phenotype and none displayed a ‘golden bunch’.

The β -CE levels in the pulp and peel of FG and FR fruit from lines with the ‘golden bunch’ phenotype were analyzed and compared with the levels in fruit from phenotypically normal lines. In the FG fruit from four lines of *ACO-MtPsy2a*, the levels of β -CE in the peel were similar to those in the three non-transgenic controls (Table 3). However, the peel β -CE levels in four *ACO-ZmPsy1* containing lines were more than ninefold higher than in fruit peel from the control lines (Table 3). Importantly, the peel β -CE levels in the *ACO-ZmPsy1* lines did not influence fruit pulp β -CE levels as the four *ACO-ZmPsy1* lines had a similar range of β -CE levels in their fruit pulp at FG to fruit from the four *ACO-MtPsy2a* lines (Table 3).

PVA analysis: sucker crop

Following the initial plant and ratoon crop assessment with single plants per line, 30 lines selected from seven promoter/transgene combinations and five wild-type lines were multiplied through suckering to a maximum of 10 replicates per line. A total of 239

transgenic and 48 wild-type plants derived from suckers were planted in a second field trial (FT-2). Each plant was harvested, and PVA levels were measured in the fruit at FG and FR by HPLC.

In all transgenic and wild-type lines, the averaged fruit PVA level was higher in the sucker crop than in either the plant or ratoon crops. The highest average PVA level was 55.0 $\mu\text{g/g dw}$ β -CE found in the FG fruit of *Ubi-MtPsy2a* line FT324 (Table 2) with one individual plant of this line reaching 73.8 $\mu\text{g/g dw}$ β -CE (data not shown). Fruit from this plant had bright orange pulp compared with the fruit of non-transgenic control plants (Figure 4h). Of the 27 transgenic lines shown in Table 2, 11 had fruit PVA levels equal to or greater than the 20 $\mu\text{g/g dw}$ β -CE target. However, the fruit PVA levels in the wild-type controls were also higher than in the plant and ratoon crops with an average of 6.0 $\mu\text{g/g dw}$ β -CE. Interestingly, the top four sucker crop lines all contained an *Ubi-Psy* promoter/transgene combination. Furthermore, six of 12 *ACO* promoter lines had PVA levels equal to or greater than target level, compared to only one *Exp1* promoter line of 9 (Table 2). Analysis of the carotenoid composition of fruit pulp from wild-type as well as transgenic fruit in the

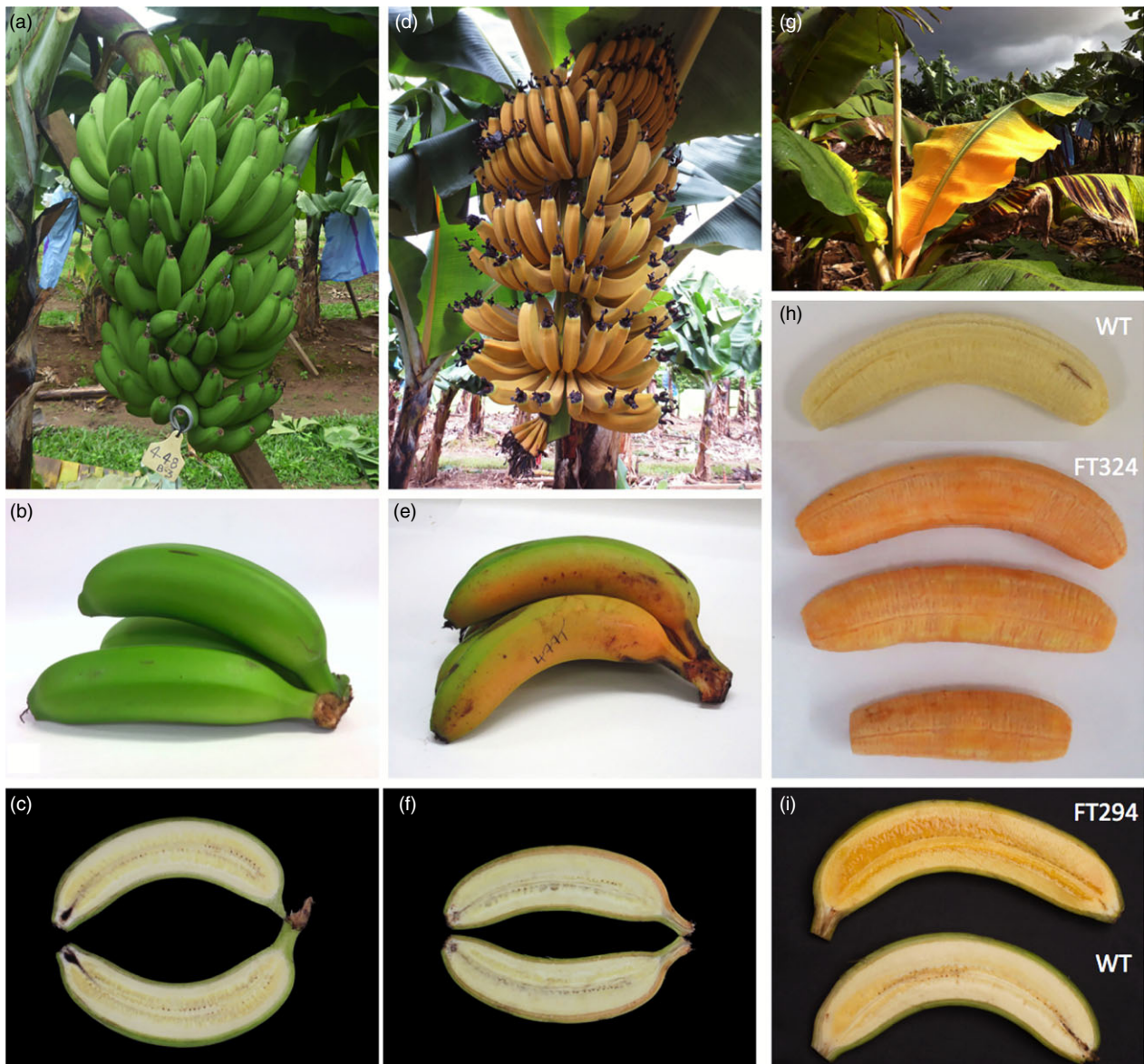


Figure 4 Characteristic phenotypes observed in wild-type and transgenic Cavendish banana lines. (a) Bunch from wild-type line FT448; (b) fruit from wild-type line FT448; (c) longitudinal section of fruit from wild-type line FT448; (d) immature bunch from ACO-*ZmPsy1* line FT477; (e) fruit from ACO-*ZmPsy1* line FT477; (f) longitudinal section of fruit from ACO-*ZmPsy1* line FT477; (g) Exp1-*ZmPsy1* line FT192; (h) Ubi-*MtApsy2a* line FT324; and (i) Ubi-*MtApsy2a* line FT294. WT, wild-type.

sucker crop revealed that, like the plant crop fruit, samples contained higher levels of α -carotene than β -carotene and in similar proportions (Figure 5).

Variation in fruit PVA levels in wild-type banana

The average PVA levels in the FG fruit of non-transgenic control plants varied considerably throughout the two field trials from a low of 1.0 $\mu\text{g/g}$ dw β -CE in March-harvested fruit in the plant crop of FT-1 to 8.1 $\mu\text{g/g}$ dw β -CE in September-harvested fruit of FT-2 (Figure 6). When analyzed together, a strong correlation was observed between the level of accumulated PVA in the fruit and the number of days from bunch emergence to harvest (bunch filling time). Indeed, fruit harvested in March had the shortest bunch filling time (94–97 days) and the lowest accumulated PVA level in the fruit compared with 142 days for September-

harvested fruit which had the highest fruit PVA levels (Figure 6). Longer bunch filling time and associated increased levels of PVA also appeared temperature dependent where fruit maturing during the cooler months had higher levels of accumulated PVA.

Discussion

Micronutrient deficiencies remain a substantial burden on the public health of populations particularly in developing countries. The 'poorest of the poor' are particularly impacted by micronutrient deficiencies because of their increasing dependence on nutrient poor single staple crops for the majority of their calorific intake (Muthayya *et al.*, 2013). There have been significant inroads into reducing VAD in children aged 6 to 59 months where worldwide prevalence has fallen from 39% in 1991 to

Two constructs containing Ubi-*MtPsy2a* and ACO-*MtPsy2a* have been transferred to the National Agricultural Research Organization (NARO) in Uganda and have been transformed into two Ugandan cultivars including EAHB.

Experimental procedures

Vector construction

The coding regions of *Musa troglodytarum* x *acuminata* cultivar Asupina phytoene synthase 2a (*MtPsy2a*; GenBank #: JX195659), *Zea mays* cultivar B73 phytoene synthase 1 (*ZmPsy1*; GenBank #: U32636) and *Pantoea ananatis* phytoene desaturase (*PaCrtI*; Genbank #: D90087) genes were used to facilitate PVA enhancement in banana. The *uidA* gene from *Escherichia coli*, encoding the enzyme β -glucuronidase (GUS), containing a catalase intron (Khanna *et al.*, 2004), was used to assess promoter activity. Each of these genes was fused to the *Agrobacterium tumefaciens* nopaline synthase (Nos) 3' transcription termination regulatory sequence (Depicker *et al.*, 1982). The banana expansin (Exp1; GenBank #: JN172931) and 1-aminocyclopropane-1-carboxylate oxidase (ACO; GenBank #: AF221107) promoters, and the maize polyubiquitin1 promoter (Ubi, Dugdale *et al.*, 2000) were characterized in banana using each of them to drive *uidA* expression, and the resulting expression cassettes: Exp1-*uidA*-nos, ACO-*uidA*-nos and Ubi-*uidA*-nos were assembled in the pBIN-19 (GenBank #: U09365) binary vector backbone. These three promoters were also used to regulate the expression of the *MtPsy2a* and *ZmPsy1* genes, resulting in the production of six expression cassettes in the pCAMBIA-2300 (GenBank #: AF234315) binary vector backbone. Expression of the *PaCrtI* gene was driven by the Ubi or Exp1 promoter, and the resulting Ubi-*PaCrtI*-nos and Exp1-*PaCrtI*-nos expression cassettes were assembled in pBIN-19. Selection of transgenic plants was mediated by the neomycin phosphotransferase II gene (*nptII*; Beck *et al.*, 1982) in both pCAMBIA-2300 and pBIN-19.

Plant transformation and regeneration

Transgenic *Musa acuminata* (AAA Group) 'Dwarf Cavendish' lines were generated via *Agrobacterium*-mediated transformation of embryogenic cell suspensions (ECS) using *A. tumefaciens* strain AGL1 (Khanna *et al.*, 2004). Binary vectors containing *MtPsy2a* or *ZmPsy1* were used for transformation of banana ECS either alone or in combination with one of the vectors containing *PaCrtI*, while vectors containing *uidA* were all used individually for transformation.

Plant material and field trials

Transgenic and wild-type banana lines established in tissue culture were transported to the field trial site at the Department of Agriculture and Fisheries (DAF) South Johnstone Research Facility (Queensland, Australia) according to conditions on the Office of the Gene Technology Regulator (OGTR) licence number DIR109. Plants were acclimatized in soil and grown in a glasshouse for 12 weeks before planting and maintenance in the field according to standard agronomic procedures. The initial field trial (FT-1) was conducted between 2009 and 2012. Promising transgenic lines and selected wild-type control lines (up to 10 sucker plants per line) were established for 12 weeks in the glasshouse before being transferred to a second field trial (FT-2) at the same facility, which commenced in September 2012.

Field sample collection and processing

Mature green (full green—FG) fruit was harvested from each plant and sent to the Centre for Tropical Crops and Biocommodities (CTCB) laboratory in Brisbane, Australia, within 48 h of harvest. When analysis of developing fruit was required, fruit was taken at 3, 6 and 9 weeks post-bunch emergence (S3, S6 and S9) prior to harvesting the entire bunch at FG. Fruit was handled under low-light conditions and was processed either immediately (for FG analysis) or at 7 days post-exposure (24 h) to ethylene for full ripe (FR) analysis. Representative fruit from the top, middle and bottom of the bunch was received at all stages of fruit development from plants transformed with *Psy* or *CrtI* genes, while fruit from the top of the bunch only was obtained from lines transformed with the *uidA* gene. Leaf samples from the field were collected from the first fully expanded leaf prior to bunch emergence. Prior to further analysis, all samples were freeze-dried in a Benchtop 4K Freeze Dryer (VirTis[®]) and homogenized in a Mini-Beadbeater-8[™] (Biospec Products) tissue disruptor.

Nucleic acid isolation

Genomic DNA (gDNA) for PCR and Southern blot analysis was isolated from 50 mg of homogenized freeze-dried leaf tissue using a modified CTAB method (Stewart and Via, 1993). Isolation of plasmid DNA (pDNA) was done using the Wizard[®] Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's instructions. Total RNA was extracted from 50 mg of homogenized freeze-dried banana fruit tissue essentially as described by Valderrama-Cháirez *et al.* (2002).

DNase treatment and complementary DNA (cDNA) synthesis

For cDNA synthesis, 3 μ g of total RNA was DNase treated using an RQ1 RNase-free DNase Kit (Promega). DNase-free RNA samples (1.8 μ g) were reverse-transcribed to cDNA using an oligo(dT20) primer and the GoScript[™] Reverse Transcription System (Promega) in 25 μ L reactions according to the manufacturer's instructions.

Polymerase chain reaction (PCR) and reverse-transcription PCR (RT-PCR)

Putatively transgenic tissue culture plants were tested under standard PCR conditions using oligonucleotide primers designed to amplify gene fragments spanning the *MtPsy2a*, *ZmPsy1* and *PaCrtI* genes and their respective promoter region and using GoTaq[®] Green master mix (Promega). Transgene-positive plants were further PCR tested for the presence of contaminating *Agrobacterium* using *VirC* gene primers (Haas *et al.*, 1995). Complementary DNA (cDNA) from PCR-positive plants was then used in RT-PCR to verify gene expression.

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCRs were done in a CFX384 Touch[™] Real-Time PCR Detection System (Bio-Rad) using GoTaq qPCR Master Mix (Promega). Each sample was analyzed in three technical replicates in addition to the inclusion of 'no template' and 'RT-negative' controls. The following amplification parameters were used: Hot-Start polymerase activation at 95 °C for 2 min, followed by 45 cycles of 10 s denaturation at 95 °C and 30 s annealing/extension at 60 °C. At the end of the reaction, a dissociation curve was

produced from 65 to 95 °C to confirm the specificity of the amplicon from each primer set. Fluorescence was recorded in real time and detected at 470 nm. Relative expression levels were calculated using the CFX Manager 3.1 (Bio-Rad) software and the Δ CT method (Schmittgen and Livak, 2008). Ct data obtained from target gene of interest (GOI) were normalized using Ct values from the two stable reference genes cyclophilin (CYP) and ribosomal protein S2 (RPS2). All primers were designed using the Primer3Plus freeware (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) (Table S1).

Southern hybridization

For determination of transgene copy number integration by Southern analysis (Southern, 1975), genomic DNA (10 µg) as well as positive control plasmid DNA (20 ng) were digested for 16 h and 1 h, respectively, by incubation at 37 °C with 20 U of restriction enzyme. Restriction enzymes were selected to only cut once within the binary vector T-DNA region without cutting the region to which the probe would hybridize. PCR based DIG-labelled probes (Roche) were designed targeting the coding regions of *MtPsy2a* and *ZmPsy1*. Digested DNA was electrophoresed, blotted and detected under standard Southern blotting conditions (Sambrook and Russell, 2001).

Carotenoid content quantification

Carotenoids were extracted from banana pulp (200 mg) or peel (25 mg) tissue and analyzed by HPLC as previously described (Buah *et al.*, 2016). Total carotenoids and β -carotene equivalents (β -CE) were expressed in µg/g dry weight (dw).

Assessment of promoter activity

β -glucuronidase (GUS) content was measured in banana pulp tissue (40 mg) by ELISA as per Dugdale *et al.* (2013), while fluorometric quantification of GUS activity was determined in leaf and peel tissue as described by Jefferson *et al.* (1987).

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Conflict of interest

Authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Determination of transgene copy number in transgenic Cavendish banana lines by Southern blot analysis.

Figure S2. Determination of transgene copy number in transgenic Cavendish banana lines by Southern blot analysis.

Figure S3. Representative HPLC chromatogram of the main carotenoids in wild-type and transgenic Cavendish banana.

Figure S4. Transgene expression analysis in selected *MtPsy2a* transgenic Cavendish banana lines by reverse transcriptase-PCR (RT-PCR).

Figure S5. Transgene expression analysis in selected *ZmPsy1* transgenic Cavendish banana lines by reverse transcriptase-PCR (RT-PCR).

Table S1. List of oligonucleotide primer sequences used for RT-PCR and qRT-PCR.