Molecular Characterisation and Carotenoid Quantification of Pro-vitamin A Biofortified Genetically Modified Bananas in Uganda

By

Ruth Mbabazi

Bachelor of Science (Hons.)

Centre for Tropical Crops and Biocommodities

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Abstract

Micronutrient deficiency affects a large proportion of the world's population. In Uganda, like in many other developing countries where a significant proportion of the population depends on a micronutrient poor food staple, micronutrient deficiency is an important health issue. It is believed that with time and effort these life-threatening deficiencies can be reduced through strategies such as biofortification. Biofortification is the enrichment of the edible parts of food crops with bioavailable essential micronutrients at concentrations high enough to impact on human health. This process can be achieved through conventional breeding, if feasible, or genetic engineering. East African Highland Banana (EAHB), a cooking banana, is a major food staple in the Great Lakes region of East Africa and even more so in Uganda. A small, sweet dessert banana 'Sukali Ndizi' is also very popular as a snack especially amongst children in Uganda. Unfortunately, 'Sukali Ndizi' and most EAHB are poor in essential micronutrients, thus populations depending on it for subsistence are at risk of severe micronutrient deficiencies, especially in rural areas. Biofortification of these bananas has therefore been proposed as a cost effective and sustainable way to address micronutrient malnutrition in rural Uganda. With financial help from the Bill and Melinda Gates Foundation, scientists at Queensland University of Technology (QUT), Australia and the National Agricultural Research Organisation (NARO), Uganda have been collaborating since 2005 to achieve this goal focussing mainly on iron and pro-vitamin A (PVA) biofortification though genetic engineering. This PhD project is part of the ongoing efforts towards the biofortification of EAHB and 'Sukali Ndizi' for increased levels of PVA.

While some banana varieties belonging to the Fe'i group can accumulate PVA carotenoids with values in excess of 7,000 µg/100g FW (fresh weight) of β-carotene equivalent (β-CE) (Englberger *et al.*, 2003), most EAHB varieties are poor in PVA carotenoids (Fungo, 2009; Fungo *et al.*, 2010). To provide a nationwide blueprint of the natural levels of PVA carotenoids present in several popular EAHB and dessert banana

cultivars growing in different geographical regions of Uganda, the first objective of the study was to measure PVA carotenoid levels in most cultivars grown in the country. Fruit from eight banana cultivars including a plantain cultivar 'Gonja Nakatansese' (GN), two dessert cultivars 'Bogoya' (BO) and 'Sukali Ndizi' (SN), four cooking-type EAHB cultivars 'Mbwazirume' (Mbz), 'Mpologoma' (Mpo), 'Nakitembe' (Nakt) and 'Nakinyika' (Nak) and one EAHB hybrid M9 were collected. Fruit at the full-green (FG) stage were then analysed for the amounts of lutein, *trans* α-carotene and *trans* β-carotene, and the β-carotene equivalent (β-CE) and total carotenoid content was calculated. The dessert bananas contained the lowest levels of β-CE (2.3±1.0 - 7.3±3.2 µg/g DW), EAHB cultivars contained between 4.6±3.0 - 10.2±6.6 µg/g DW while the plantain cultivar contained 30.7±8.9 µg/g DW. The composition of the carotenoids in the different banana cultivars was found to vary considerably. More than 50% of the total carotenoids present in green EAHB banana cultivars were found to comprise α- and β-carotene, making them good candidate cultivars for PVA biofortification. In the dessert cultivar, SN, more than 50% of total carotenoids were comprised of the non-PVA carotenoid lutein, while in the plantain, β-carotene was the major constituent of the carotenoids (around 70%) following by α-carotene and only small amounts of lutein (around 2%). The effect of geographical location and/or altitude on the carotenoid content in full-green fruit was also examined. Whereas no correlation between altitude and PVA carotenoid concentration was observed, significant differences in β-CE levels were found in fruit from the dessert cultivar SN ($p<0.05$), the EAHB hybrid M9 ($p<0.05$) and the EAHB cultivars Mbz ($p<0.001$) growing in different agricultural regions of Uganda.

Storing and/or ripening can have an impact on the accumulation levels of carotenoids in the banana fruit. Therefore, the carotenoid concentration in the fruit of the eight cultivars at various ripening stages was assessed. For the EAHB cultivars tested, a consistent increase in β-CE was observed during 14 days of storage from FG with fold increment of 1.88 and 2.80-fold observed in cultivars Mpo and Nakt, respectively. Further, fruit from Nakt showed a consistent increase in the concentration of all three

carotenoids tested (lutein, *trans* α-carotene and *trans* β-carotene) during storage. Analysis of fruit from the dessert banana SN and the plantain GN, revealed a severe decrease in β-CE during ripening with significant accumulation of the non-PVA carotenoid lutein ($p<0.001$). Further, the levels of all three carotenoids tested were found to decrease significantly in SN during ripening at $p<0.001$. Overall, ripening significantly increased the levels of the non-PVA carotenoid lutein in both dessert bananas, SN and BO, as well as in the plantain GN at $p<0.001$.

Since EAHB and plantains are generally boiled or steamed prior to consumption in Uganda, the effect of these processes on carotenoid retention values in full-green fruit from these cultivars was assessed. The β-CE retention levels after exposure to the different processing regimes ranged from 55.4 to 93.9% for boiling and 57.3 to 79.6% for steaming. Although the amount of both α- and β-carotene in all cultivars tested was found to decrease after boiling and steaming, these reductions were mainly evident after steaming in cultivars Mbz, Mpo and Nakt for α-carotene and Mbz and Nak for β-carotene.

The second objective of this study was to characterise and analyse 71 transgenic plants (28 different lines) of the dessert banana, 'Sukali Ndizi', and the EAHB hybrid M9 that had been previously generated at NARO. These two banana cultivars had been transformed with either a *Zea mays* phytoene synthase 1 (*ZmPsy1*) gene or a Fe'i type banana (Asupina spp.)-derived phytoene synthase 2 (*APsy2a*) gene under the control of a banana derived expansin 1 promoter (Exp1), and were growing in a confined field trial at NARO. One of the two variants of the *ZmPsy1* transgenes were used, *Psy1B*73 from maize cv. B73 and *Psy1Q*60 from maize cv. Q60 the proteins of which only differ by a single amino acid. Leaf samples were taken from each plant and the presence of the respective transgene was confirmed by PCR. To enable the selection of only the elite lines for further studies, fruit from all 28 transgenic lines was sampled at the full ripe (FR) and full green (FG) stages, and analysed using high-performance liquid

chromatography (HPLC) to analyse the levels of carotenoid accumulation. The PVA carotenoid content of transgenic 'Sukali Ndizi' lines ranged from 0.2-6.8 µg/g DW while the levels in the non-GM controls ranged from 0.5-1.4 µg/g DW. With the EAHB M9 transgenic plants, the PVA carotenoid content ranged from 5.6-33.1 µg/g DW with the one non-GM control containing 5.8 µg/g DW. Based on these results, eight transgenic lines (2 x SN and 6 x M9) with a PVA carotenoid content greater than 2 µg/g DW (for SN) and 5 µg/g DW (for M9) were subjected to further analyses to investigate the effect of phytoene synthase (*Psy*) transgene expression levels on PVA carotenoid accumulation during fruit development and across successive generations.

Full-green stage fruits from the two transgenic SN lines were shown to have between 5.3-16.7-fold more PVA compared to non-GM control fruit. The increase in PVA levels commenced early on during fruit development and was maintained through to maturity of the fruit. Using qPCR, no direct correlation was observed between the levels of transgene expression and PVA accumulation in these lines. Analysis of the PVA levels in the fruit during four crop generations revealed a substantial increase in PVA accumulation from the mother plant to the first ratoon (R1). Although the higher PVA levels seen in the R1 generation were not maintained in successive generations, the PVA levels were still higher than those of the non-transgenic control plants over the 4 generations.

All M9 transgenic lines expressing *Psy1Q60* also showed a consistent increase in PVA accumulation during bunch development. Fruit at the full-green stage from the 4 transgenic M9 lines was shown to have between 0.96-1.9-fold more PVA compared to non-GM control fruit. Similar to observations made in the 'Sukali Ndizi' cultivar, no correlation was observed between the pattern and level of transgene expression and the pattern and levels of PVA accumulation.

Finally, the transgenic plants were evaluated for phenotypic abnormalities and agronomic performance. For almost all of the agronomic traits evaluated, the performance of the 8 elite transgenic lines was similar to that of the non-GM controls. The only exception to this was with bunch weight whereby a correlation was seen between elevated β-CE levels and reduced bunch weight. In terms of phenotypic abnormalities, all plants of the two transgenic SN lines displayed a "golden leaf" phenotype. This phenotype only associated with the Exp1-*Psy1B73* promoter-gene combination with none of the other transgenic lines (including cultivar M9) containing the Exp1-*APsya2a* ever displaying this phenotype. The final objective of this study was to investigate the role of the *Psy2a* gene in carotenoid accumulation during fruit development in both the EAHB cultivar 'Mpologoma' and the Fe'i type banana 'Asupina'. Full-green fruit from 'Asupina' was shown to accumulate up to 131.4±13.9 µg/g DW β-CE compared to 17.8±5.1 µg/g DW for 'Mpologoma'. With 'Asupina', the accumulation of PVA carotenoids occurred during a very short period of time at a very late stage of fruit development. No correlation was observed between accumulation of carotenoids and *Psy2a* mRNA levels in either cultivar.

This study provides a comprehensive insight on the levels of PVA carotenoids present in dessert and East African highland banana cultivars grown in Uganda. The results from this study also deliver a platform for further research aimed at biofortifying EAHB and 'Sukali Ndizi' through genetic engineering in Africa.

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List of abbreviations

Abbreviation	Description
cDNA	complementary deoxyribonucleic acid
CTAB	cetyltriethylammonium bromide
DNA	deoxyribonucleic acid
EAHB	East African Highland Bananas
EAR	estimated average requirement
EDTA	ethylene-diamine tetraacetic acid
gDNA	genomic deoxyribonucleic acid
HPLC	high performance liquid chromatography
NIH	National Institute of Health
*npt*II	neomycin phosphotransferase
pBIN	binary vector - pBINPLUS
pCAM	binary vector - pCAMBIA
PCR	polymerase chain reaction
PVA	pro-vitamin A
PVP	polyvinylpyrrolidone
RAE	retinol activity equivalents
RDA	recommended daily allowance
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	tris-ethylene-diamine tetraacetic acid
UBOS	Uganda Bureau of Statistics
UNICEF	United Nations Children's Fund
USDA	United States Department of Agriculture
VAD	vitamin A deficiency
WHO	World Health Organisation

Units	Description
°C	degrees Celsius
cm	centimetre(s)
DW	dry weight
g	gram(s)
g	relative centrifugal force
hr(s)	hour(s)
(k)bp	(kilo)base pairs
kCal	kilo calories
kg	kilogram(s)
L	litre(s)
M	Molar
mg	milligram(s)
min(s)	minute(s)

mL	millilitres(s)
MW	molecular weight
mW	milliWatts
nm	Nanometers
OD	optical density
ρmol	Picomoles
rpm	revolutions per minute
sec	Seconds
V	Volts
v/v	volume to volume
w/v	weight to volume
λ	lambda
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometre

Institution names	
CTCB	Centre for Tropical Crops and Biocommodities
NARO	National Agricultural Research Organisation
QUT	Queensland University of Technology

Declaration

The work contained within this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made.

Date: 10/06/2015

Signature:

QUT Verified Signature

Ruth MBABAZI

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Chapter 1 - Literature review

1.1 Banana and plantains

1.1.1 Botany and genetics

Bananas are large monocotyledonous and herbaceous plants belonging to the genus *Musa*, family *Musaceae*, which comprises around 40 different species (Simmonds, 1962; Purseglove, 1972; Stover and Simmonds, 1987). The plant has a pseudostem composed of compact overlapping leaf sheaths. The basal stem is condensed and only elongates through the pseudostem at flowering to eject the inflorescence. Most banana plants propagate vegetatively through suckers that arise from the basal stem of the mother plant (Purseglove, 1972; Stover and Simmonds, 1987).

Edible bananas were derived through the processes of natural hybridisation between the two seeded wild diploid progenitor subspecies *Musa acuminata* Colla (AA) and *M. balbisiana* Colla (BB) (Karamura, 1998). Triploid cultivars constitute most of the world banana production and are found as either dessert or cooking varieties in the AAA, AAB and ABB genomic groups. They are usually sturdier and bigger than their ancestors but are usually seedless, sterile and parthenocarpic. ABB genotypes are mainly cooking bananas (Bluggoe type), AAB are plantain and AAA are dessert bananas (Daniells *et al.*, 2001; Karamura and Mgenzi, 2004). The majority of bananas grown in East Africa are called the East African highland bananas (EAHB), which are a unique type of cooking banana endemic to East Africa and belong to the AAA-EA subgroup.

In Uganda, approximately 90% of all bananas grown are EAHB which belong to 4 clone sets of the Lujugila-Mutika subgroup, also known as 'Matooke' (Karamura, 1998). The remaining 10% include mainly the plantain and apple bananas ('Sukali Ndizi'), which are of the AAB genome type (Pillay *et al.*, 2001).

1.1.2 The importance of banana

Bananas are an economically important crop worldwide. They are cultivated in over 130 countries throughout tropical regions and in some subtropical regions in Asia, Africa, Latin America, the Caribbean and the Pacific (Frison and Sharrock, 1999). In 2006, the reported area cultivated worldwide was 4.2 million hectares (Mha) of bananas and 5.4 Mha of plantains, with a world production of 107.1 million metric tonnes of bananas and 37.7 million metric tonnes of plantains (FAO, 2011). Banana varieties such as *Musa textilis* are also important for fibre production and ornamental purposes (Frison and Sharrock, 1999).

Banana is a staple food crop in the Great Lakes region of East Africa and, in Uganda, East African Highland bananas (EAHB) are a major staple food crop and an important source of household income (Rubaihayo, 1991; Adeniji *et al.*, 2010). They form an integral part of the diet for most rural and urban families but are also an important source of household income in countries including Uganda, Kenya, Rwanda, Burundi, the Democratic Republic of Congo and Tanzania (Rubaihayo, 1991; Adeniji *et al.*, 2010). In banana growing areas, they contribute the bulk of food intake and a banana meal is often used as the weaning food for children (Kikafunda *et al.*, 1996). It is estimated that 75% of Ugandan farmers grow bananas (Ssebuliba *et al.*, 2006) contributing about 7% of global bananas and plantains production (FAO, 2011). Uganda grows and consumes 220-250 kg/person/year and, as such, is ranked the largest banana consumer in the world (Tushemereirwe *et al.*, 2006). In the FAO statistics in 2012, plantains and bananas were ranked among the top five food commodities in Africa (Table 1.1) based on international commodity prices (FAO, 2012).

In Uganda, bananas are consumed in various forms. The adopted processing procedures include steaming, boiling, roasting, frying and, in the case of dessert bananas, ripening. Banana juice is also common in rural areas, sometimes fermented to produce a local brew. Bananas are also increasingly being processed into dried chips and banana flour (Bukusuba *et al.*, 2008).

Table 1.1. Value of commodities production in 2012 for the African region

Rank	Commodity	Production (USD $ x1000)
1	Cassava	15,553,269
2	Meat indigenous, cattle	14,413,336
3	Yams	11,789,547
4	Milk, whole fresh cow	10,372,954
5	Plantains and bananas	9,463,562
6	Rice, paddy	7,034,898
7	Tomatoes	6,629,178
8	Maize	6,610,467
9	Meat indigenous, chicken	6,583,229
10	Meat indigenous, sheep	4,665,727

Source: (FAO, 2012)

1.1.3 Nutritional value of bananas

Banana and plantain are a rich source of carbohydrates such as sugars and starch (INIBAP, 2001; Muzanila and Mwakiposa, 2003). Other macronutrients include low levels of protein and fat, which can vary depending on the variety and method of processing (Table 1.2). Indeed, studies conducted on products derived from bananas showed that banana flour has a higher energy and protein content compared to steamed or boiled bananas (Muzanila and Mwakiposa, 2003). Potassium is commonly the most abundant mineral in bananas, reaching up 400mg per 100g serving on dry matter basis. The different varieties of bananas under cultivation in Uganda have varying levels of micronutrients (Bukusuba *et al.*, 2008), which may be influenced by the environment in which the banana variety is grown. Pro-vitamin A carotenoid (β- and α-carotene) concentration in selected Ugandan banana varieties have been reported to be in the range of 50.6 to 513.7 µg/100g FW (fresh weight) while the concentration of Fe and Zn were determined to be in the ranges 0.112 to 0.608 mg/100g FW and 0.054 to 0.539 mg/100g FW, respectively (Table 1.3) (Fungo, 2009; Fungo *et al.*, 2010).

The β-carotene content in most of the selected Ugandan banana varieties (Table 1.3) was (Englberger *et al.*, 2003; Englberger *et al.*, 2003). The recommended daily intake of β-carotene for an adult man and woman are 10.8mg and 8.4mg, respectively (Table 1.4). These values are calculated from the ratio of 12µg of β-carotene to 1 µg retinol, referred to as retinol activity equivalents (RAE) (Institute of Medicine (US) Panel on Micronutrients, 2002). The RAE provides a measure of ingested pro-vitamin A (PVA) carotenoids that are converted into metabolically active retinol available for storage and utilisation (van Lieshout *et al.*, 2003). Besides β-carotene, the amount of other vitamin A-active carotenoids equivalent to 1 RAE is 24µg (Institute of Medicine (US) Panel on Micronutrients, 2002). According to the RAE value, the levels of PVA carotenoid in Ugandan banana varieties are too low to meet the

Table 1.2. Macronutrient composition for selected banana and plantain foods

Component	Fresh banana	Banana flour	Chips
Crude protein (g/100g DW)	5.14	4.04	2.4
Crude fat (g/100g DW)	6.4	0.64	6.81
Ash (g/100g DW)	-	3.22	-
Carbohydrate (g/100g DW)	72.06	77.29	87.11
Crude fibre (g/100g DW)	3.0	2.15	1.26
Potassium (K) (mg/100g)	400	-	-
Calcium (Ca) (mg/100g)	-	36.9	-
Phosphorus (P) (mg/100g)	-	41.45	-

DW = dry weight. Calculations based on dry matter content.

Sources: (Muzanila and Mwakiposa, 2003; Yomeni *et al.*, 2004; Muranga *et al.*, 2007).

Table 1.3. Micronutrient composition of some banana varieties in Uganda

Variety	β-carotene (µg/100g FW)	Fe (mg/100g FW)	Zn (mg/100g FW)
'Nakitembe'	513.7	0.509	0.369
'Tereza'	245.7	0.231	0.294
'Nakhaki'	448.8	0.608	0.539
'Kibuzi'	428.9	0.257	0.054
'Mpologoma'	146.4	0.292	0.222
'Sukali Ndizi'	50.6	0.112	0.159

Source: (Fungo, 2009; Fungo *et al.*, 2010).

Table 1.4. Dietary reference intakes of Vitamin A by life stage group

	DRI values (µg RAE[a]/day)					
	EAR[b]		RDA[c]		AI[d]	UL[a,f]
	males	females	males	females		
Life stage group						
0 to 6 months					400	600
7 to 12 months					500	600
1 to 3 years	210	210	300	300		600
4 to 8 years	275	275	400	400		900
9 to 13 years	445	420	600	600		1,700
14 to 18 years	630	485	900	700		2,800
19 to 30 years	625	500	900	700		3,000
31 to 50 years	625	500	900	700		3,000
51 to 70 years	625	500	900	700		3,000
>70 years	625	500	900	700		3,000
Pregnancy						
≤ 18 years		530		750		2,800
19 to 50 years		550		770		3,000
Lactation						
≤ 18 years		885		1,200		2,800
19 to 50 years		900		1,300		3,000

[a]**RAE** = Retinol activity equivalent. 1 µg RAE = 1 µg retinol, 12 µg β-carotene, and 24 µg α-carotene or β-cryptoxanthin. The RAE for dietary pro-vitamin A carotenoids in foods is twofold greater than retinol equivalents (RE), whereas the RAE for preformed vitamin A in foods is the same as RE.

[b]**EAR** = Estimated Average Requirement

[c]**RDA** = Recommended Dietary Allowance

[d]**AI** = Adequate Intake

[e]**UL** = Tolerable Upper Intake Level

[f]The UL for vitamin A applies only to preformed vitamin A (eg. Retinol, the form of vitamin A found in animal foods, most fortified foods, and supplements). It does not apply to vitamin A derived from carotenoids.

Source: http://hungermath.wordpress.com/2012/12/23/u-s-rda-for-vitamin-a-in-retinol-activity-equivalents/

daily dietary intake levels for β-carotene which are required to meet dietary requirements of healthy individuals in a particular age and gender group.

In addition, the form in which the banana is cooked also affects the nutritional composition since processing is known to change the chemical composition of nutrients in food (Muzanila and Mwakiposa, 2003). In studies on β-carotene bioaccessibility in orange fleshed sweet potato which examined the effect of processing on the β-carotene isomers, bioavailability of β-carotene increased in the order: raw<baked<steamed<deep fried (Tumuhimbise *et al.*, 2009).

1.1.4 Reliance on a micronutrient poor diet

In Uganda, bananas form an integral part of the diet of women and children and are sometimes used as a weaning food for children (Kikafunda *et al.*, 1996). As described previously, most banana and plantain cultivars grown in Uganda have inadequate levels of vitamin A but also other key micronutrients such as iron (Fe), iodine and zinc (Zn) (Fungo *et al.*, 2007; Fungo *et al.*, 2010). As a result, populations which rely heavily on banana as a staple food, fail to meet the recommended dietary allowances (RDA) of these essential elements and vitamins. For example, in Uganda vitamin A deficiency (VAD) amongst children under the age of five was has high as 27.9% nationally while the 3 main banana growing areas of Central, Western and Eastern Uganda had 21.8%, 28.6% and 27.6% VAD respectively (UBOS, 2006; Fungo, 2009). According to that same survey conducted in 2001, VAD has a higher prevalence in women (15-49 years) with as much as 51.9% nationally and 56.5%, 54.7% and 51.7% in Central, Western and Eastern Uganda respectively. Interestingly these numbers dropped below 45% for women and 16% for children in urban areas, exhibiting the influence that reliance on a micronutrient poor staple like banana can have on VAD.

1.2 Micronutrients and their deficiencies in the human diet

1.2.1 Causes and effects of micronutrient deficiencies

Micronutrient malnutrition has a direct effect on public health as it affects physical growth and development, and increases morbidity and mortality. Furthermore, micronutrient deficiencies have been reported to have long term effects in society whereby children affected in the first 2 years after birth develop health problems later in life (Victora *et al.*, 2008). Therefore, the prevention of maternal and child malnutrition is a long-term investment that benefits both present and future generations.

Micronutrient poor diets are the main cause of micronutrient deficiencies (Victora *et al.*, 2008). Therefore, the provision of safe and effective amounts of dietary micronutrients is vital for the prevention of diseases associated with micronutrient deficiency. Some micronutrients, such as zinc and selenium, are critical to the prevention of common infections. Zinc plays a key role in the prevention of diarrhoea and pneumonia, and is involved in most of the body's enzymatic processes, whereas selenium has been reported to play a major role in the maintenance of defences against infection and modulation of growth and development (Black *et al.*, 2008). Other major micronutrients that are important for human health include iron, iodine and vitamin A. In the human body, iron serves as a carrier of oxygen from the lungs to the tissues by red blood cell haemoglobin. It also acts as a transport medium for electrons within cells and plays an integrated part of important enzymatic reactions. Iodine is vital in the synthesis of thyroid hormones by the thyroid gland (Institute of Medicine (US) Panel on Micronutrients, 2002) while vitamin A is important for growth and development, the maintenance of the immune system and good vision. Vitamin A deficiency (VAD) has been reported as one of the most common dietary problem affecting children worldwide, with 1.2 million deaths annually among children aged 1 to 4 (Humphrey *et al.*, 1992; WHO, 2009). Research has shown that VAD increases child mortality by as much as five times, mostly affecting the 0 to 5 years age group (Humphrey *et al.*, 1992; West, 2002; Maziya-Dixon *et al.*, 2006; Danneskiold-Samsøe *et al.*, 2013). In Uganda,

VAD was reported in some parts of the country mainly as a result of excessive reliance on a vitamin A-poor staple diet (Harvey *et al.*, 2010).

1.2.2 The role of carotenoids in health

Plant dietary carotenoids such as lycopene, lutein, β- and α-carotene are essential requirements for human and animal nutrition. β-carotene is the most potent dietary precursor of vitamin A, the deficiency of which leads to xerophthalmia, blindness and premature death (Mayne, 1996). Other carotenoids have been shown to alleviate age-related diseases, probably because of their powerful properties as lipophilic antioxidants. For example, zeaxanthin and lutein offer protection against macular degeneration (Seddon *et al.*, 1995). High intake of tomatoes is associated with reduced incidence of prostate cancer (Giovannucci, 1999). Their consumption is correlated to reduction in the amount of DNA damage in white blood cells and prostate tissues of prostate cancer victims (Chen *et al.*, 2001). Critical to the determination of the potential role of carotenoids in prevention of chronic diseases is a better understanding of the relative bioavailability of carotenoids from foods. In particular, the two critical areas of research focus to alleviate vitamin A deficiency in populations have been; 1) biosynthesis of carotenoids, with emphasis to bio-accessibility of β-carotene, in food, and 2) bioavailability of the different carotenoids obtained from food and possible factors affecting carotenoid bioavailability in the body (Bresnahan *et al.*, 2012).

1.2.3 Vitamin A

Vitamin A is comprised of a group of highly unsaturated, nutritionally active hydrocarbon compounds. They include retinol, retinal, retinoic acid and several pro-vitamin A carotenoids, among which β-carotene, α-carotene and β-cryptoxanthin are the most important (Damodaran *et al.*, 2007). In a healthy diet, the daily dietary requirement of vitamin A is estimated at 75% retinol intake obtained from foods of animal origin, such as eggs, milk, butter, and liver, and 25% pro-vitamin A carotenoids obtained from foods of plants origin, such as sweet potatoes, pumpkin, carrots,

tomatoes, peaches, nectarines, apricots, mangoes, lettuce and some greens, broccoli, and asparagus (Mangels *et al.*, 1993; Castenmiller and West, 1998; Castenmiller *et al.*, 1999; Belitz, 2004). Retinal is formed as a result of the cleavage of the carotenoid β-ring by the β-carotene 15,15'-monooxygenase enzyme in the human digestive system (Andersson and Lindqvist, 2002). Vitamin A is vital for several physiological and developmental functions of the human body including vision, immune function, bone metabolism, skin health, gene transcription, embryonic development and reproduction, haematopoiesis, and as an antioxidant (Paul and Bramley, 2004; Kimura *et al.*, 2007; Beyer, 2010). Thus, consumption of diets insufficient in PVA carotenoids may lead to related physiological abnormalities.

1.2.4 Vitamin A deficiency (VAD)

The role of vitamin A in the diet cannot be understated and several studies have been conducted on the prevalence of vitamin A deficiencies and its consequences on public health (West *et al.*, 2002; SUMMIT, 2008). A WHO study published in 2009 on the prevalence of VAD as a public health problem by country during the period from 1995 to 2005 in preschool-age children and pregnant women clearly showed the importance of VAD and its incidence in sub-Saharan Africa and developing countries like Uganda (Figure 1.1) (WHO, 2009). During that period, 190 millions preschool-age children and 19.1 pregnant women were affected by VAD (Table 1.5). Effects of severe VAD include clinical signs of xerophthalmia (dryness of the conjunctiva and cornea) (WHO, 1998) and in Uganda, VAD has been associated with blindness, higher disease incidences among children under the age of 5 years, and increased maternal mortality in expectant mothers (Kikafunda *et al.*, 1996). Serum retinol is used as a biochemical indicator of vitamin A status in population nutrition studies to reflect the level of retinol binding protein (RBP), where 0.7 µmol/L of retinol is equivalent to a concentration of 0.825 µmol/L of RBP (Gorstein *et al.*, 2007). Using these criteria, a VAD studies conducted in Uganda in 2006 found that 20% of the children in the studied

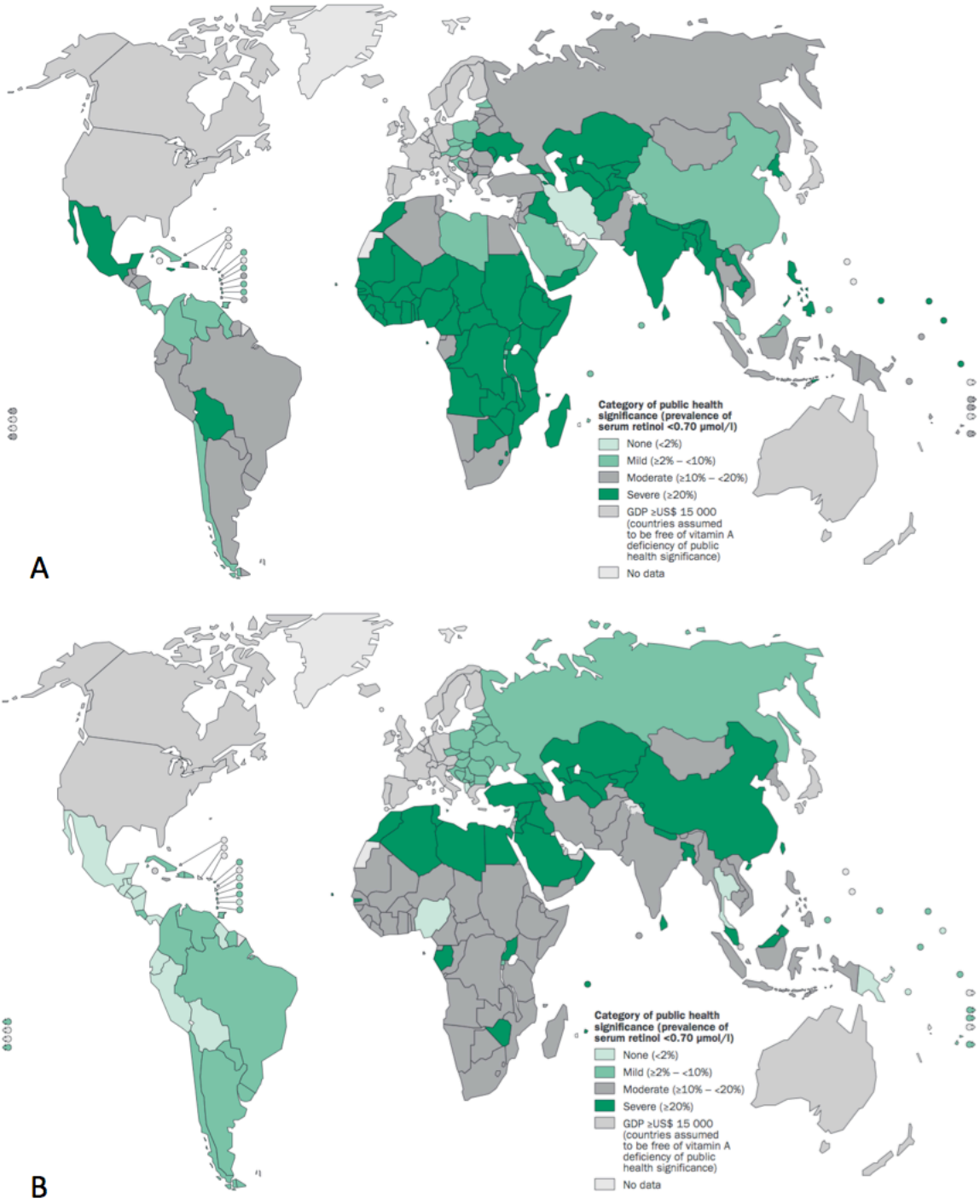

Figure 1.1. Biochemical vitamin A deficiency (retinol) as a public health problem by country 1995-2005.
A, preschool-age children and B, pregnant women. Countries and areas with survey data and regression-based estimates.

Source: (WHO, 2009)

Table 1.5. Prevalence of serum retinol <0.70 µmol/L and number of individuals affected among preschool-age children and pregnant women in populations of countries at risk of vitamin A deficiency 1995-2005, globally and by WHO region

WHO region	Preschool age children[a]		Pregnant women	
	Prevalence[b] (%)	# affected (millions)	Prevalence[b] (%)	# affected (millions)
Africa	44.4 (41.3-47.5)[c]	56.4 (52.4-60.3)	13.5 (8.9-18.2)	4.18 (2.73-5.63)
Americas	15.6 (6.6-24.5)	8.68 (3.70-13.7)	2.0 (0.4-3.6)	0.23 (0.04-0.41)
South-East Asia	49.9 (45.1-54.8)	91.5 (82.6-100)	17.3 (0.0-36.2)	6.69 (0.00-14.0)
Europe	19.7 (9.7-29.6)	5.81 (2.87-8.75)	11.6 (2.6-20.6)	0.72 (0.16-1.29)
Eastern Mediterranean	20.4 (13.2-27.6)	13.2 (8.54-17.9)	16.1 (9.2-23.1)	2.42 (1.38-3.47)
Western Pacific	12.9 (12.3-13.5)	14.3 (13.6-14.9)	21.5 (0.0-49.2)	4.90 (0.00-11.2)
Global	**33.3 (31.1-35.4)**	**190 (178-202)**	**15.3 (7.4-23.2)**	**19.1 (9.30-29.0)**

[a] Population subgroups: Preschool-age children (<5 years); Pregnant women (no age range defined).
[b] Numerator and denominator excludes countries with a 2005 GDP ≥ US$ 15,000.
[c] 95% Confidence intervals.

population had VAD (UBOS, 2006). The same study conducted 5 years earlier presented slightly higher values for children VAD, however the data presented for women VAD then was much higher (around 50% in 2001 compared to around 20% in 2006). East and Central regions of Uganda, which heavily rely on banana, still had the highest proportion of children and women with VAD (32% and 31% respectively). Due to the change in the methodology for measuring vitamin A status in the 2000-2001 and 2006 UDHS surveys, the report warn that any changes observed between the two surveys may be due more to differences in the methodologies than to true changes in the population (UBOS, 2006).

Globally, vitamin A, iron and zinc deficiencies are **r**eported to have the largest percentage of disease burden amongst the micronutrients that are known to have a negative impact on public health (Black *et al.*, 2008). To address VAD, a number of interventions have been carried out, however, these have presented some major limitations in developing countries.

1.2.5. Strategies to alleviate micronutrient deficiency and their limitations

The problem of malnutrition and micronutrient malnutrition is critical in developing countries where it is responsible for premature death, poor growth among children, disability, and productive inefficiency (HarvestPlus, 2007). Micronutrient deficiencies can be combated through policy and behavioural interventions (Bryce *et al.*, 2008; Loo-Bouwman *et al.*, 2009). Policy interventions include nutrient supplementation and nutrient fortification of foods, such as breakfast cereals, iodised salt, margarine, flour or oil. Behavioural interventions focus on individual knowledge, attitude and practice. They include breast feeding, complementary feeding and diet diversification. Supplementation, fortification and diet diversification interventions provide a mechanism to correct specific micronutrient deficiencies with relatively simple behaviour change by the consumer. Using these interventions, there are population studies that indicate controlled effect on clinical and subclinical forms of micronutrient

malnutrition associated with iodine and vitamin A (Semba *et al.*, 2005). In addition, increased doses of iron, folic acid, vitamin A, iodine and zinc supplementation result in reduced infant mortality rates among study populations (SUMMIT, 2008). Adoption of supplementation, if effectively adopted, can reduce the disease burden in the most vulnerable of populations (Bouis, 2002; SUMMIT, 2008). In developed countries and urban centres in the developing world where fortified foods such as breakfast cereals are widely consumed, low levels of micronutrient deficiencies are often reported. Adoption of supplementation and food fortification in an effort to control micronutrient malnutrition among populations have yielded positive impacts, though due to a number of shortcomings, these interventions have been inadequate in reaching the goals set by international organisations (WHO/FAO, 2004; Victora *et al.*, 2008; WHO, 2009). However, these interventions are mainly urban based, where the population is in vicinity of the service providers and they fail to reach rural communities that are most in need.

Several factors determine the feasibility and effectiveness of the different intervention strategies. These include health infrastructures, the economy, political stability and the access to suitable methods of nutrient fortification. Although effective in developed countries, interventions are often not reproducible in the developing world where the situation is often aggravated by limited incomes, insufficient food intake, high burdens of social responsibilities on women and in various cases, limited amounts of essential nutrients in the major staples (Bryce *et al.*, 2008; SUMMIT, 2008). Countries with highest levels of micronutrient deficiencies are also countries where dietary diversity is poor, the use of processed food is limited, and the availability of centrally milled staple food to use as a vehicle for food fortification is restricted. In Uganda for example, the level of VAD among children varies according to habitation whereby children in urban communities have low prevalence of VAD compared to those from rural communities (UBOS, 2006). Therefore, controlling VAD requires not only vitamin A supplementation

but also new approaches targeting staple food crops to improve on the daily micronutrient consumption of rural poor communities.

1.3. Biofortification as a strategy to alleviate VAD

Communities in the developing world are highly dependent on specific foods, referred to as staple foods. Major staple crops in sub-Saharan Africa include cassava, sweet potato, banana, cocoyam, maize, sorghum, millet, wheat and rice (Nestel *et al.*, 2006). Unfortunately, these foods normally contain limited amounts of essential micronutrients. Therefore, improving the micronutrient content of key staple foods can greatly contribute to the reduction of micronutrient malnutrition in targeted populations.

Biofortification is the delivery of micronutrients via micronutrient-dense crops. It offers a cost effective and sustainable approach, complementing other interventions by reaching rural populations. Biofortification relies on the plant's biosynthetic or physiological capacity to produce or accumulate the desired nutrients (Mayer *et al.*, 2008). Bioavailable micronutrients in the edible parts of staple crops at concentrations high enough to impact on human health can be obtained through conventional breeding provided that sufficient genetic variation for a given trait exists, or through genetic engineering. There are various efforts by crop breeding programs to enrich the major food staples in developing countries targeting the most important micronutrients including iron, pro-vitamin A, zinc and folate.

Biofortification of staple foods as a dietary intervention provides sufficient daily dietary intake since staples provide a major proportion of the daily diet (Nestel *et al.*, 2006). The intervention is cost effective, given that recurrent costs are low once investments in developing the seed stock have been made. The intervention is highly sustainable as production of fortified crops can continue in the absence of funding and the products can be easily accessed by undernourished communities in remote areas.

Biofortification approaches have been deployed to improve the availability of key micronutrients in plants. This has been achieved through adoption of conventional breeding and genetic engineering techniques.

1.3.1. PVA biofortification through conventional breeding

Conventional breeding has been widely adopted as the method of choice to increase the nutritional content of staple crops in an effort to alleviate VAD, especially in communities of sub-Saharan Africa. For example, orange-flesh sweet potato lines have been successfully bred with improved PVA content (Delia and Kimura, 2004; Laurie *et al.*, 2009; Hotz and McClafferty, 2007), in addition to rice and maize with improved PVA, iron and zinc content (Bouis, 2002; Gregorio, 2002; Maneesha *et al.*, 2008; Aluru *et al.*, 2011). Other crops such as cassava and corn have also been targeted in breeding programs (Delia and Kimura, 2004; Sayre *et al.*, 2011).

1.3.2. PVA biofortification through genetic engineering

When the target crop is reproductively sterile and/or the desirable trait is not available among the target crop genetic resources, genetic engineering becomes a better approach for biofortification than conventional breeding (Vuylsteke *et al.*, 1998; Swennen and Vuylsteke, 2001). For example, the genetic improvement of cultivated banana varieties is hampered by crop sterility and the long cropping cycle which poses a major challenge in banana breeding programs (Vuylsteke *et al.*, 1998). In Uganda, successful primary crosses have mainly been made when the wild species of banana, *Musa acuminata* spp. *burmannicoides* 'Calcutta 4' (a diploid) is used as a male parent in crossing programs (Ssebuliba *et al.*, 2006). Although 'Calcutta 4' possesses disease resistance traits, it does not possess most desired nutrient traits such as high PVA carotenoid content, thus limiting its use in conventional genetic improvement programs for enhanced nutrient content.

Several plant species have been genetically modified with the aim of improving their PVA carotenoids content. These include sorghum, canola and rice for improved carotenoid content (Shewmaker *et al.*, 1999; Ye *et al.*, 2000; Paine *et al.*, 2005; Lipkie *et al.*, 2013), cassava for improved levels of PVA carotenoids (Sayre *et al.*, 2011; Ihemere *et al.*, 2012; Leyva-Guerrero *et al.*, 2012), potato for increased total carotenoids accumulation (Diretto *et al.*, 2006; Diretto *et al.*, 2007; van Eck *et al.*, 2007; Diretto *et al.*, 2010), tomato plant for enhanced fruit carotenoids and flavonoids (Parry and Horgan, 1992; Fray *et al.*, 1995; Berg, 1999; Fraser *et al.*, 2002; Seymour *et al.*, 2002; Davuluri *et al.*, 2005; Morris *et al.*, 2006) and carrot for improved ketocarotenoid accumulation (de Jong *et al.*, 2009).

1.4. Carotenoids as precursors of Vitamin A

1.4.1. Carotenoids in plants

Carotenoids are lipid-soluble plant pigments, of which there are estimated to be over 600 different types in nature (Yeum and Russell, 2002). They represent a diverse group of pigments, contributing to the red, orange and yellow colours found in many flowers, fruits and vegetables. In plants, carotenoids support photosynthesis processes by serving as accessory pigments to harvest light for photosynthesis (Britton, 1995; Lu and Li, 2008). In addition, the coloration formed in flowers and fruits due to carotenoid accumulation attract animals to enhance pollen and seed dispersal (Howitt and Pogson, 2006). The accumulated carotenoids in plants also form the major source of the main precursor of vitamin A in the human diet, namely the pro-vitamin A (PVA) carotenoid, β-carotene (Bramley, 2002; Howitt and Pogson, 2006; Wiebke and Bock, 2009).

In plants, carotenoids are synthesised and sequestered in plastids (Fraser and Bramley, 2004). They are derived from five-carbon isoprene units, joined together to form a 40-carbon isoprenoid molecule that contains up to 15 conjugated double bonds (Hyoungshin *et al.*, 2002; Fraser and Bramley, 2004). PVA carotenoids in edible plant parts such as roots, leaves, shoots, fruit and flowers are the main source of vitamin A in

the human diet (Fraser and Bramley, 2004). For example, raw leafy green vegetables such as lettuce and spinach are common source of PVA carotenoids (Table 1.6).

1.4.2. Biosynthesis of carotenoids in plants

Pro-vitamin A carotenoids, such as β-carotene, form one of the over 600 structures of carotenoids produced by plants and other photosynthetic organisms (Yeum and Russell, 2002). In chloroplasts, carotenoids and tocochromanols (tocopherols and tocotrienols-Vitamin E), are the two most abundant lipid-soluble antioxidants (Cunningham and Gantt, 1998). The two groups share a common pathway in their biosynthesis called the non-mevalonate pathway or 2-C-methyl-D-erythritol 4-phosphate pathway (MEP) that starts with the reaction between pyruvate and glyceraldehyde-3-phosphate (Figure 1.2). The MEP pathway is used by many eubacteria, green algae, and chloroplasts of higher plants. The enzyme 1-deoxy-D-xylulose-5-phostaphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) regulate the first step in the MEP pathway leading to the production of 2-C-methyl-D-erythritol 4-phosphate (MEP). Then a series of reactions occurs until the enzyme 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) leads to the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Giuliano *et al.*, 2008). Interestingly another route allow for the production of IPP and DMAPP in eucaryotes, archaebacteria, and cytosols of higher plants and is called the mevalonate (MVA) pathway.

The key intermediate 20-carbon molecule geranylgeranyl diphosphate (GGPP) is then produced by the condensation of three molecules of IPP and one molecule of DMAPP by the enzyme geranyl-geranyl diphosphate (GGPP) synthase (DellaPenna and Pogson, 2006; Clotault *et al.*, 2008; Lu and Li, 2008). The first committed step in plant carotenoid biosynthesis is the condensation of two molecules of GGPP to produce phytoene by the enzyme phytoene synthase (PSY) (Figure 1.3). GGPP is also the precursor for numerous other groups of metabolites, including tocopherols, chlorophylls, phylloquinones and gibberellins. Phytoene is produced as a 15-cis isomer, which is subsequently converted

Table 1.6. Carotenoid concentration of raw leafy green vegetables, fruits, roots and seeds

Species	Carotenoid (µg/100g fresh weight)					
	Total	Zeaxanthin	Lutein	α-carotene	β-carotene	Lycopene
Brussel sprouts	1163	-	610	-	553	-
Green bean	940	-	494	70	376	-
Broad bean	767	-	506	-	261	-
Broccoli	2533	-	1614	-	919	-
Green cabbage	139	-	80	-	59	-
Lettuce	201	-	110	-	91	-
Parsley	10,335	-	5812	-	4523	-
Pea	2091	-	1633	-	458	-
Spinach	9890	-	5869	-	4021	-
Watercress	16,632	-	10,713	-	5919	-
Apricot	2196	31	101	37	1766	-
Banana	126	4	33	50	39	-
Carrot (May)	11,427	-	170	2660	8597	-
Carrot (Sept)	14,693	-	283	3610	10,800	-
Orange	211	50	64	Nd	14	-
Pepper	2784	1608	503	167	416	-
Peach	309	42	78	Tr	103	-
Sweet corn	1978	437	522	60	59	-
Tomato	3454	-	78	-	439	2937

Values include cis and trans-isomers. Nd= not detected and Tr= trace
Sourced: (Institute of Medicine (US) Panel on Micronutrients, 2002).

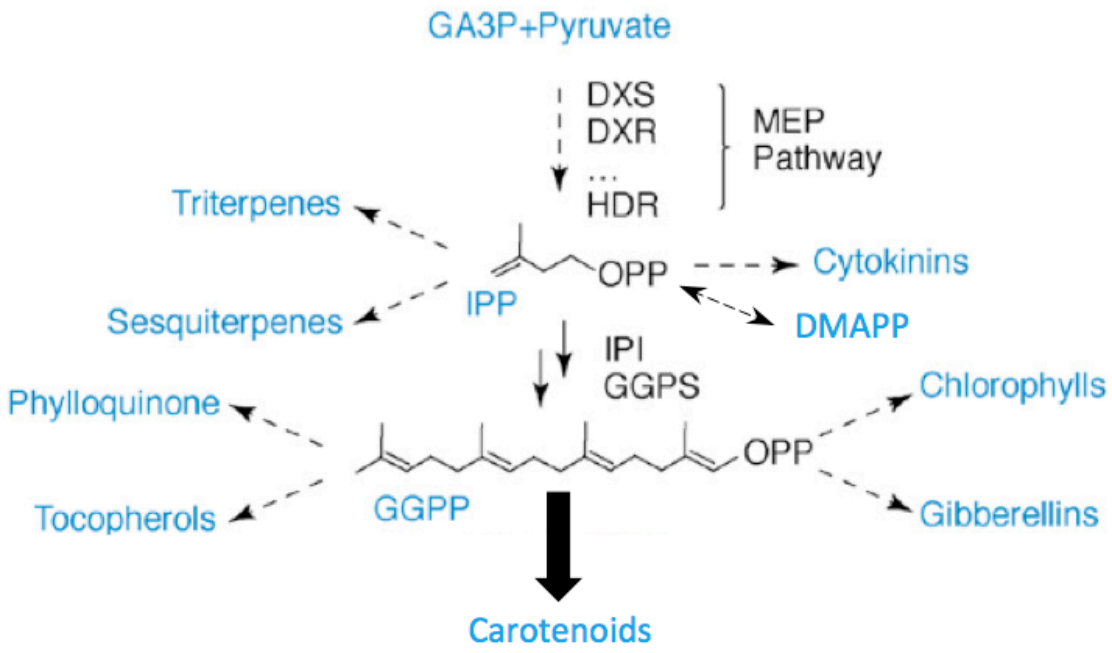

Figure 1.2. Overview of the MEP pathway in plastids. Names of compounds are in blue, and names of enzymes are in black. 1-deoxy-D-xylulose 5-phosphate synthase (DXS); 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR); 2C-methyl-D-erythritol 4-phosphate (MEP); 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR); isopentenyl diphosphate (IPP); dimethylallyl diphosphate (DMAPP); geranylgeranyl diphosphate synthase (GGPS); isopentenyl diphosphate isomerase (IPI); geranylgeranyl diphosphate (GGPP). Source: (Giuliano *et al.*, 2008).

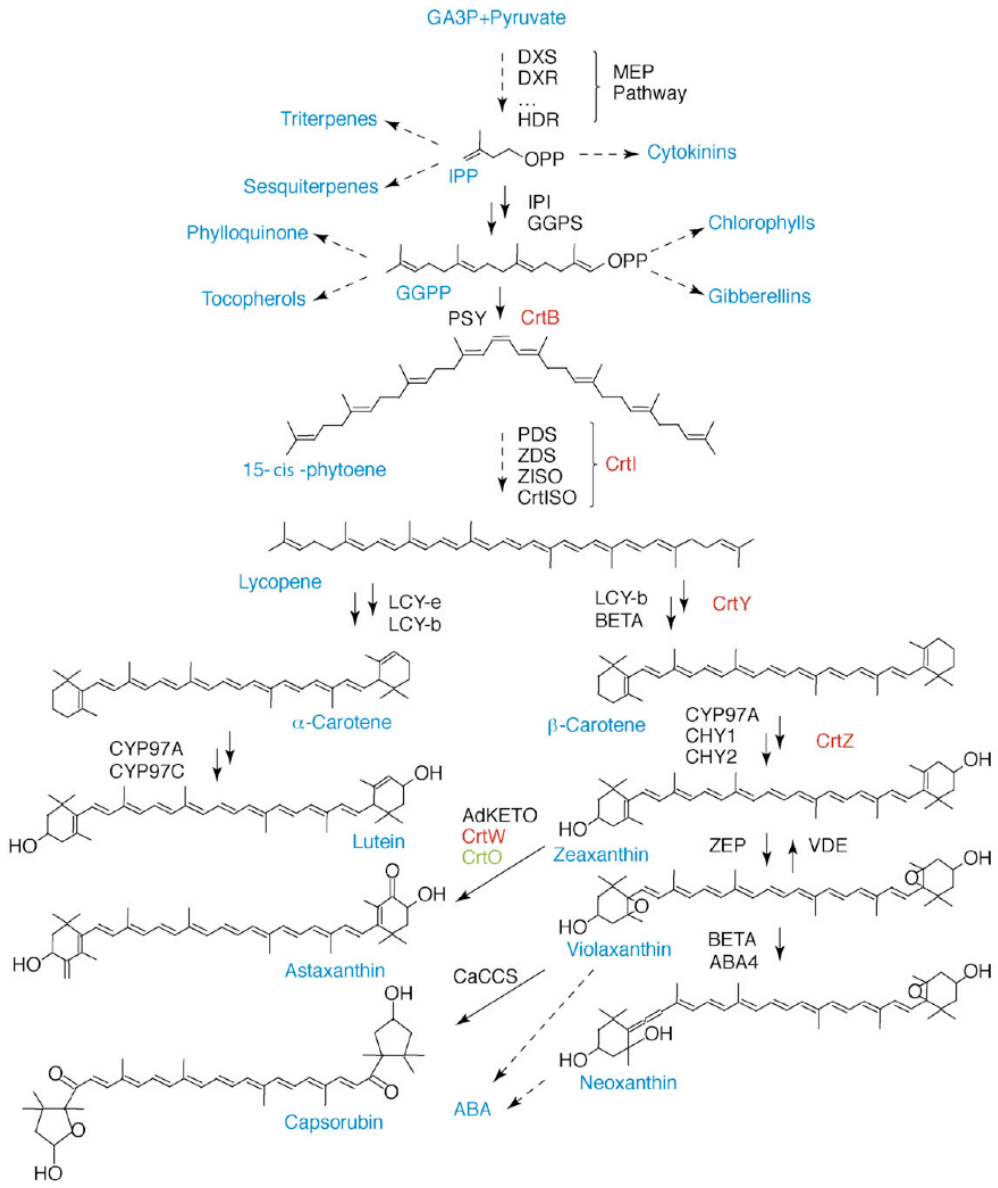

Figure 1.3. The carotenoid biosynthetic pathway. Names of compounds are in blue, and names of enzymes are in black (plant enzymes), red (bacterial enzymes) and green (CrtO from green algae and cyanobacteria). Geranylgeranyl diphosphate (GGPP); phytoene synthase (plant, PSY); phytoene synthase (bacterial, CrtB); phytoene desaturase (plant, PDS); z-carotene desaturase (plant, ZDS); ζ-carotene isomerase (plant, ZISO); carotene isomerase (plant, CrtISO); phytoene desaturase/isomerase (bacterial, CrtI); Ɛ-cyclase (plant, LCY-e); β-cyclase (plant, LCY-b); β-cyclase (bacterial, CrtY); cytochrome P450 carotene hydroxylases (plant, CYP97A, CYP97C); chromoplast-specific β-cyclase (plant, BETA); non-heme carotene hydroxylases (plant, CHY1, CHY2); β-carotene hydroxylase (bacterial, CrtZ); ketolase (plant, AdKETO); ketolase (bacterial, CrtW); ketolase (algal/cyanobacterial, CrtO); zeaxanthin epoxidase (plant, ZEP); violaxanthin de-epoxidase (plant, VDE); abscisic acid (ABA); capsanthin/capsorubin synthase (plant, CaCCS). Source: (Giuliano *et al.*, 2008).

to all-trans isomer derivatives. Two plant desaturases, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), catalyse similar dehydrogenation reactions by introducing four double bonds to form poly-*cis*-compounds (Fig 1.3). The isomerisation of poly-*cis*-lycopene into lycopene is then regulated by the enzymes zeta carotene isomerase (ZISO) and carotenoid isomerase (CRTISO) (Giuliano *et al.*, 2008).

After lycopene synthesis, there are two main branches, forming either the α-carotene group or the β-carotene group that both require the cyclising of the terminal ends of lycopene. β-carotene is formed by action of a single enzyme called lycopene β-cyclase (*LCY*-b), which introduces a β-ring at both ends of lycopene in a sequential two-step reaction. β-carotene can then be hydroxylated to β-cryptoxanthin and finally to zeaxanthin (Kim *et al.*, 2009).

Epoxidation in green tissues can eventually turn zeaxanthin to violaxanthin through the action of zeaxanthin epoxidase, while the reverse reaction is catalysed via the enzyme violaxanthin de-epoxidase as part of the xanthophyll cycle. Its role is to protect the photosynthetic apparatus from photodamage caused by light-induced oxidative stress (Verhoeven *et al.*, 1999; Bassi and Caffarri, 2000). On the other branch, α-carotene and lutein production requires the action of two enzymes, LCY-b and ε-cyclase (LCY-e). One β- and one ε-ring are introduced into lycopene to form α-carotene which is then converted to zeinoxanthin (α-cryptoxanthin) by a β-ring hydroxylase and finally hydroxylated by an ε-ring hydroxylase to produce lutein (Giuliano *et al.*, 2008). A multitude of other carotenoids such as ketocarotenoid and keto-xanthophylls with unique biosynthetic routes exist in some plants (Umeno and Arnold, 2003; Umeno *et al.*, 2005; Lu and Li, 2008).

1.4.3 Manipulating the carotenoid biosynthesis pathway in plants

Tomato and canola plants have been widely used as model plants for the manipulation of the carotenoid biosynthetic pathway to improve carotenoid content in these plants.

Overexpression of the bacterial phytoene desaturase gene *CrtI* in tomato resulted in a 3-fold elevation in β-carotene levels although no differences in the total carotenoid levels were found compared with the non-transgenic lines (Römer *et al.*, 2000). Similarly, transformation of tomato plants with *CrtB* (using a polygalacturonase promoter and the *Psy1* transit sequence to target the CrtB protein to the chromoplast) resulted in a 2-4-fold increase in fruit carotenoids, with no effect on other isoprenoids (Fraser *et al.*, 2002). When the same gene was used to transform canola, the mature seeds were reported to contain up to 50-fold increase in carotenoid content, with α- and β-carotenes being the most predominant (Ravanello *et al.*, 2003). The best example of the practical exploitation of the carotenoid biosynthesis pathway is "Golden Rice". By co-expression of the maize phytoene synthase 1 (*Psy1*) and the bacterial phytoene desaturase (*CrtI*) in rice endosperm, increased concentrations of β-carotene were achieved (Ye *et al.*, 2000; Paine *et al.*, 2005). In maize, overexpression of the *Psy1* gene increased total carotenoid content by 133-fold (Zhu *et al.*, 2007) while in kumquat (*Citrus japonica*), expression of the Citrus *Psy* gene increased β-carotene content by 2.5 fold (Zhang *et al.*, 2009). The *DXS* gene that catalyses the first reaction in the MEP pathway has also been used to enhance carotenoid levels in model plants such as Arabidopsis and tomato (Lois *et al.*, 2000; Estévez *et al.*, 2001; Enfissi *et al.*, 2006). More importantly, its expression in potato caused a 2-fold increase in tuber carotenoid content, with a 6-7-fold increase in phytoene content relative to the control (Morris *et al.*, 2006)

In addition to the transgene used, the choice of promoter used to control gene expression is also important as this influences the dynamics, level and tissue specificity of gene expression (Fraser and Bramley, 2004). In the studies listed above, a variety of different types of promoters were used. For example the expression of the bacterial genes *CrtB* and *CrtI* were under the control of a seed- and a fruit-specific promoter in canola and tomato, respectively (Shewmaker *et al.*, 1999; Römer *et al.*, 2000). In some instances, constitutive expression of the transgenes has been preferred. For example,

over-expression of an endogenous lycopene β-cyclase gene in tomato, which led to increased β-carotene production, was achieved using a constitutive promoter (D'Ambrosio *et al.*, 2004).

1.4.4 Carotenoid bioavailability for improved nutrition

A better understanding of the relative bioavailability of carotenoids from foods is vital to the determination of the potential role of carotenoids in the human diet. Bioavailability of carotenoids in the human body is influenced by a number of factors directly affecting food digestibility, namely micellarisation and bioaccessibility (Schumann *et al.*, 1997; Chitchumroonchokchai *et al.*, 2004; Chitchumroonchokchai and Failla, 2006). Studies investigating possible factors affecting bioavailability of food in the body have been carried out both *in-vitro* and using animal models. These factors have been found to include the status of food (cooked or raw), type of carotenoid (the more polar the compound the more bioavailable), food processing method, food matrix in which the carotenoid is incorporated, nutrient status of the host, the complexity of food composition and interactions with other food compounds (Castenmiller *et al.*, 1999; Hof *et al.*, 2000; Boileau *et al.*, 2002; Chung *et al.*, 2004; Hess *et al.*, 2005; Ryan *et al.*, 2008; Colle *et al.*, 2010; Bresnahan *et al.*, 2012).

Carotenoids are primarily found in complex with protein in nature. Thus, release from the food matrix is an important initial step in the metabolite transfer process. Accordingly, it is important to consider influential factors such as food type and processing when determining the bioavailability of carotenoids in the body (Boileau and Erdman, 2004). Studies on the effects of cooking on carotenoid content and bioavailability in food have confirmed that processing can lead to the degradation, isomerisation, or enhanced or reduced bioavailability of carotenoids (Castenmiller and West, 1998; Boileau *et al.*, 2002; Faulks and Southon, 2005; Schieber and Carle, 2005; Bresnahan *et al.*, 2012). Breshnahan *et al.* (2012) reported that bananas contain high levels of resistant starch. This type of starch is resistant to digestion in the human gut

(Faisant *et al.*, 1995; Faisant *et al.*, 1995), and is thus thought to limit nutrient bioavailability from banana fruit (Tanumihardjo and Howe, 2005; Menezes *et al.*, 2011). However, ripening and thermal processing was shown to reduce resistant starch concentrations in bananas (Bresnahan *et al.*, 2012), thereby enhancing carotenoid bioavailability in banana fruit. Previous bioavailability studies examining the effects of cooking on carotenoids in food revealed that, although cooking reduced the β-carotene content in the food (Bernhardt and Schlich, 2006; Hornero-Méndez and Mínguez-Mosquera, 2007), the process enhanced β-carotene transfer to micelles, thus an improvement in micellarisation of carotenes was observed (Edwards *et al.*, 2002; Hedren *et al.*, 2002; Livny *et al.*, 2003; Tyssandier *et al.*, 2003; Chandrika *et al.*, 2006; Veda *et al.*, 2006; Hornero-Méndez and Mínguez-Mosquera, 2007; Ryan *et al.*, 2008).

The bioavailability of nutrients provides a measure of recommended dietary allowance (RDA) represented by the average daily dietary intake levels of a particular nutrient required for a given age or gender group. For example, the bioavailability of PVA carotenoids provides a measure of RDA for vitamin A, measured in terms of retinol activity equivalents (RAE) (van Lieshout *et al.*, 2003). The Food and Agricultural Organisation (FAO) and WHO reports suggested that in oil, 6 μg of β-carotene carries the activity of 1 μg of retinol, while a ratio of 12 μg of β-carotene: 1 μg of retinol has been suggested for complex foods (WHO, 1998). However, more recent studies based on the factors influencing bioavailability of β-carotene and its subsequent bio-conversion into retinol suggest that 21 μg of β-carotene are required to produce 1 μg of retinol (West, 2000; West *et al.*, 2002). Based on the WHO, the RDA for vitamin A intake has been estimated at 1300 μg of RAE for lactating mothers, 770 μg for pregnant women, 700 μg for adult females, 900 μg for men and 400-500 μg for children below 14 years (National Instututes of Health, 2005). Thus food biofortification to improve pro-vitamin A content in foods will contribute to the average daily dietary intake levels of vitamin A required for a given population group.

1.5. Banana biofortification to combat VAD in Uganda

In Uganda, EAHBs are estimated to contribute between 16-31% of total calorie intake, and 25% of carbohydrate in a predominantly banana diet (Muzanila and Mwakiposa, 2003). Unfortunately, despite the worldwide diversity in bananas (Swennen and Vuylsteke, 2001; Kiggundu *et al.*, 2003; Davey *et al.*, 2007; Garzón *et al.*, 2012), most edible banana and plantain cultivars grown in East Africa have low levels of micronutrients such as pro-vitamin A, Fe and Zn (Fungo, 2009; Fungo *et al.*, 2010). Consequently, a national demographic survey carried out in Uganda in 2006 indicated that most banana consuming areas are deficient in vitamin A and iron (UBOS, 2006) emphasizing the problem of reliance on a micronutrient poor food staple. In Uganda, most of the locally grown banana cultivars have very low pro-vitamin A carotenoid content with, in the worst cases, more than 50 times lower levels than some cultivars grown in Papua New Guinea (Fungo *et al.*, 2010) and the Micronesian Islands (Englberger *et al.*, 2003; Englberger *et al.*, 2003). In light of the societal benefits derived from bananas and the nutritional variations attributed to the crop from previous studies, banana form a key crop for improvement to provide the required public health intervention in low income communities that rely on banana as a staple crop.

1.5.1. Genetic modification of EAHB

Genetic improvement of cultivated banana varieties through conventional breeding is hampered by the crop sterility and the long cropping cycle which poses a major challenge in banana breeding programs (Swennen and Vuylsteke, 2001). Despite these challenges, *Musa* breeding programs still try to develop a wide range of improved varieties through a combination of conventional breeding and the use of biotechnology techniques. Approaches adopted include the use of rapidly developing genomics and proteomics technologies (May *et al.*, 1995; Sagi *et al.*, 1995; Escalant and Panis, 2002). In addition, the development of molecular markers and marked-assisted selection methods has improved the efficiency of selection of improved cultivars.

The direct genetic transformation of *Musa* has the potential to result in major positive impacts on banana and plantain improvement. There are two routinely used methods for the genetic transformation of banana namely particle bombardment (Sagi *et al.*, 1995; Sagi *et al.*, 1998; Remy *et al.*, 2000; Tripathi, 2003) and *Agrobacterium* mediated transformation (May *et al.*, 1995; Khanna *et al.*, 2004; Acereto-Escoffié *et al.*, 2005; Tripathi *et al.*, 2005). Using these techniques, banana plants have been genetically modified for resistance to a range of pests and diseases such as the banana weevil (Gold *et al.*, 2002), fungal diseases (Chakrabarti *et al.*, 2003; Ferreira *et al.*, 2004; Hwang and Ko, 2004; Pei *et al.*, 2005; Paul *et al.*, 2011) and nematodes (Atkinson *et al.*, 2004). Lately, genetic transformation of banana has focused on improvement for micronutrient content mainly driven by the need to address the ill health issues associated with micronutrient deficiencies prevalent in communities for which cooking banana are a staple diet (Bouis *et al.*, 2003).

1.5.2. Banana21

The Banana21 is a collaborative research project between the Centre for Tropical Crops and Biocommodities at Queensland University of Technology (QUT) in Australia and the National Banana Research Program of the National Agricultural Research Organisation (NARO) of Uganda. The project, funded by the Bill and Melinda Gates Foundation as part of the a Grand Challenge in Global Health program, is aimed at tackling micronutrient deficiencies In Uganda through increasing the levels of pro-vitamin A and iron in the fruit of the staple food of Uganda, the East African Highland banana.

Since 2005, QUT has demonstrated in Australia that PVA levels could be dramatically increased in 'Cavendish' banana using only a phytoene synthase gene. Two types of phytoene synthases were used in parallel, a *Psy1* gene from maize (*Psy1B73* and *Psy1Q60*) previously successfully used in the Golden rice project (Ye *et al.*, 2000; Paine *et al.*, 2005) as well as a *Psy2a* gene isolated from a high β-carotene banana cultivar, 'Asupina' (Fe'i group) (James Dale personal communication). Since 2005, QUT has been

evaluating a large number of promoter/*psy* combinations to generate elite transgenic 'Cavendish' banana lines with enhanced levels of PVA in the fruit. In 2009, the first Australian field trial (AFT1) of genetically modified 'Cavendish' banana harbouring genes targeting enhancement of PVA carotenoids was established. Lines with β-carotene equivalent levels reaching almost 19 µg/g (dry weight) were identified which represented a 11-fold increase over wild-type PVA baseline levels (James Dale personal communication).

Following the successful proof of concept regarding the potential of the *Psy* genes to enhance PVA carotenoids in *Musa* spp., the technology has been progressively transferred to Uganda using Ugandan selected varieties. Therefore, as part of the efforts to enhance micronutrients in bananas, NARO in partnership with QUT has generated a number of transgenic banana plants using the three transgene *APsy*2*a*, *Psy1B73* and *Psy1Q60* under the control of the banana derived expansin 1 promoter (Exp1). Transgenic banana plants were generated for two cultivars, an East African Highland Banana (EAHB) hybrid (M9), and a dessert banana ('Sukali Ndizi'), each containing a single gene, *APsy*2*a* or *ZmPsy*1 (James Dale personal communication). Selected lines were planted in a confined field trial for evaluation at NARL-NARO, Uganda.

This PhD project aimed to characterise transgenic banana lines of EAHB hybrid M9 and 'Sukali Ndizi' generated for enhanced PVA biosynthesis and accumulation. In addition, a PVA content comparative study was carried out on non-genetically modified bananas of various popular cultivars collected from different geographical regions of Uganda. The objectives of the study were to:

1. Establish a baseline study of PVA accumulation in the fruit pulp of 8 wild-type banana cultivars commonly grown in Uganda namely 'Sukali Ndizi', 'Bogoya', Gonja, 'Nakitembe', 'Mbwazirume', 'Mpologoma', 'Nakinyika', and an elite EAHB hybrid called M9.

2. Identify putative elite transgenic banana lines of both ‘Sukali Ndizi’ and EAHB hybrid M9 expressing the phytoene synthase genes *APsy2a* or *Psy1* from the confined field trial by assessing the level of PVA accumulation in all the line.

3. Perform the full molecular characterisation of a selection of elite PVA biofortified transgenic banana lines of cultivar ‘Sukali Ndizi’ and EAHB hybrid M9 and assess the expression levels of transgenes in these lines.

4. Quantify both the accumulation of PVA carotenoids as well as the expression levels of the respective transgenes across fruit development and successive generations in the selected elite lines.

5. Quantify and compare the expression of the phytoene synthase gene 2a (*Psy2a*) gene as well as the accumulation levels of PVA carotenoids during fruit development in the non-transgenic EAHB cultivar, ‘Mpologoma’ and the high β-carotene Fe’i type banana, ‘Asupina’.

Chapter 2 - General materials and methods

2.1 General methods

2.1.1. Bacterial culture methodologies

2.1.1.1. Preparation of competent *E. coli* cells

E. coli strain JM109 cells were inoculated in 5mL of LB medium and incubated at 37^{o}C, with shaking (Inoue *et al.*, 1990). Once optimal growth was reached at optical density 0.5-0.7 (O.D 600nm = 0.5-0.7), the cells were centrifuged at 9,000 *g*, 4^{o}C for 10 min. The pellet was gently re-suspended in 200mM of chilled calcium chloride and incubated on ice for 30 min. The centrifugation step was repeated and cells were re-suspended in 5mL of 80mM calcium chloride. Cells were left on ice for 1 hr before they were used for transformation.

2.1.1.2. Transformation of *E. coli*

Plasmid DNA (100ng) was added to the competent cells, the tube was gently swirled and tapped for thorough mixing and incubated on ice for 30 mins (Dower *et al.*, 1988). The cells were heat-shocked at 42^{o}C in a water bath for 30 seconds then quickly returned on ice for 2 min. Subsequently, 500µL of pre-warmed LB media was added and the culture incubated for 2 hrs at 37^{o}C with shaking. After incubation, 100µL of the culture was plated on selective LB medium containing 100 µg/mL of kanamycin and incubated overnight at 37^{o}C. A single bacterial colony was picked the next day and cultured overnight in 5mL of LB medium containing 100 µg/mL kanamycin and incubated at 37^{o}C with shaking. The generated recombinant bacterial culture was used for plasmid purification and restriction digestion to confirm presence and integrity of the plasmid.

2.1.1.3. Preparation of chemically competent *Agrobacterium tumefaciens* strain AGL1

A single colony of *Agrobacterium tumefaciens* strain AGL1 cells was inoculated in 50mL of antibiotic supplemented YMB media and incubated for 2 days at 28°C, with shaking at 250rpm (Lazo *et al.*, 1991). The cells were centrifuged at 9,000*g* for 5 mins and the

supernatant discarded. The pellet was re-suspended in 10mL of ice-cold distilled water and centrifuged for 5 mins at 9,000*g* and 4°C. The pellet was then re-suspended in 10mL 0.15M $CaCl_2$ and centrifuged at 6,000*g* for 5 min. The supernatant was discarded and the pellet re-suspended in 1mL of chilled 20mM $CaCl_2$ to ease the uptake of the plasmid by the bacterial cells. Aliquots (100µL) of the competent cells were then used for transformation with the different vectors.

2.1.1.4. Transformation of *Agrobacterium tumefaciens* strain AGL1

Individual binary vectors (100ng) received from Queensland University of Technology were treated to 100µL of competent AGL1 cells. The mix was incubated on ice for 30 min, snap frozen in liquid nitrogen for 1 min and thawed for 2 mins at 37°C. LB medium (500µL) was added and the culture incubated at 28°C for 4 hrs with gentle shaking at 200 rpm. A 100µL aliquot of the culture was plated on YMA medium containing 250 µg/mL carbenicillin, 25 µg/mL rifampicin and 100 µg/mL kanamycin and incubated for 3 days at 28^{o}C. A plate streaked with non-transformed AGL1 cells on selection was included as a negative control while a plate with untransformed AGL1 on plain YMA was used to check for viability of the competent AGL1. After 3 days of incubation at 28°C, single colonies were picked and grown in YMB supplemented with antibiotic selection in preparation for transformation of banana embryogenic cell suspensions (ECS).

2.1.2. Plant tissue culture and post-tissue culture methodologies

2.1.2.1. Embryogenic cell suspensions (ECS) and genetic transformation

Embryogenic cell suspensions of banana cultivars 'Sukali Ndizi' and EAHB hybrid M9 were initiated and maintained in liquid MA2 (Appendix 2) medium according to a previously described procedure with some minor modifications (Cote *et al.,* 1996) until required for transformation. Established banana ECS of 'Sukali Ndizi' (Ndizi 256) and hybrid M9 (498) were stably transformed using an Agrobacterium-mediated transformation technique previously described by Khanna *et al.,* (2004).

Four days after sub-culturing in fresh liquid MA2 medium, ECSs were collected, sieved through a 500µm mesh and allowed to settle in a sterile 50mL Falcon tube for 10 min. The cells were resuspended in a sieved cells volume (SCV):MA2 media ratio of 3:1 prior to equal volume distribution into 15mL Falcon tubes and transferred to a rotary shaker at 200 rpm. To the ECS aliquots was added 10mL of pre-induced *Agrobacterium* cell suspension supplemented with 0.02% Pluronic F68 and gently mixed by inversion ten times. Mixtures were centrifuged at 200g for 3 mins in a bench top centrifuge Universal 32R (Hettich) and the same operation (mix and spin) was repeated a second time. After the second spin, cells were gently mixed as previously described and allowed to rest for 30 mins at 27^{o}C. Excess liquid was then removed to a 3:1 ratio, the cells gently mixed and the cells were then aspirated onto 55-mm diameter glass fibre filters sitting on Whatman paper to drain most of the excess liquid. The glass fibre filters with the cells were then transferred to bacterial co-culture media (BCCM) in 60mM Petri dishes for 3 days of co-cultivation at 22^{o}C in the dark.

2.1.2.2. General plant tissue culture

After 3 days of co-cultivation in the dark, inoculated ECSs were washed 3 times with liquid MA2 medium supplemented with 200 µg/mL Timentin and plated on glass filter paper (Khanna *et al.*, 2004). The filter discs were transferred to semi-solid MA3 media (appendix 2) supplemented with 200 µg/mL Timentin and 50 µg/mL kanamycin and kept at 25^{o}C in the dark. The cells on glass filters were sub-cultured on fresh media every 14 days. After 3 months of selection, embryos were transferred to semi-solid RD1 media supplemented with 200 µg/mL Timentin and 100 µg/mL kanamycin for one month to allow embryo development and maturation. Mature embryos were transferred to germination medium MA4 (appendix 2) supplemented with 200 µg/mL Timentin and 100 µg/mL kanamycin. Germinated shoots were transferred to MS media supplemented with 200 µg/mL Timentin and 200 µg/mL kanamycin to enable root formation. Well-rooted plantlets were put on multiplication media and at this stage leaf samples were collected and analysed by PCR for presence of the transgenes. Plants that tested

positive for the respective transgene were further multiplied with monthly subcultures to get 10 clones for every line. For every line generated, one clone was maintained as a mother plant in tissue culture and the rest were rooted.

2.1.2.3. Acclimatisation procedures, hardening and glasshouse conditions

Rooted plants were washed free of growth medium then weaned into plastic cups containing sterilised soil. These plants were placed in humid chamber and kept at a constant temperature of 25^{o}C for a period of 2 weeks. Subsequently, hardened plants were transferred to 200mM diameter pots and kept in the glasshouse at a controlled temperature of 27^{o}C and under natural light conditions for 8 weeks before being transferred to the confined field trial (CFT).

2.1.3. Nucleic acid extraction and purification

2.1.3.1. Plasmid purification

Plasmid DNA was isolated from *E. coli* cultures using a modified alkaline lysis protocol (Sambrook and Russell, 2001). A single bacterial colony from each of the constructs was incubated in 10mL of selective LB medium overnight at 37^{o}C, with shaking. A 1.5mL aliquot of the overnight culture was then centrifuged at 9,000*g* for 2 min. The pellet was suspended in 100µL of solution I using gentle vortexing. Freshly prepared Solution II (200µL) was added and the contents mixed rapidly by inverting the tube. The lysate was incubated on ice for 5 mins before adding 150µL of ice-cold solution III, mixed gently and the tube returned to ice for 10 min. The lysate was then centrifuged at 9,000*g* for 5 mins at 4^{o}C and the supernatant was pipetted into a fresh Eppendorf tube. An equal volume of phenol:chloroform (1:1) solution was added and the Eppendorf was centrifuged at 9,000*g* for 2 mins at room temperature. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the collected aqueous layer in a fresh tube and the tube was centrifuged at 9,000*g* for 2 mins at 4^{o}C. For precipitation of the DNA, the aqueous layer was collected into a new tube to which 2 volumes of ethanol and 0.1 volumes of sodium acetate (3M) was added, mixed and incubated for 2 hrs at -

20°C. The DNA was recovered after centrifugation at 9,000*g* for 10 mins at 4°C and washed with 70% ethanol. The pellet was air dried and re-suspended in 30µL of sterile water and a 5µL aliquot was checked by electrophoresis through a 1% agarose gel after restriction digestion.

2.1.3.2. DNA extraction from banana leaves

Total nucleic acid (TNA) was extracted using a cetyltrimethyl ammonium bromide (CTAB) method (Caffarri *et al.*, 2001). The CTAB extraction buffer was preheated to 65°C for 10 min. Banana leaf tissue (100mg) was crushed using a mortar and a pestle and mixed with 10 volumes of CTAB extraction buffer. The sample paste (750µL) was transferred into a 1.5mL Eppendorf tube and the sample heated at 65°C for 30 min. An equal volume (750µL) of chloroform: isoamylalcohol ($CHCl_3$:IAA, 24:1) was added, vortexed for 1 min and centrifuged at 14,000*g* for 10 min. The top aqueous phase (500µL) was transferred into a new 1.5mL Eppendorf tube. To the aqueous layer, 1 µL of RNAseA (10 mg/mL) was added and incubated for 60 mins at 37°C. The treated samples were mixed with an equal volume of $CHCl_3$:IAA (24:1) vortexed for 1 min and centrifuged at 14,000*g* for 10 min. The top aqueous phase (500µL) was transferred into a new 1.5mL Eppendorf tube. The DNA was precipitated by adding 0.6 volumes of cold (-20°C) isopropanol and incubated at -20°C for 1 hr. The samples were centrifuged at 14,000*g* at 4°C for 10 mins to obtain a pellet. The pellet was washed in 0.5mL of 70% ethanol by vortexing and then centrifuged for 5 mins at 14,000 *g*. Ethanol was removed and the pellet was vacuum dried for 10 min. The dried pellet was suspended in 100µL RNAse free water and stored at -20°C. Extractions were diluted 1:100 fold in RNAse free water before being used in PCR amplifications. The quantity and purity of nucleic acids in solution was measured using a NanoDrop. The purity of DNA was measured from the absorbance ratio A_{260}/A_{280} in the range 1.8-2.0. The DNA concentration was calculated by taking 1 OD_{260} unit equal to 50 µg/mL. All samples were then diluted to a concentration of 10 ng/µL before the PCR assay.

2.1.3.3. Extraction and purification of total RNA from banana pulp

Total RNA was extracted from banana fruit pulp using a cetyltrimethyl ammonium bromide (CTAB) method (Wang and Vodkin, 1994). CTAB extraction buffer (RNA) was preheated to 65^{o}C for 10 min. Frozen tissue (100mg) was crushed in a mortar using a pestle and the powder transferred into a 2mL cryovial tube. Extraction buffer (900µL) was added, vortexed for 1 min and centrifuged at 14,000*g* for 5 min. The top aqueous phase (800µL) was transferred into a new 1.5mL Eppendorf tube and the polysaccharides in the sample were precipitated by the addition of 77µL of 5M potassium acetate (pH 5.2) and 175µL of absolute ethanol. After vortexing for 1 min and centrifugation at 14,000*g* for 5 min, the top layer (700µL) was transferred into a new 2mL Eppendorf tube and extracted with 700µL of $CHCl_3$:IAA (49:1 v/v), vortexed for 1 min and centrifuged at 14,000*g* for 5 min. The $CHCl_3$:IAA extraction was repeated once and the top layer (600µL) was transferred into new 1.5mL tube. RNA was recovered from the supernatant by adding 12M LiCl (233µL) (to a working concentration of 3M LiCl), mixed gently by inversion and precipitated overnight at -20^{o}C. The samples were then centrifuged at 20,000*g* at 4^{o}C for 45 min. RNA pellet was washed with 1mL of chilled 70% ethanol, dried under vacuum for 10 mins and resuspended in 50µL of RNAse free water. Total RNA (1-3 µg) was purified using a RNeasy Plant Mini Kit, according to the manufacturer's instructions. The quantity and purity of nucleic acids in solution was measured using a NanoDrop. The purity of RNA was estimated from the absorbance ratio A_{260}/A_{280} in the range 1.8-2.0. The RNA concentration was calculated by taking 1 OD_{260} unit equal to 40 µg/mL.

2.1.4. Nucleic acid amplification and manipulation

2.1.4.1 Polymerase chain reaction (PCR)

PCR were prepared in 20µL reactions using GoTaq® green master mix according the manufacturer's instructions. Each reaction contained 1X GoTaq® green, 10ρmol of each forward and reverse primer, RNAse free water and 200ng of DNA template. PCRs were carried out in a PTC-200 DNA Engine thermal cycler (Bio-Rad), using the following cycling

conditions: denaturation at 94°C for 2 mins prior to 35 cycles at 94°C for 30 s, 50ºC (depending on primer set) for 30 s and 72°C for 1 min/kbp. A final extension step of 72°C for 5 mins was included.

2.1.4.2. Agarose gel electrophoresis

DNA samples and PCRs were resolved by electrophoresis through 1% (w/v) agarose gels prepared in TAE buffer and stained with 2.5µL of ethidium bromide (10 mg/mL). DNA size marker (0.7 µg) was included on each gel for comparison, electrophoresis was run at 80 V for 40 mins after which results were observed under UV light.

2.1.4.3 First strand cDNA synthesis

SuperScriptTM III first strand cDNA synthesis kit (Invitogen) was used to synthesize cDNA according to the manufacturer's instructions. A RNA/primer Oligo(dT) mix was made in 20µL reactions to which was added a volume containing 3µg of total genomic RNA, 1µL of 50µM Oligo(dT) primer, 1µL of 10mM dNTP mix and up to 10µL DEPC-treated water. After mixing and centrifugation at 9,000*g* for 10 sec, the reactions were incubated at 65°C for 5 min. The mixture was then immediately placed on ice for at 1 min. Separately, a cDNA synthesis mix was prepared by adding 2µL of 10X Reverse Transcriptase (RT) buffer, 4µL of 25mM $MgCl_2$, 2µL of 0.1M DTT, 1µL of RNaseOUT (40U/µL) and 1µL of SuperScriptTM III RT (200U/µL). To each RNA/primer Oligo(dT) mix, 10µL of cDNA synthesis mix was added, gently mixed, collected by brief centrifugation at 9,000*g* for 10 sec and incubated at 50°C for 50 min. The reaction was terminated at 85°C for 5 min, immediately chilled on ice and collected by centrifugation at 9,000*g* for 10 sec. To each tube, RNase H (1µL) was added and incubated at 37°C for 20 min. The cDNA synthesis reaction mix was stored at -20°C for qPCR quantification. The quality of cDNA was checked using 1µL of the cDNA in a PCR with banana *Actin* specific primers (5'-ACCCTGTATTGCTCACCGAAG-3' and 5'-GCATAAAGGGAAAGAACAGC-3') designed from Musa actin (Genebank accession AF285176) to give a 160 bp amplicon. PCR amplification conditions were 3 mins at 95°C to denature the DNA followed by 35 cycles

of 30 sec at 95^{o}C, 1 min at 55^{o}C and 1 min at 72^{o}C. This was followed by a final extension step of 10 mins at 72^{o}C. cDNA concentration in the RT mix was quantified using a Nanodrop.

2.1.4.4 Quantitative real-time PCR (qRT-PCR)

First strand cDNA (10ng) was used as template for all RT-qPCR assays, using Ubiquitin (Ubi) and Ribosomal protein S (RS4) genes as reference genes (RG), and specific primers for the maize phytoene synthase 1 B73 (*Psy1B73*) gene and the 'Asupina' phytoene synthase 2a (*Apsy2a*) gene. The transcripts were quantified in duplicates on StepOne^{TM} Real Time PCR System (Applied Biosystem®) using SYBR-Green 1 chemistry following the manufacturer's instruction. A total reaction volume of 20µL was used, comprising of 50ng of cDNA, 10µL of SYBR Green master mix and 0.5µM of each primer. Cycling conditions used were, an initial DNA denaturing step for 10 mins at 95^{o}C, followed by 40 cycles of 15 sec at 95^{o}C, 1 min at 60^{o}C and 20 sec at 72^{o}C. Melting curves of the PCR products were acquired by an extra cycle of 15 sec at 95^{o}C followed by 1 min at 55^{o}C. Samples included: 1) transgenic plants, for treated experiments, 2) control or non-transformed plants, 3) No-template control and 4) no-amplification control. Gene expression profile was calculated from the obtained quantification cycle (C_T) values obtained (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Data for gene expression profile from untreated (UT), baseline experiments were treated as a normalised individual data point using the equation:

$$2^{-\Delta CT} = 2^{-[CT\ UT - CT\ RG]} \quad \text{.....(i)}$$

Relative gene expression profile from treated (TD) transgenic experiments was normalised against a reference point (RP) sample using the equation:

$$2^{-\Delta\Delta CT} = 2^{-[(CT\ TD - CT\ RG\text{-}TD) - (CT\ RP - CT\ RG\text{-}RP)]} \quad \text{..... (ii)}$$

2.1.5. Pro-vitamin A analysis

2.1.5.1 Samples preparation

Fruit samples were selected from the top, the middle and the bottom of the bunch. The selected fingers were peeled, the pulp diagonally cut into two sections and further sliced into smaller cross sections and mixed thoroughly. From the mixed composite sample, sub-samples were taken and used fresh, ripe or processed according to a particular experiment. All fruit samples were freeze-dried under vacuum and manually crushed in a mortar and stored at -80^{o}C before pro-vitamin A analysis.

2.1.5.2 Carotenoids extractions from banana fruit pulp

The carotenoids extraction procedure from freeze-dried banana fruits for quantification by HPLC was derived from (Rodriguez-Amaya and Kimura, 2004) with some modification. In brief, from each freeze-dried fruit sample, 200mg of powder was weighed into 2mL vial to which 2mL of acetone was added together with 100µL of α-tocopherol acetate internal standard from a working stock concentration of 1 mg/mL made in acetone. The mixture was thoroughly mixed by vortexing before centrifugation at 4,000*g* for 5 mins to separate the solution from the solid residues. The supernatant (2mL) was collected into a fresh 15mL Falcon tube. Acetone extraction was repeated twice and the 3 x 2mL fractions pooled. To separate the organic phase from the aqueous phase, 2mL of petroleum ether:diethyl ether PE:DE (2:1 v/v) was added to the pooled fractions and vortexed thoroughly. To this, a solution of 1% NaCl was added up to a 14mL mark and mixed well before centrifugation at 4,000*g* for 10 min. The upper organic phase was transferred to 2 x 1.5mL Eppendorf tubes. Extraction of the organic phase was repeated with 1mL of PE:DE. To monitor the recovery of the carotenoid extraction process, 100µL α-tocopherol acetate (1 mg/mL) internal standard was aliquoted into 4 separate 1.5mL Eppendorf tubes. The organic phase was dried by evaporation in a vacuum desiccator in the dark. The dried organic phase was re-dissolved in 200µL of solvent C [methanol:TBME (1:1, v/v)] at room temperature and aliquoted into a 1.5mL HPLC vial for HPLC analysis.

2.1.5.3 Analytical methods for carotenoid quantification

2.1.5.3.1. β-carotene calibration curve

A β-carotene stock solution was first made by dissolving 3mg of β-carotene standard (SIGMA) into 1mL of HPLC grade chloroform. A β-carotene working solution was then prepared by dissolving 15µL of the β-carotene stock solution into 10mL acetone in a 10mL volumetric flask. A range of standard samples were made with increasing amount of β-carotene by aliquoting into 1.5mL tubes 25, 50, 100, 200, 400, 600, 800 and 1000µL of the working solution. The aliquots were dried under vacuum and resuspended in 200µL of solvent C [methanol:TBME (1:1, v/v)] and a 10µL aliquot was injected into the HPLC system (Agilent 1200, Agilent Technologies, Australia) equipped with a 5µm C30 reversed-phase column (Polymeric 5µm C30 stationary phase, 4.6mm (i.d.) x 250mm, PrincetonSPHER C30 200A 5U: 250 x 4.6mm: Part No.: 250046-07574) using a gradient system for analysis. In addition, concentrations of the working solution were also determined using the Lambert-Beer equation (Equation iii) and the OD at 450nm (Appendix 1).

$c = E/\varepsilon$ ……(iii)[1]

2.1.5.3.2. Analysis of pro-vitamin A carotenoids in banana fruit pulp using HPLC

All extracts were analysed in duplicate (technical replicates) using the HPLC system described above. Aliquots of 10µL of the sample were auto-injected into the HPLC column and all signals recorded 450 and 250nm for carotenoid isomers and α-tocopherol acetate respectively on a UV detector (Rodriguez-Amaya and Kimura, 2004). Separation was performed at a column temperature of 24^{o}C using a linear gradient from 100% to 57% of solvent C within 32 mins at a flow rate of 1 mL/min. The signal for all carotenoids was monitored from a multiwave detector at 450nm (being the λ_{max} absorbance for β-carotene in solvent C) while the signal for α-tocopherol was monitored at 285 nm. Integration and quantification of non-pro-vitamin A (PVA) carotenoids such as lutein and PVA carotenoids such as β-carotene and α-carotene were derived from the

[1] Lambert-Beer equation whereby; E: extinction ε: molar extinction coefficient, c: concentration, d: distance =1

area under the peak and calculated against the β-carotene standard curve equation using Agilent ChemStation Rev. B.04.01 [481] software (Copyright © Agilent Technologies 2001-2008). The amounts of total carotenoids (TC), PVA carotenoids and β-carotene equivalent (β-CE) content were obtained from analyses of lutein, all trans-β-carotene (TBC), α-carotene (AC) and cis-β-carotene (CBC) using the equations:

PVA = TBC + AC + CBC……(iv)

TC = Lutein + PVA ……(v)

β-CE = TBC + ½ (AC + CBC) ……(vi)

Percentage recovery of carotenoids was monitored using a correction factor generated from recovery of a known amount of α-tocopherol acetate pre-introduced in each sample as an internal standard prior to extraction.

The percent micronutrient retention was calculated based on dry weight of the processed and unprocessed fruit (Rodriguez-Amaya and Kimura, 2004), where;

$$\% \text{ Retention} = \frac{\text{Carotenoid content per g of processed fruit (dry basis)}}{\text{Carotenoid content per g of raw fruit (dry basis)}} \times 100 \quad \text{(vii)}$$

2.1.6. Statistical analysis

The data sets were organised using Excel (Microsoft) and analysed using one-way analysis of variance (ANOVA) followed by LSD Post Hoc test with statistical significance reported at a level of $p<0.05$ using the software GenStat V. 12 Statistical (VSN International Ltd).

Chapter 3 - Profile and stability of pro-vitamin A carotenoids in East African Highland Banana, 'Apple' and 'Plantain' cultivars

3.1. Introduction

Vitamin A deficiency (VAD) is a major public health problem in the developing world affecting over 190 million children under the age of five years, particularly in Africa and South East Asia (WHO, 2009). VAD leads to serious chronic diseases such as night blindness, keratomalacia and bronchopulmonary dysplasia (Strobel *et al.*, 2007; Ahmed *et al.*, 2012). Humans cannot synthesize vitamin A *de novo*, so they rely on their diet for an adequate supply which can be from animal origin in the form of retinol and/or plant origin in the form of pro-vitamin A (PVA) carotenoids (Fitzpatrick *et al.*, 2012).

Bananas are an economically important crop worldwide. It is also an important cash and staple food crop providing starch and potassium in the diets of many people living in tropical and sub-tropical regions. In the Great Lakes region of East Africa, cooking bananas are a staple food crop and, as such, they form an integral part of the diet for most rural and urban families. However, most agronomically important cultivars contain low levels of critical micronutrients such as iron and PVA (Hardisson *et al.*, 2001; Ekesa *et al.*, 2012), which are essential dietary requirements, especially for children as well as pregnant and breast feeding women (Ahmed *et al.*, 2012; Fitzpatrick *et al.*, 2012). Therefore, it is not surprising that a national demographic survey carried out in Uganda in 2006 showed that populations in most regions where banana are consumed as the primary source of carbohydrate are deficient in critical micronutrients such as iron and vitamin A (UBOS, 2006).

Although the physical, chemical and biological properties and functions of carotenoids have been widely documented, there is considerably less information available on the

carotenoid content of some tropical fruits, both at the crop and cultivar level. In banana, several studies have shown that carotenoid content can vary not only between cultivars and but also in fruits from similar cultivars harvested from different geographical locations (Englberger *et al.*, 2003; Englberger *et al.*, 2003; Englberger *et al.*, 2007; Davey *et al.*, 2009). For example, 'Cavendish' bananas grown in Hawaii have been reported to contain an average of 55.7 μg β-carotene and 84.0 μg α-carotene per 100g of fresh tissue (Wall, 2006) while 'Cavendish' bananas growing in Australia are reported to contain 50-64 μg β-carotene and 93-123 μg α-carotene per 100g of fresh tissue (Englberger *et al.*, 2007). Since PVA carotenoids are light and heat sensitive, it is possible that some of the variation in reported PVA levels might be due to the processing procedures used in the studies. Such procedures may also affect the nutritional composition of the final food product (Bresnahan *et al.*, 2012; Ekesa *et al.*, 2012).

The objective of the research described in this chapter was to (i) determine the carotenoid content in fruit from representative banana cultivars commonly grown in the different geographical regions of Uganda and (ii) to assess the effect of different processing methods on the carotenoid content in the fruit pulp.

3.2. Materials and Methods

3.2.1. Methods

3.2.1.1. Sampling sites

Fruit samples were collected from different locations within selected banana growing areas in the central, western and eastern regions of Uganda (Figure 3.1). In each region, samples were collected at low (1000-1299 m), middle (1300-1599 m) and high (1600-2100 m) altitudes.

3.2.1.2. Banana cultivars and sampling procedure

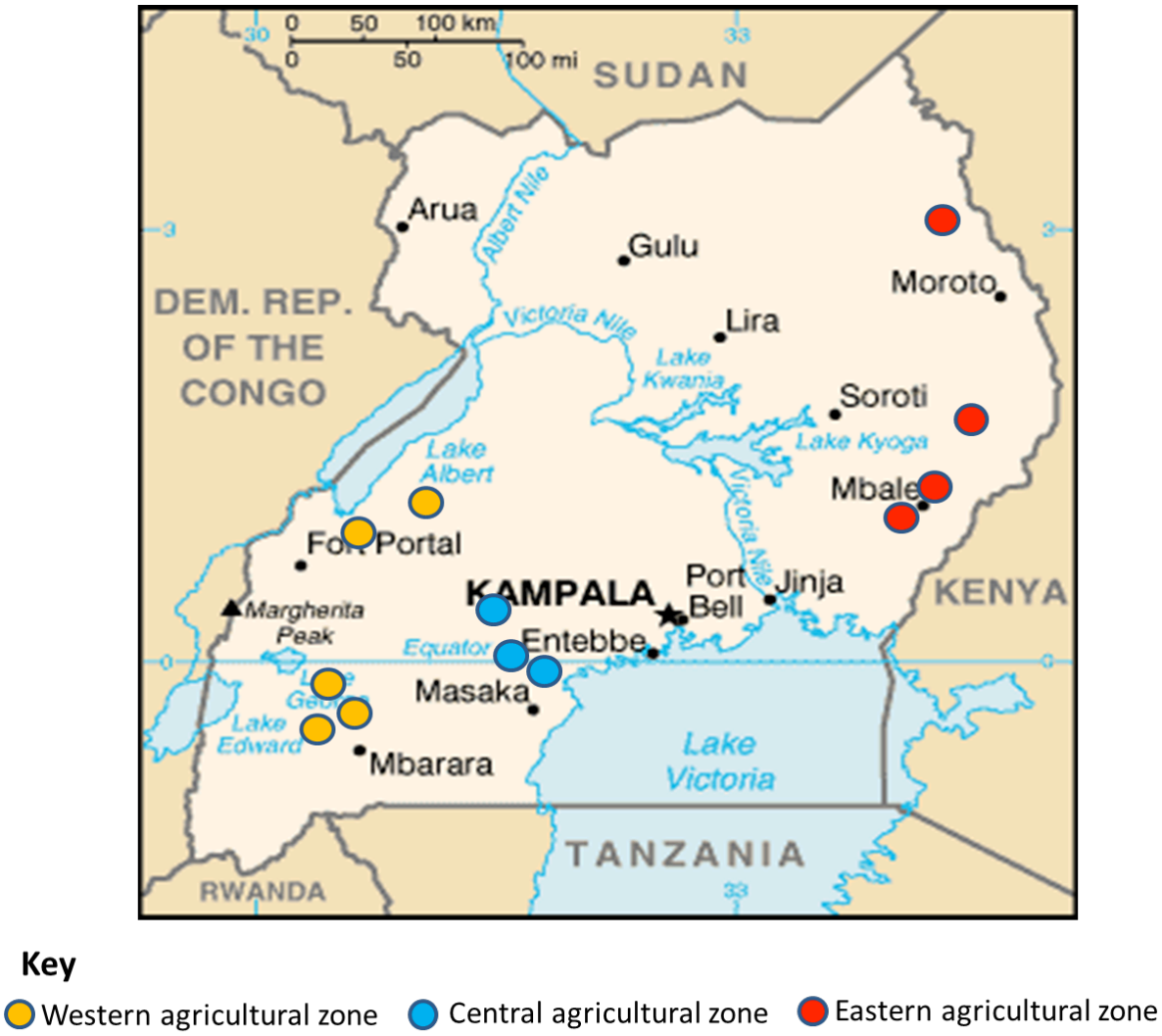

Figure 3.1. Location of the banana sampling sites in the central, western and eastern regions of Uganda.

Cultivars included in this study were representative of farmer preferred and popular varieties. Fruits from eight banana cultivars including a plantain cultivar (*Musa* spp. AAB group - 'Gonja Nakatansese'), two dessert cultivars (*Musa* spp. AAB group - 'Bogoya' and 'Sukali Ndizi'), four cooking-type East African Highland Banana (EAHB - *Musa* spp. AAA-EA - 'Mbwazirume', 'Mpologoma', 'Nakitembe' and 'Nakinyika') and one EAHB hybrid (*Musa* spp. AAA-EA - M9), were used in this study. A total of 222 banana bunches from the three different regions, west, east and central of the country (Figure 3.1, Table 3.1) were harvested for analysis. Harvested fruits were stored at room temperature in a well-aerated room.

For all cultivars, a composite sample was taken which consisted of three fingers (one fruit from the top, one from the middle and one from the bottom of each bunch). For EAHB cultivars, a composite sample was taken at three different storage times, namely Day 1 (full green), Day 7 and Day 14 after harvest. This timeframe represents, on average, the fruit storage time before consumption as adopted for on-farm consumption and trading. For dessert and plantain cultivars, a composite sample was only taken at full green (Day 1 after harvest) and full ripe (Soltani *et al.*, 2010). The fruit samples were processed according to locally adopted banana-processing techniques, boiling and steaming for 15 mins and 1 hr, respectively, and stored as described in Chapter 2.4.1.2.

3.2.1.3. Banana sample processing and preparation

Samples for analysis were prepared as follows. The selected fingers were peeled, the pulp diagonally cut into two sections and further sliced into smaller cross sections and mixed thoroughly. Samples derived from all eight cultivars at the different sampling stages were left unprocessed prior to carotenoid content analysis. However, Day 1 (full green) samples of the EAHB cultivars and the plantain cultivar were also processed by two different cooking methods (boiling and steaming) before being analysed for carotenoid content. As such, pre-weighed sub-samples were taken from the mixed

Table 3.1. Banana samples collected per cultivar

Banana (*Musa* spp.) cultivars		Sample location	Number of samples (Bunches) per region	Total number of samples (Bunches)
Dessert cvs	'Sukali Ndizi' (SN)	East-high	8	30
		East-low	9	
		West-middle	8	
		Central-low	5	
	'Bogoya' (BO)	East-high	12	30
		East-low	5	
		West-middle	7	
		West-low	1	
		Central-low	5	
EAHB cvs	'Mbwazirume' (Mbz)	East-high	15	28
		West-high	2	
		West-middle	6	
		Central-low	5	
	'Mpologoma' (Mpo)	East-high	2	30
		East-low	6	
		West-high	4	
		West-middle	10	
		Central-low	8	
	'Nakinyika' (Nak)	West-middle	7	29
		Central-middle	4	
		Central-low	18	
	'Nakitembe' (Nakt)	East-high	10	32
		West-middle	7	
		Central-middle	3	
		Central-low	12	
EAHB hybrid	M9	East-high	2	26
		East-low	4	
		West-high	1	
		West-middle	7	
		West-low	1	
		Central-middle	3	
		Central-low	8	
Plantain	'Gonja Nakatansese' (GN)	West-high	2	17
		West-middle	14	
		Central-middle	1	
Total number of samples				**222**

composite full-green sample derived from the EAHB and plantain cultivars and subjected to steaming and boiling. For each sub-sample, fresh weight was recorded before and after processing. For steaming, fruits were wrapped in banana leaves, tied with banana fibre and steamed on a charcoal stove in a saucepan for one hour. For boiling, fruits were directly placed in 400mL of boiling water for 15 mins. After processing, all fruit samples were lyophilised (freeze dried) and manually crushed in a mortar before being stored at -80^{o}C.

3.2.1.4. Carotenoid quantification and data analysis

Carotenoid content was quantified as described in Chapter 2.4. The obtained data was grouped in different data processing categories, which included: full green, cultivar, processing type, storage, agro-ecological zones and altitude. The different data sets were individually analysed using statistical tools described in Chapter 2.1.6.

3.3. Results

3.3.1. Carotenoid content in unprocessed Ugandan banana cultivars at the full-green stage

Samples taken from the 222 full-green banana bunches from the 8 cultivars were analysed by HPLC to determine the amounts of lutein, trans α-carotene and trans β-carotene and to subsequently calculate the β-carotene equivalent (β-CE) and total carotenoid content (Table 3.2). Results provide the mean values and variations for all analyses carried out across cultivars. The total carotenoid and β-carotene equivalent levels ranged from 6.3±1.4 to 37.0±10.7 µg/g DW and 2.3±1.0 to 30.7±8.9 µg/g DW, respectively, across all cultivars analysed. Fruit from the plantain 'Gonja Nakatansese' (GN) had β-CE levels (30.7 µg/g DW) higher than other cultivars and was followed by 'Nakinyika' (Nak), 'Nakitembe' (Nakt), 'Mpologoma' (Mpo), 'Mbwazirume' (Mbz), 'Bogoya' (BO), M9 and 'Sukali Ndizi' (SN) (Table 3.2). No significant difference was found in the levels of β-CE present in the four EAHB cultivars tested. Interestingly, the level of β-CE in the EAHB hybrid M9 was closer to that found in the SN dessert banana while BO, the other dessert banana, had amounts closer to the EAHB cultivars Mbz and Mpo

Table 3.2. HPLC analysis - Carotenoid concentrations in the fruit pulp of unprocessed full green bananas

Cultivar	Total Carotenoids	Carotenoids (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
SN	6.3±1.4^{a}	3.2±1.6^{cde}	1.6±0.8^{a}	1.5±0.7^{a}	-	2.3±1.0^{a}
BO	14.4±6.1^{bcd}	3.4±1.5^{de}	7.3±3.7^{bcd}	3.7±1.4^{bc}	-	7.3±3.2^{bc}
M9	9.6±5.5^{b}	2.7±1.2^{bcd}	4.7±3.3^{b}	2.2±1.4^{ab}	0.1±0.1^{a}	4.6±3.0^{b}
Mbz	13.9±6.6^{bc}	2.1±0.7^{b}	6.9±3.4^{bc}	4.9±2.9^{cd}	-	8.3±4.5^{cd}
Mpo	16.8±10.0^{cde}	2.4±0.5^{bc}	9.0±6.0^{cde}	5.4±4.1^{d}	-	9.9±6.9^{cd}
Nak	19.9±8.8^{cde}	4.0±1.6^{e}	10.0±5.0^{cde}	5.8±2.8^{d}	0.1±0.1^{a}	10.8±5.2^{d}
Nakt	17.1±9.8^{cde}	2.4±0.9^{bc}	8.9±5.6^{cde}	5.7±3.9^{d}	0.1±0.1^{a}	10.2±6.6^{d}
GN	37.0±10.7^{f}	0.8±0.5^{a}	10.8±3.4^{de}	25.2±7.3^{e}	0.2±0.2^{a}	30.7±8.9^{e}

Values are means ± SD. SN - 'Sukali Ndizi' (n= 30), M9 - EAHB hybrid (n= 26), BO - 'Bogoya' (n= 30), Mbz - 'Mbwazirume' (n= 28), Mpo - 'Mpologoma' (n= 30), Nak - 'Nakinyika' (n= 29), Nakt - 'Nakitembe' (n= 32) and GN - 'Gonja Nakatansese' (n= 17). DW - dry weight. One-way ANOVA. Values with the same letter are not significantly different at 5% LSD.

The concentration of both α-carotene and β-carotene was found to be the highest in the plantain GN. As shown in Figure 3.2, the carotenoid composition varied considerably between the dessert banana (SN), the plantain (GN) and all of the EAHB. The major carotenoid constituent in SN was found to be lutein with only around 25% comprising α- and β-carotene. In contrast, the main carotenoid constituent in all EAHB cultivars was α-carotene, followed by β-carotene and lutein. In the plantain (GN), β-carotene was the major constituent of the carotenoids (around 70%) following by α-carotene and only small amounts of lutein (around 2%).

3.3.2. Effect of boiling and steaming on the carotenoid content of full-green banana samples

Since EAHB and plantains are generally boiled or steamed prior to consumption in Uganda, a study was done to determine the carotenoid retention values in fruit samples that were subjected to these processes.

The β-CE retention levels after processing ranged from 55.4 to 93.9% for boiling and 57.3 to 79.6% for steaming (Table 3.3). Although boiling and steaming fruit both resulted in a loss in carotenoids (β-CE), these losses were mainly evident in steamed fruit from the EAHB cultivars Mbz and Mpo, and plantain GN. With both boiling and steaming, a significant reduction ($p<0.001$) in β-CE was only seen with fruit from the plantain GN showing poor retention capacity. Further, with the exception of Nak and GN, the amount of the non-PVA carotenoid lutein detected in all cultivars was found to be higher after boiling while no significant differences were observed after steaming. In contrast, the amount of α- and β-carotene detected in all cultivars tested was found to be lower after boiling and steaming. These reductions were high after steaming in cultivars Mbz, Mpo and Nakt for α-carotene and Mbz and Nak for β-carotene. In the case of plantain cultivar GN, significant reductions ($p<0.001$) in both α- and β-carotene were seen after steaming and boiling.

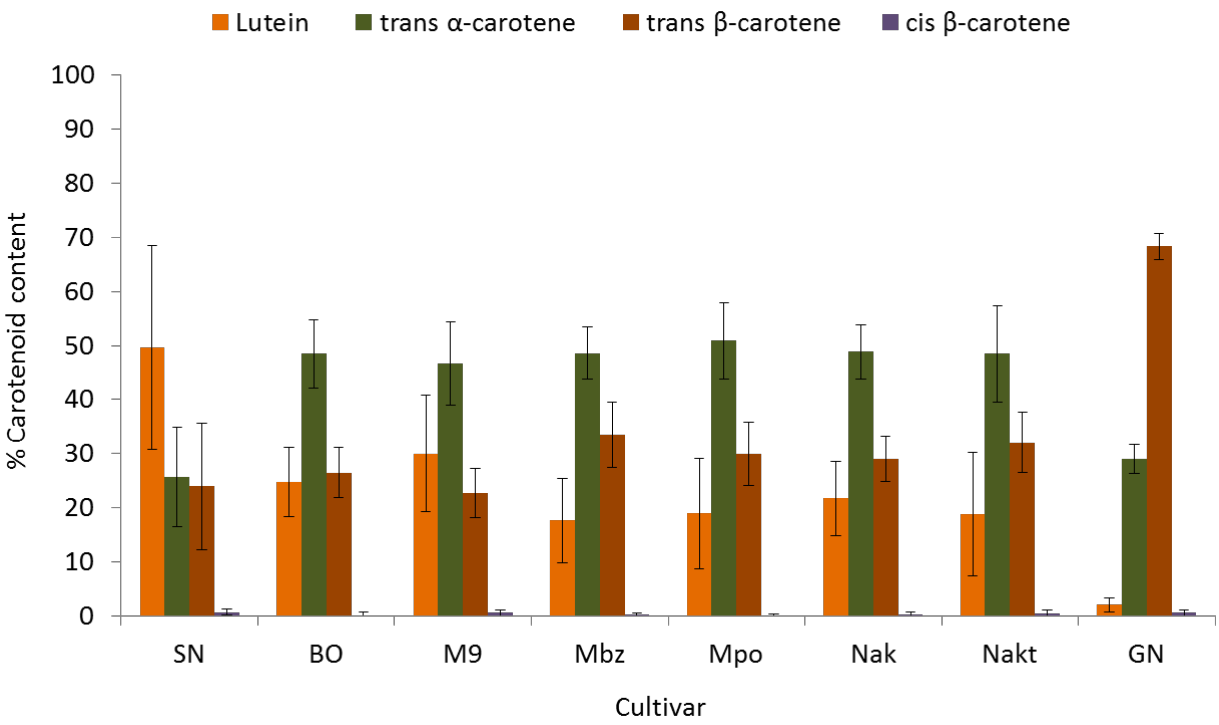

Figure 3.2. Percentage accumulation of individual carotenoids in the flesh of full green banana from popular cultivars in Uganda. Values are means ± SD. Percentage (%) carotenoid content calculated based on total carotenoid content in the fruit pulp. SN - 'Sukali Ndizi' (n= 30), M9 - EAHB hybrid (n= 26), BO - 'Bogoya' (n= 30), Mbz - 'Mbwazirume' (n= 28), Mpo - 'Mpologoma' (n= 30), Nak - 'Nakinyika' (n= 29), Nakt - 'Nakitembe' (n= 32) and GN - 'Gonja Nakatansese' (n= 17).

Table 3.3. HPLC analysis - Carotenoid concentrations in banana fruit pulp processed by steaming or boiling

Cultivar	Sample	Carotenoids (µg/g DW)					β-CE retention values (%)
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE	
M9	FG	2.7±1.2[a]	4.7±3.3[a]	2.2±1.4[a]	0.1±0.1[a]	4.6±3.0[a]	-
	BOILED	3.7±0.9[b]	3.7±1.7[a]	1.7±0.7[a]	0.1±0.1[a]	3.6±1.6[a]	78.3
	STEAMED	2.7±0.5[a]	3.5±1.4[a]	1.7±0.6[a]	0.2±0.1[a]	3.5±1.3[a]	76.1
Mbz	FG	2.1±0.7[a]	6.9±3.4[a]	4.9±2.9[b]	-	8.3±4.5[b]	-
	BOILED	2.6±0.4[a]	5.6±2.1[a]	3.9±1.9[ab]	0.2±0.1[a]	6.8±2.9[ab]	79.8
	STEAMED	2.2±0.4[a]	4.8±1.6[a]	3.3±1.4[a]	0.4±0.2[b]	5.9±2.2[a]	67.9
Mpo	FG	2.4±0.5[a]	9.0±6.0[a]	5.4±4.1[a]	-	9.9±6.9[a]	-
	BOILED	4.0±0.9[b]	8.4±4.2[a]	5.1±2.3[a]	0.1±0.1[a]	9.3±4.3[a]	93.9
	STEAMED	2.6±0.6[a]	5.9±2.7[a]	3.8±1.8[a]	0.5±0.3[b]	7.0±3.2[a]	68.7
Nak	FG	4.0±1.6[a]	10.0±5.0[a]	5.8±2.8[a]	0.1±0.1[a]	10.8±5.2[a]	-
	BOILED	4.0±1.0[a]	7.9±3.1[a]	4.7±1.7[a]	0.3±0.2[b]	8.8±3.2[a]	81.5
	STEAM	3.4±1.0[a]	7.9±3.5[a]	4.4±1.7[a]	0.4±0.2[b]	8.6±3.4[a]	79.6
Nakt	FG	2.4±0.9[a]	8.9±5.6[a]	5.7±3.9[a]	0.1±0.1[a]	10.2±6.6[a]	-
	BOILED	3.5±1.0[b]	7.7±3.9[a]	5.2±2.8[a]	0.2±0.1[ab]	9.2±4.7[a]	90.2
	STEAMED	2.0±0.9[a]	6.2±3.0[a]	4.4±2.5[a]	0.3±0.2[b]	7.7±3.9[a]	75.5
GN	FG	0.7±0.5[a]	10.8±3.4[b]	25.2±7.3[b]	0.2±0.2[a]	30.7±8.9[b]	-
	BOILED	0.6±0.3[a]	4.8±1.9[a]	14.4±3.6[a]	0.4±0.2[a]	17.0±4.3[a]	55.4
	STEAMED	0.5±0.5[a]	5.1±2.6[a]	14.5±4.8[a]	1.1±0.7[b]	17.6±5.5[a]	57.3

Values are means ± SD. M9 - EAHB hybrid (n= 26), Mbz - 'Mbwazirume' (n= 28), Mpo - 'Mpologoma' (n= 30), Nak - 'Nakinyika' (n= 29), Nakt - 'Nakitembe' (n= 32) and GN - 'Gonja Nakatansese' (n= 17). FG, full green mature fruit and DW, dry weight. One-way ANOVA. Values with the same letter are not significantly different at 5% LSD.

3.3.3. Carotenoid content in unprocessed Ugandan banana cultivars during storage/ripening

Storing and/or ripening can have an impact on the accumulation levels of carotenoids in the banana fruit. Therefore, the carotenoid concentration in the fruit of the eight cultivars at various ripening stages was assessed.

For the EAHB cultivars tested, a consistent increase in β-CE was observed during storage from FG to D14 with of 1.88 and 2.80-fold increment observed in cultivars Mpo and Nakt, respectively (Table 3.4). Further, fruit from Nakt showed a consistent increase in the concentration of all three carotenoids tested (lutein, trans and cis α-carotene and trans β-carotene) during storage.

Analysis of fruit from the dessert banana SN and the plantain GN, revealed a decrease in β-CE during ripening from FG to FR (Table 3.5). The levels of all three carotenoids tested were found to decrease significantly ($p<0.001$) in SN during ripening. However, ripening significantly ($p<0.001$) increased the levels of the non-PVA carotenoid lutein in both dessert bananas, SN and BO, as well as in the plantain GN.

3.3.3. Carotenoid content in banana fruit from different agricultural zones in Uganda

Bananas and plantains are grown throughout Uganda but are more prominent in the east, central and western districts where banana consumption is the highest. To determine whether geographical location and/or altitude affected the accumulation of carotenoids, the carotenoid composition of full-green fruit collected from popular dessert, EAHB and plantain cultivars growing in three different agricultural zones and from low, middle and high altitudes, was analysed.

Table 3.4. HPLC analysis - Changes in carotenoid concentration in EAHB fruit pulp during storage

Cultivar	Sample	Carotenoids (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
M9	FG	2.7±1.4^{b}	4.0±1.0^{a}	2.1±0.6^{a}	0.1±0.1^{a}	4.1±1.0^{a}
	D7	2.1±0.1^{a}	4.6±0.2^{a}	2.1±0.3^{a}	0.8±0.1^{b}	5.1±0.4^{a}
	D14	2.9±1.2^{b}	6.9±1.7^{b}	2.9±0.6^{b}	0.9±0.2^{b}	7.2±1.6^{b}
Mbz	FG	2.0±0.7^{a}	6.5±1.3^{ab}	4.4±0.7^{a}	-	7.7±1.2^{a}
	D7	2.0±0.3^{a}	5.6±1.0^{a}	4.4±0.5^{a}	2.6±0.7^{a}	9.8±1.3^{b}
	D14	2.1±0.2^{a}	7.7±0.6^{b}	5.7±1.6^{b}	3.5±1.4^{b}	13.1±3.2^{c}
Mpo	FG	2.8±0.8^{b}	9.5±1.9^{a}	5.4±0.9^{a}	-	10.2±1.6^{a}
	D7	3.2±0.2^{b}	11.2±0.8^{a}	6.3±0.7^{a}	1.5±0.2^{a}	13.5±1.1^{b}
	D14	2.4±0.1^{a}	15.8±1.5^{b}	9.7±2.3^{b}	1.6±0.4^{a}	19.2±2.3^{c}
Nak	FG	3.3±1.8^{a}	9.7±1.0^{b}	5.7±0.5^{b}	0.1±0.1^{a}	10.6±0.5^{b}
	D7	4.2±0.1^{a}	8.0±0.2^{a}	3.9±0.1^{a}	1.3±0.0^{b}	9.2±0.2^{a}
	D14	3.9±1.5^{a}	11.5±2.4^{c}	5.8±0.3^{b}	1.8±0.2^{c}	13.3±1.1^{c}
Nakt	FG	2.8±0.7^{a}	8.7±3.8^{a}	4.5±1.8^{a}	0.1±0.1^{a}	8.9±3.5^{a}
	D7	3.5±0.2^{ab}	12.4±0.2^{ab}	7.5±0.3^{b}	1.7±0.1^{b}	15.4±0.5^{b}
	D14	4.2±0.8^{b}	18.5±0.9^{b}	13.2±0.7^{c}	2.4±0.1^{c}	24.9±0.4^{c}

Values are means ± SD. M9 - EAHB hybrid (n= 4), Mbz - 'Mbwazirume' (n= 4), Mpo - 'Mpologoma' (n= 4), Nak - 'Nakinyika' (n= 4), and Nakt - 'Nakitembe' (n= 4), and GN - 'Gonja Nakatansese'. FG, full green mature fruit; D7, 7 days of storage and D14, 14 days of storage. DW, dry weight. One-way ANOVA. Values with the same letter are not significantly different at 5% LSD.

Table 3.5. HPLC analysis - Changes in carotenoid concentration in dessert and plantain banana fruit pulp during ripening

Cultivars	Sample	Carotenoids (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
SN	FG	3.2±1.6[a]	1.6±0.8[b]	1.5±0.7[b]	-	2.3±1.0[b]
	FR	7.3±1.3[b]	0.6±0.4[a]	0.5±0.1[a]	-	0.8±0.3[a]
BO	FG	3.4±1.5[a]	7.3±3.7[a]	3.7±1.4[a]	-	7.3±3.2[a]
	FR	4.7±1.6[a]	8.4±3.1[a]	4.5±1.1[b]	0.1±0.2	8.7±2.5[a]
GN	FG	0.7±0.5[a]	10.8±3.4[a]	25.2±7.3[b]	0.2±0.2[a]	30.6±8.9[b]
	FR	2.7±0.9[b]	8.8±3.5[a]	17.5±7.8[a]	0.3±0.2[a]	21.9±9.5[a]

Values are means ± SD. SN - 'Sukali Ndizi' (n= 30), BO - 'Bogoya' (n= 30), and GN - 'Gonja Nakatansese' (n= 17). FG, full green mature fruit and FR, full ripe fruit. DW, dry weight. One-way ANOVA. Values for the analysed variable with the same letter are not significantly different at 5% LSD.

HPLC analysis of the carotenoid content in fruit samples collected from all three different agricultural zones revealed a significant variation in β-CE concentration in cultivars SN ($p<0.05$), M9 ($p<0.05$) and Mbz ($p<0.001$) (Table 3.6). The greatest variation in β-CE levels was observed in cultivar Mbz, and this was largely due to the significant difference between the β-carotene ($p<0.001$) contents in fruits obtained from the three zones, followed by α-carotene ($p<0.05$).

No significant differences were seen in β-CE levels in the banana fruit obtained from same cultivars at different altitudes in these agricultural zones except for M9 and GN at high and middle altitudes (Table 3.7).

Table 3.6. HPLC analysis - Carotenoid concentration in fruit from full-green bananas from different agricultural zones

Cultivar	Zone	Carotenoids (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
SN	Central	4.6±1.5[b]	1.3±0.9[a]	0.8±0.1[a]	-	1.5±0.5[a]
	East	2.3±0.7[a]	1.9±0.7[a]	1.8±0.6[b]	-	2.7±0.8[b]
	West	4.2±1.8[b]	1.4±0.9[a]	1.1±0.7[b]	-	1.8±1.2[ab]
M9	Central	3.0±1.2[a]	6.0±3.2[b]	2.7±1.5[b]	0.1±0.1[a]	5.7±3.2[b]
	East	2.2±1.1[a]	1.9±0.9[a]	1.0±0.5[a]	-	1.9±1.0[a]
	West	2.6±1.4[a]	5.0±3.3[b]	2.4±1.2[b]	0.1±0.0[a]	4.9±2.8[b]
BO	Central	3.5±1.9[a]	7.6±4.4[a]	3.5±1.7[a]	-	7.3±3.9[a]
	East	3.0±1.1[a]	7.2±4.2[a]	3.6±1.4[a]	-	7.1±3.5[a]
	West	4.1±1.7[a]	7.3±2.9[a]	4.1±1.2[a]	0.1±0.2	7.8±2.5[a]
Mbz	Central	2.2±0.6[a]	8.2±2.2[b]	5.1±1.5[b]	0.1±0.1	9.2±2.5[b]
	East	1.9±0.7[a]	5.2±2.8[a]	3.3±1.7[a]	-	5.9±3.1[a]
	West	2.5±0.6[a]	9.2±3.6[b]	7.7±3.1[b]	-	12.3±4.8[b]
Mpo	Central	2.1±0.6[a]	10.2±7.7[a]	6.2±6.1[a]	-	11.3±9.9[a]
	East	2.6±0.5[a]	11.0±5.5[a]	5.9±2.8[a]	-	11.4±5.5[a]
	West	2.5±0.4[a]	7.3±5.0[a]	4.6±3.4[a]	-	8.3±5.7[a]
Nak	Central	3.5±1.4[a]	9.6±5.6[a]	5.6±3.1[a]	0.1±0.1[a]	10.5±5.8[a]
	West	5.6±0.8[b]	11.3±2.8[a]	6.3±1.6[a]	0.1±0.1[a]	11.9±3.0[a]
Nakt	Central	2.8±1.0[b]	10.5±5.7[a]	6.0±3.2[a]	0.1±0.1[a]	11.4±6.0[a]
	East	1.8±0.7[a]	6.1±4.8[a]	4.2±4.3[a]	-	7.3±6.6[a]
	West	2.3±0.9[ab]	9.3±5.8[a]	7.3±4.5[a]	0.1±0.1[a]	12.0±7.3[a]
GN	Central	0.5	12.9	24.4	-	31.2
	West	0.8±0.5	10.7±3.5	25.3±7.5	0.2±0.2	30.7±9.2

Values are means ± SD. SN - 'Sukali Ndizi', M9 - EAHB hybrid, BO - 'Bogoya', Mbz -'Mbwazirume', Mpo - 'Mpologoma', Nak - 'Nakinyika', Nakt - 'Nakitembe' and GN - 'Gonja Nakatansese'. DW - dry weight. One-way ANOVA. Values for the analysed variable with the same letter are not significantly different at 5% LSD.

Table 3.7. HPLC analysis - Carotenoid concentration in fruit from full-green bananas growing at different altitudes

Cultivar	Altitude	Carotenoid (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
SN	Low	3.2±1.8^{b}	1.8±0.9^{a}	1.4±0.7^{ab}	0.1±0.1^{a}	2.3±1.2^{a}
	Middle	4.1±1.5^{b}	1.4±0.9^{a}	1.1±0.6^{a}	-	1.8±1.0^{a}
	High	2.1±0.7^{a}	1.7±0.4^{a}	1.8±0.6^{b}	0.1±0.1^{a}	2.7±0.7^{a}
M9	Low	2.3±1.5^{ab}	4.3±3.3^{b}	2.0±1.3^{b}	0.1±0.1^{a}	4.1±3.1^{ab}
	Middle	3.2±1.1^{b}	6.0±3.3^{b}	2.7±1.5^{b}	0.1±0.1^{a}	5.7±0.9^{b}
	High	2.3±0.3^{a}	2.1±0.6^{a}	1.3±0.5^{a}	-	2.4±2.9^{a}
BO	Low	3.5±1.8^{ab}	8.0±2.8^{a}	4.0±1.0^{a}	0.1±0.2	8.0±2.3^{a}
	Middle	4.2±1.4^{b}	6.9±3.8^{a}	3.8±1.5^{a}	-	7.3±3.4^{a}
	High	2.9±1.1^{a}	6.8±4.4^{a}	3.4±1.5^{a}	-	6.8±3.7^{a}
Mbz	Low	2.2±0.5^{a}	8.2±2.9^{a}	5.1±2.7^{a}	0.1±0.0^{a}	9.2±4.2^{a}
	Middle	2.3±0.6^{a}	8.0±2.2^{a}	6.9±1.5^{a}	0.1±0.0^{a}	10.9±2.5^{a}
	High	2.0±0.8^{a}	6.1±3.8^{a}	4.1±3.0^{a}	-	7.1±4.8^{a}
Mpo	Low	2.3±0.4^{a}	10.3±5.4^{a}	6.0±3.7^{a}	-	11.1±6.2^{a}
	Middle	2.4±0.7^{a}	6.2±6.4^{a}	4.1±4.8^{a}	-	7.2±7.9^{a}
	High	2.5±0.4^{a}	10.8±4.9^{a}	6.3±2.5^{a}	-	11.7±4.9^{a}
Nak	Low	3.6±1.5^{a}	9.3±4.8^{a}	5.3±2.6^{a}	0.1±0.1^{a}	10.0±5.0^{a}
	Middle	4.7±1.4^{a}	11.0±5.2^{a}	6.5±2.8^{a}	0.1±0.1^{a}	12.0±5.4^{a}
Nakt	Low	2.9±0.8^{b}	12.0±5.7^{b}	6.8±4.4^{a}	0.1±0.1^{a}	12.9±7.2^{a}
	Middle	2.4±1.1^{ab}	7.8±4.9^{ab}	6.0±2.9^{a}	0.1±0.1^{a}	10.0±5.3^{a}
	High	1.8±0.7^{a}	6.1±4.8^{a}	4.2±4.3^{a}	-	7.3±6.6^{a}
GN	Middle	0.8±0.5^{a}	11.3±3.2^{b}	26.3±6.9^{b}	0.2±0.2^{a}	32.0±8.4^{b}
	High	0.4±0.1^{a}	6.9±3.1^{a}	17.2±6.7^{a}	0.1±0.2^{a}	20.7±8.1^{a}

Values are means ± SD, n ≥ 3 replicates. SN - 'Sukali Ndizi', M9 - EAHB hybrid, BO - 'Bogoya', Mbz - 'Mbwazirume', Mpo - 'Mpologoma', Nak - 'Nakinyika', Nakt - 'Nakitembe' and GN - 'Gonja Nakatansese'. DW - dry weight. One-way ANOVA. Values with the same letter are not significantly different at 5% LSD.

3.4. Discussion

Carotenoid accumulation levels in different banana cultivars have been reported to vary widely (Englberger *et al.*, 2003; Davey *et al.*, 2009). Characterisation of this variation in biofortification target cultivars may provide valuable information to assist conventional and molecular breeding programs. In this study, levels of accumulated carotenoids were evaluated in (i) green and ripe dessert bananas (SN - 'Sukali Ndizi' and BO - 'Bogoya'), (ii) green, stored (7 and 14 days) and processed (steamed and boiled) EAHB (Mbz - 'Mbwazirume', Mpo - 'Mpologoma', Nak - 'Nakinyika', and Nakt - 'Nakitembe') and EAHB hybrid (M9), and (iii) green, ripe and processed plantain bananas (GN - 'Gonja Nakatansese').

Variations in the concentrations of individual carotenoids were observed across all cultivars included in this study. Previous studies have shown that the predominant PVA carotenoids present in bananas are α-carotene and all trans β-carotene while very small concentrations of the cis β-carotene isomers are present as well as important amounts of the non-PVA carotenoid, lutein (Englberger *et al* 2003a). This is fortuitous since carotenoids with the highest vitamin A activity are all-trans β-carotene followed by all trans α-carotene (Deming *et al.*, 2002). In this study, it was shown that more than 50% of the total carotenoids present in green EAHB banana cultivars comprised of α- and β-carotene. As such, EAHB are a good candidate for PVA biofortification.

The high variation (over 40%) in PVA carotenoid content in samples obtained from different cultivars corresponds to findings of a study examining the micronutrient content of banana and plantain (*Musa* spp.) fruit from West and Central Africa (Davey *et al.*, 2007). Similar variation has also been observed in other crops including citrus (Ikoma *et al.*, 2001), tomato (Bramley, 2002; Giuliano *et al.*, 2003), and pepper (Bouvier *et al.*, 1998), and may be a result of developmental changes in carotenoid metabolic processes. The observed variation also confirms the earlier reported genetic variation in different varieties of the same crop (Hulshof *et al.*, 2000; Morris *et al.*, 2004; Ortiz-

Monasterio *et al.*, 2007). Genetic variation may arise from the interaction between several genes of largely unknown function combined with environmental influences that control trait variation, in this case carotenoid accumulation (Carlborg and Haley, 2004; Evans *et al.*, 2006).

Substantial amounts of the non-PVA carotenoid, lutein, equivalent to over 50% of total carotenoids, were also detected in the cultivar SN, with other cultivars containing a minimum of 50% less lutein. By acting as quenchers of reactive oxygen species in the body (Beutner *et al.*, 2001), non-PVAC such as lutein have strong anti-oxidative properties and have been proven to be associated with a number of health benefits (Gliszczyńska-Swigło *et al.*, 2006; Grassmann *et al.*, 2007; Di Vaio *et al.*, 2008; Zanfini *et al.*, 2010). The results from the SN cultivar indicate a α-carotene favoured branching at the cyclase step with a subsequent high level of hydroxylation of PVA compound into lutein by cytochrome P450 carotene hydroxylases. The results are underscored by observations in maize, which suggest that, as much as the cyclase enzyme is effective in controlling the pathway branching, the enzymatic actions do not necessarily result in enhanced accumulation of PVA carotenoids due to hydroxylation of PVAC to non-PVAC (Vallabhaneni *et al.*, 2009; Yan *et al.*, 2010). Similarly, the proportionally high β-carotene content in GN, compared to other cultivars indicates a β-carotene favoured cyclase branching with reduced hydroxylation activity downstream.

A number of studies have shown the importance of food processing to improve carotenoid bioaccessibility and bioavailability (Ryan *et al.*, 2008). In a banana feeding study with Mongolian Gerbils, cooked bananas were reported to have a 2-fold increase in bioconversion of hepatic retinol compared to their raw counterparts (Bresnahan *et al.*, 2012). Mild processing conditions can be sufficient to disrupt the fruit pulp tissue due to the relatively tender nature of banana tissue. Cooking and processing has been reported to increase the chemical extractability of carotenoids in some tissue due to its disruptive effect on the carotene-protein complexes, but also due to the inactivation of

carotene oxidizing enzymes (Lessin and Schwartz, 1997; van het Hof *et al.*, 1998). Indeed, heat treatments, such as boiling and steaming, help to release bound carotenoids and enable them to be more readily extracted using solvents. As such, controlled steaming and boiling processes adopted in the current study could have made carotenoids present in the banana pulp tissue matrix readily available for extraction. After processing, cis-β-carotene isomers comprised ≤ 3% of total PVA in all cultivars. Minimum carotenoid destruction, as represented by a high β-CE retention capacity of more than 70%, was obtained in EAHB cultivars and M9, with relatively low isomerisation of the trans β-carotene to cis-β-carotene. More importantly, it is reassuring to see that two of the cultivars currently being used for our biofortification efforts at NARO, 'Nakinyika' and EAHB hybrid M9 have PVA retention of 81.5 and 78.3% after boiling and 79.6% and 76.1% after steaming respectively. In addition, this study showed that, with only the exception of the Mbz cultivar, no significant differences in β-CE levels were observed between EAHB cultivars after boiling or steaming. Cultivars 'Nakitembe' and 'Nakinyika' retained over 75% of their β-carotene content after processing. Although, 'Gonja Nakatansese' (GN), a plantain type, contained high levels of all trans-β-carotene and α-carotene in the fruit pulp at full green, it only retained slightly more than half of the content after boiling and steaming. The results obtained (Table 3.3) indicate that level of PVA carotenoid degradation in the GN cultivar is less affected by isomerisation with a possibility of micronutrient degradation via oxidative processes induced by the heating process (Kidmose *et al.*, 2006). Considering that in Africa plantains are traditionally either roasted or fried, this phenomenon can only be exacerbated by these methods making plantain an unsuitable target crop for biofortification. The part of the study underscores the effect of food processing on carotenoid content where vigorous processing conditions such as frying, roasting and extended cooking often adopted in banana preparation in Uganda are likely to destroy carotenoids in the food.

This study has clearly demonstrated the importance that storage can have on the accumulation of carotenoids in the fruits of EAHB cultivars. Indeed, all EAHB fruits stored over a period of 14 days showed an increase in β-CE content although these results were only significant for two cultivars, Mpo ($p<0.05$) and Nakt ($p<0.001$). During storage and ripening, physiological changes such as a transition from amyloplasts to chromoplasts occur in the banana tissue (Emter *et al.*, 1990). The banana fruit pulp tissue undergoes changes in texture from a hard starch rich matrix to a softer texture rich in simple sugars (Emter *et al.*, 1990). These physiological changes affect metabolite movement and availability within the cells and lead to a continuous dissociation of the carotene-protein complexes in the tissue, thus increasing the levels of free carotenoids extracted from the fruit pulp. This may render the carotenoids more readily available for extraction, thus explaining the higher β-CE levels observed after 7 and 14 days of storage (for EAHB) and subsequently at the full ripe stage (for dessert bananas and the plantain).

The results obtained from this study indicate that, both within and between different banana genotypes, there are differences in carotenoid accumulation in fruit pulp. For example, plantain 'Gonja Nakatansese' and apple banana, 'Sukali Ndizi', both with an AAB genome, differed significantly from each other in their carotenoid accumulation across all parameters measured. Similar differences in carotenoid accumulation were also seen in comparisons between the two AAB genome cultivars and the EAHB containing an AAA genome. In conclusion, carotenoid accumulation in bananas is not directly related to the plant genotype and to establish the carotenoid accumulation profile and pattern in the fruit pulp, carotenoid accumulation across fruit maturity in a given cultivar needs to be investigated. This study was critical in determining the basic pro-vitamin A contribution of the banana to a population that predominantly feed on a banana diet. The data generated forms a basis to support current efforts towards improving the nutrient content in popular EAHB cultivars in Uganda through biofortification approaches.

Chapter 4 - Molecular, biochemical and phenotypic characterisation of pro-vitamin A biofortified transgenic banana Lines of 'Sukali Ndizi' and East African Highland Banana hybrid, M9

4.1. Introduction

Micronutrient deficiencies and vitamin A deficiency (VAD) in particular are one of the world's major public health problems. In Uganda and other African countries where cooking bananas are the staple food, clinical vitamin A deficiency occurs in more than 15% of children under 5 years and women of child-bearing age (Kikafunda *et al.*, 1998). Despite very effective and widely deployed strategies such as food fortification and supplementation these deficiencies continue. In rural populations of Uganda where East African Highland bananas (EAHB) form a major and sometime unique part of the diet, 20 μg/g DW β-CE is the minimum amount of pro-vitamin A required to alleviate VAD (James Dale personal communication). This target level is based on (i) providing 50% of the Estimated Average Requirement (EAR), (ii) an average daily consumption of 300g of cooked bananas, (iii) a 70% retention of pro-vitamin A through the traditional cooking process and (iv) a 6:1 bioconversion ratio of β-carotene to retinol.

The results presented in the previous chapter showed that, although some EAHB cultivars such as 'Nakitembe' and 'Mpologoma' can, after 14 days in storage, reach levels near/above the 20 μg/g DW β-CE target, most EAHB cultivars barely reach half the necessary amounts. Therefore, biofortification of acceptable EAHB cultivars with enhanced levels of pro-vitamin A (or other micronutrients) is now believed to be the most effective approach to reach affected populations that are recalcitrant to the previously mentioned strategies. Biofortification of crop plants can be achieved through either conventional breeding, where the necessary traits are available within the

accessible “breeder’s gene pool”, or through genetic modification as exemplified by Golden Rice (Ye *et al.*, 2000; Paine *et al.*, 2005).

As a key strategy to address the continuing levels of VAD in Uganda, researchers at the National Agricultural Research Organisation (NARO) have genetically modified banana in an attempt to increase the level of pro-vitamin A in the fruit at harvest. Two banana cultivars, ‘Sukali Ndizi’, a small sweet dessert banana and M9, an East African Highland banana (EAHB) hybrid have been genetically transformed with either a *Zea mays* phytoene synthase 1 (*Psy1B73* or *Psy1Q60*) gene or a Fe’i type banana (*Asupina* spp)-derived phytoene synthase 2 (*APsy*2*a*) gene under the control of a banana derived expansin 1 promoter (Exp1) (James Dale personal communication).

The control of gene expression is a crucial regulatory mechanism in plant physiological processes and is therefore pivotal for successful genetic modification of plants for pro-vitamin A biofortification. At the cellular level, transgene expression may be influenced by factors such as the integration site of the transgene in the plant genome, rearrangement of the transgene, copy number of the integrated transgene and the growth environment (Yoshida and Shinmyo, 2000). Gene integration in the plant genome can also lead to adverse changes in agronomic traits including plant height, yield, number of flowers per plant, grain quality, and resistance/susceptibility to diseases, insects, pests, cold, drought and salt (Jain, 2001).

The results presented in this chapter report on the molecular characterisation of transgenic lines of ‘Sukali Ndizi’ and M9 that were generated at NARO. In addition, the relative expression of the two phytoene synthases and their effect on the accumulation of PVA carotenoids in the fruit pulp of the two banana cultivars grown under confined field trial conditions was also investigated. Finally, agronomic performance evaluation of transformed plants was done to evaluate desirable yield components and ensure that

they were retained throughout the tissue culture, transformation and regeneration processes.

4.2. Materials and methods

4.2.1 Transformation constructs

Binary plasmids, pOPT-K, pOPT-J and pCAM-Exp-PSYB73 (Fig. 4.1), each containing a banana-derived expansin 1 promoter controlling the expression of either an 'Asupina' phytoene synthase 2a gene (*APsy2a*) or two variants of a *Zea mays* phytoene synthase 1 gene (*Psy1B*73 and *Psy1Q*60) were supplied by Distinguished Professor James Dale (QUT, Brisbane, Australia). Plasmid pART-TEST7, containing the green fluorescent protein (*GFP*) reporter gene under the control of the CaMV 35S promoter (Fig. 4.1), was also used as control and was kindly provided by Dr. Harjeet Khanna (QUT). Binary plasmids were transformed into chemically competent *E. coli* JM109 cells using a heat shock method and into *Agrobatcterium tumefaciens* AGL1 strain using electroporation.

4.2.2. Stable transformation of banana

Although the transformation of banana cells was done by other researchers at NARO, the methodology is still presented here as background information. Binary vectors for banana transformation were transferred into *Agrobacterium* strain AGL1 as previously described (Chapter 2.1.1.4). Embryogenic cell suspensions (ECS) of banana cultivars, 'Sukali Ndizi' (*Musa* spp. AAB group) and and EAHB hybrid M9 (*Musa* spp. AAA group) were stably transformed using a centrifugation assisted *A. tumefaciens*-mediated transformation technique described by Khanna *et al.* (2004). Initially, 150mg of sieved cell volume (SCV) was transformed, in triplicate, with each of the test binary plasmids and the control plasmids containing *GFP*. Each transformation batch was subsequently divided into three co-cultivation plates. For the first month, cells were sub-cultured every two weeks onto fresh BL media supplemented with 200 mg/L Timentin (to control *Agrobacterium*) and 50 mg/L kanamycin as the selecting antibiotic. Cells were then moved to supplemented BL media.

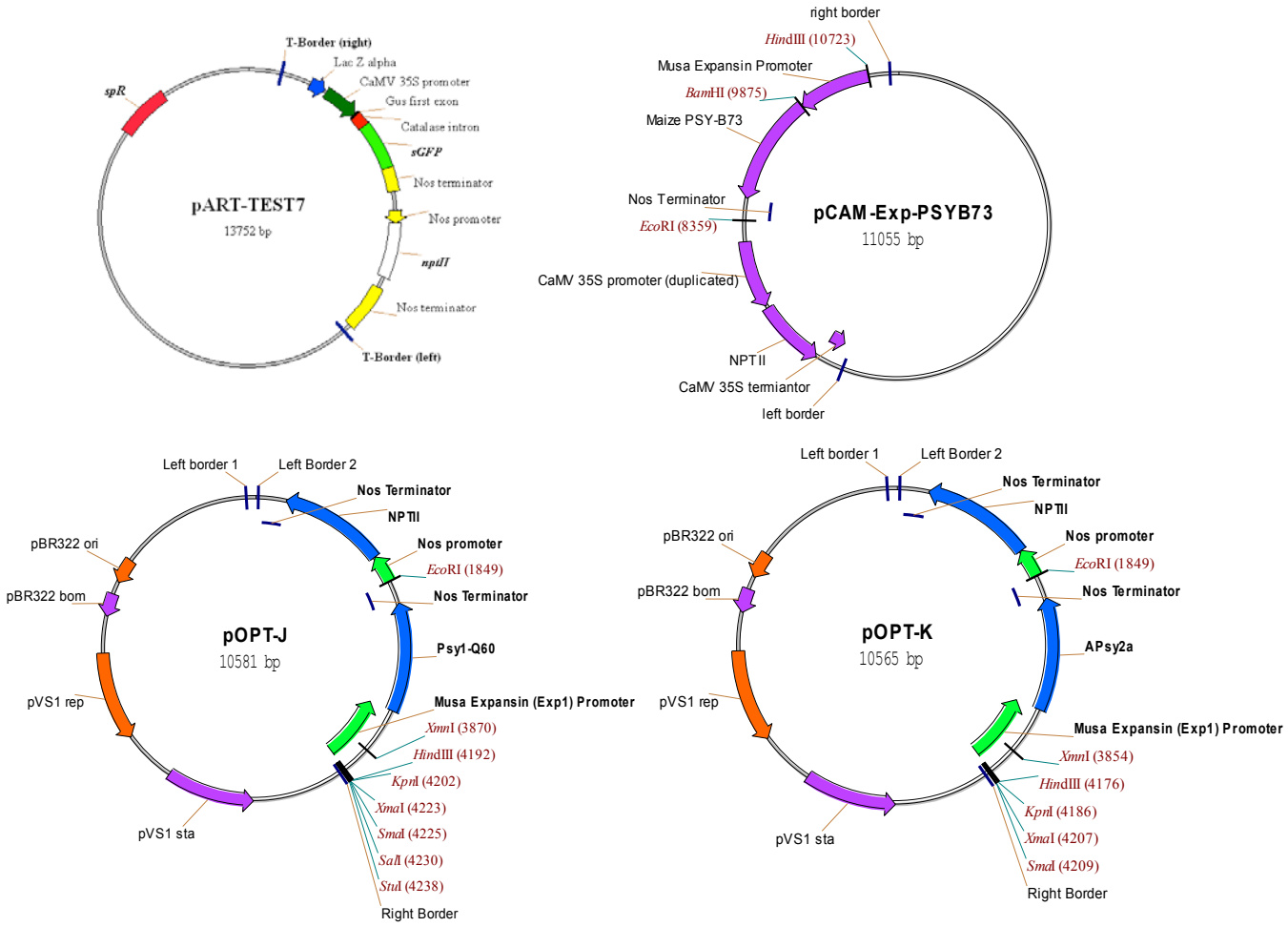

Figure 4.1. Schematic representation of plasmids. Plasmids pART-TEST7 (containing the green fluorescent protein (GFP) reporter gene under the control of the CaMV 35S promoter), pCAM-Exp-PSYB73 and pOPT-J (containing two variants of a *Zea mays* phytoene synthase 1 gene (*Psy1B*73 and *Psy1Q*60) under the control of the expansin 1 promoter) and pOPT-K (containing the *'Asupina'* banana-derived phytoene synthase 2a gene (*APsy2a*) under the control of the expansin 1 promoter) were used for transformation of banana embryogenic cell suspensions.

4.2.3. Plant acclimatisation, greenhouse and confined field trial conditions

4.2.3.1. Plant acclimatisation and greenhouse establishment

Tissue-cultured transgenic and non-transgenic banana plants were deflasked and their roots were washed free of growth medium. They were then potted into seedling tubes using Searles® Seed Raising Mixture and the tubes were placed in a humid chamber and kept at a constant temperature of 25^{o}C for a period of 2 weeks. Hardened plants were transferred into the glasshouse at a controlled temperature of 27^{o}C and under natural light conditions for 8 weeks before being transferred to the confined field trial (CFT).

4.2.3.2. Confined field trial, approvals and growth conditions

The CFT site is located at the National Agricultural Research Laboratories (NARL), Kawanda. The site is delimited by the GPS coordinates N 0 24.900, E 32 32.053 (COI); N 0 24.902, E 32 32.101 (CO4); N 0 24.778, E 32 32.102 (CO3) and N 0 24.778, E 32 32.051 (CO2) (Figure 4.2). The planting area boundaries were established by planting a border row of non-transgenic banana plants around the CFT. Transgenic plants were transferred from the greenhouse to the CFT in accordance with the National Biosafety Committee (NBC) approval decision document No. 2/2009. Plants were grown for 36 months during which time they were regularly monitored and fruit samples collected for analysis as required.

4.2.4. Sample collection

Fruit from wild-type control and genetically modified (GM) plants in the CFT were harvested at different stages depending on cultivar and availability of samples. Samples from 'Sukali Ndizi' transgenic plants were collected from the mother plant (MP) and three successive ratoon crops (R1, R2 and R3) at full green (FG) and full ripe (FR). In addition, samples were collected from R1 at several different stages of bunch development designated S6, S9, S12 and S15 representing week 6, 9, 12, and 15 post bunch emergence, respectively. Samples were also collected from non-GM control plants in the CFT. Samples from EAHB hybrid M9 transgenic plants were only collected

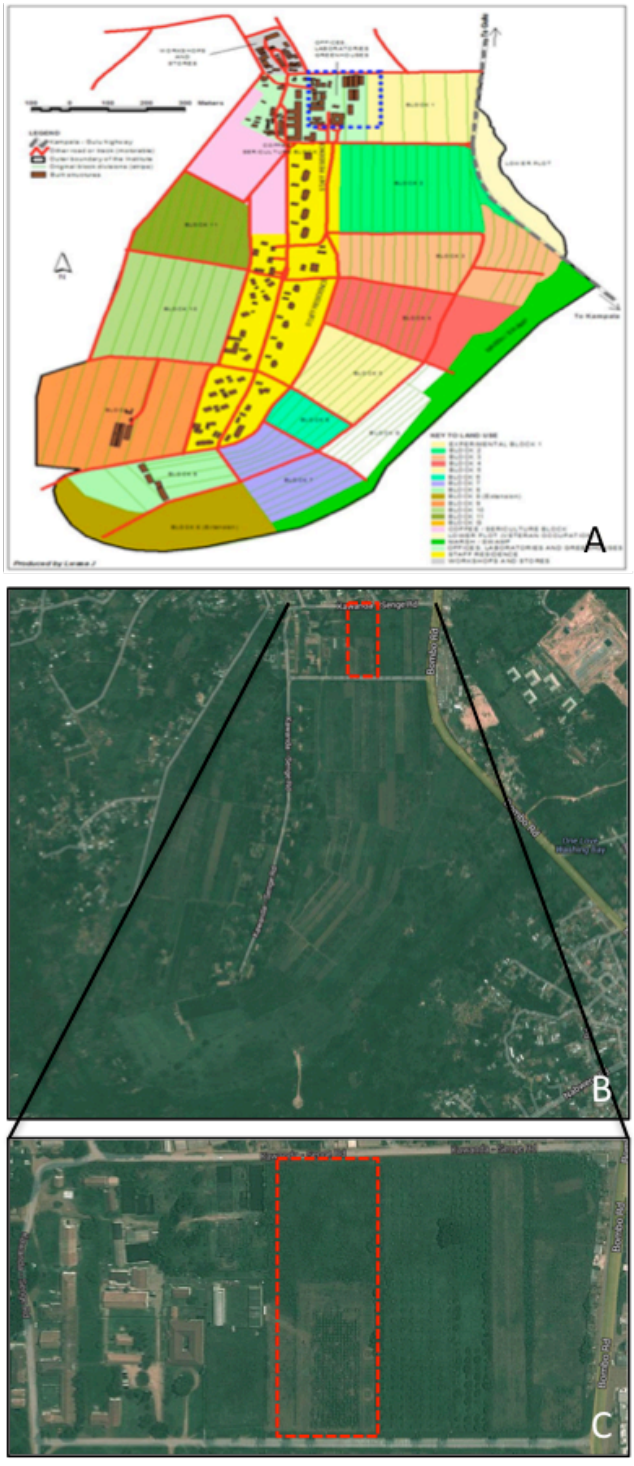

Figure 4.2. Aerial view of the Kawanda Agricultural Research Station. A, map of KARS, B, aerial view and C, National Agricultural Research Organisation (NARO) with CFT site delimited by the red dotted line.

from the mother crop at FG and FR as well as developmental stages S3, S6 and S10 representing week 3, 6 and 10 post bunch emergence, respectively.

4.2.5. Sample preparation, carotenoid extractions and analysis

Sample preparation, carotenoid extraction and analysis of carotenoids by HPLC were carried out according to the procedure outlined in Chapter 2.1.5.

4.2.6. Molecular characterisation

4.2.6.1. Primer design

Primers for PCR and qPCR were designed using the online software Primer3 (http://www-genome.wi.mit.edu) to anneal at 58^{o}C and amplicon size was maintained below 200 bp as recommended for SYBR green I reactions (Roche) (Table 4.1). The reference genes used in the experiment, Ubiquitin (UBQ) and Ribosomal protein S (RS4), were selected based on qPCR expression data analysis using geNorm v3 for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm). Primer specificity was validated by semi-quantitative RT-PCR and by the absence of primer dimers following electrophoresis of the products through 2% agarose gels. Each PCR product was cloned and sequenced to confirm it's identify.

4.2.6.2. Isolation of total DNA from banana leaf tissue and PCR screening of transgenic plants

Total DNA was isolated from banana leaf tissue using a modified CTAB protocol essentially as described by Stewart and Via (1993) (Stewart and Via, 1993) (Chapter 2.2.3.1). The screening of transgenic plants for the presence of transgenes was done by PCR in a PCR BIORAD thermal cycler using GoTaq®Green (Promega). All reaction mixes (20 μL) were as described in Chapter 2.1.4.1 and contained approximately 200ng of total gDNA template and specific primers (Table 4.1). Initially, all extracts were tested for DNA quality using banana *Actin* gene-specific primers. All positive plants were subsequently tested with primer set VCF-AGL FWD/Rev to identify false positive plants

Table 4.1. Primer sequences for PCR and qPCR analysis

Experiment	Primer	Target/location	Primer Sequence 5'→3'	Accession No.
PCR	EXPint-FWD	Banana expansin 1 promoter	GCCACCAACTTGTCTCTTTCC	AY083168.1
	*APsy*2*a* Rev	'Asupina' *Psy2a*	TTGTACCTCGATTTCCGCAGGTC	JX195659.1
	*ZmPsy*1 Rev	Maize *Psy1*	TCTAGAGTCGACTCACTAGGTCTGGCCATT	AY324431.1
	Actin FWD/Rev	Banana genomic Actin	CTGGTGATGGTGTGAGCCAC CATGAAATAGCTGCGAAACG	
	VCF-AGL FWD/Rev	VirC operon in Ti plasmid	GCCTTAAAATCATTTGTAGCGACTTCG TCATCGCTAGCTCAAACCTGCTTCTG	
qPCR	*ZmPsy*1 Fwd/Rev	Maize *Psy1B73* and *Psy1Q60*	ATAGCACAGCATCCTCACTT AGCAACTTCTTCCCTTTACC	AY324431.1
	*APsy*2*a* FWD/Rev	'Asupina' *Psy2a*	CGACGAACTCTATCTCTACTG CTGAGTATGTTGGTGAGTTGA	JX195659.1
	RS4 FWD/REV	*Musa* spp. RS4 reference gene	TGAGAGTGGCTTGACCCTGA GTGACATTTAGTCGTCTGCTGG	HQ853247
	UBQ FWD/REV	*Musa* spp. UBQ reference gene	GGCACCACAAACAACACAGG AGACGAGCAAGGCTTCCATT	HQ853254

which still contained *Agrobacterium*. Extracts testing positive for actin and negative for *Agrobacterium* were then tested using transgene-specific primers to detect the presence of the transgene in each line.

4.2.6.3. RNA extraction and RT-PCR

RNA was extracted from 100mg of fresh leaf tissue using an "in house" RNA protocol as described in Chapter 2.2.3.2. Approximately 1 μg total RNA was treated with RNase-free DNase (Promega) (Chapter 2.2.3.2) and cDNA synthesis done according to Chapter 2.2.4. PCR (Chapter 2.3.1.1) was then done as previously described with the appropriate gene-specific primers (Table 4.1).

4.2.6.4. Expression analysis and q(RT)PCR

To determine the expression level of *Psy1B*73 and *APsy2a* in the fruit pulp of transgenic banana plants, total RNA was extracted from the banana fruit pulp as described in Chapter 2.2.3.2. This was followed by first-strand cDNA synthesis according to Chapter 2.2.4. Expression analysis was carried out using a quantitative real-time PCR approach, Chapter 2.3.1.3. The transcript amounts were normalised to the average value of 2 reference genes (RS4 and UBQ) and the data calculated using the ΔΔCT method and expressed relative to the appropriate reference samples (either developmental stage S6 or mother plant (MP)) depending on the experiment. Briefly, to obtain the gene expression levels, the relative levels were computed by dividing the normalised expression of the gene by the normalised expression of the same gene in the reference sample (RS) as described by equation below:

$$2^{-\Delta\Delta CT} = 2^{-[(CT\ TD - CT\ RG\text{-}TD) - (CT\ RS - CT\ RG\text{-}RS)]} \quad \text{(viii)}$$

4.2.6.5 Agronomic and phenotypic evaluation

Transgenic banana lines in the CFT were monitored for any abnormal phenotypic characteristics and any postharvest changes related to the expression of the transgenes.

Agronomic characteristics that were monitored in the experiment included middle girth, plant height, total leaf number, number of clusters, bunch weight, bunch height, bunch circumference, leaf colour, pulp colour, time to fruit maturity and fruit colour.

4.3. Results

4.3.1. Banana transformation and regeneration

Embryogenic cell suspensions (ECS) of banana cultivars, 'Sukali Ndizi' and and EAHB hybrid M9 were stably transformed by researchers at NARO with phytoene synthase genes *APsy2a* (pOPT-K), *Psy1Q*60 (pOPT-J) and *Psy1B*73 (pCAM-Exp-PSYB73) using a centrifugation assisted *A. tumefaciens*-mediated transformation method. As mentioned previously, the expression of these genes was controlled by the banana-derived expansin 1 promoter. The green fluorescent protein (*GFP*) reporter gene (pART-TEST7), under the control of CaMV 35S promoter, was also included as a transformation control.

After three days of co-cultivation with *Agrobacterium*, cells from all experiments were washed and transferred to BL selection media. When control cells transformed with the *GFP* reporter gene were examined under a fluorescence microscope, large numbers of green fluorescent foci were observed indicating GFP expression (data not shown) and successful transformation. Plantlets were regenerated after 12 weeks on BL media, 12 weeks on embryo induction media (M3) and a further 4 weeks on germination media (MA4).

A total of 71 putative transgenic plants were regenerated from all the transformation experiments. These plants were initially established in the glasshouse for a period of 10 weeks then transplanted into the confined field trials (CFT).

4.3.2. Confined field trials (CFTs) and transgene detection by PCR

A total of 71 putative transgenic plants were planted in the CFTs with 28 and 43 planted in CFT-1 and CFT-2, respectively. To test these plants for the presence of the respective transgene, leaf samples were collected from all plants in both CFTs. Total gDNA was

extracted from the leaves of putatively transformed SN and EAHB hybrid M9 banana plantlets and used in a PCR with *Actin* (housekeeping gene)-specific primers to initially assess the quality of the extracts. Extracts testing positive for actin were then tested using transgene-specific primers to detect the presence of the transgene in each line. Further, to avoid the possibility of detecting false positives due to the presence of residual *Agrobacterium*, any plant testing positive for the transgene was subsequently screened by PCR for the presence of the virC complementation group of the *Agrobacterium tumefaciens* Ti plasmid. No plants tested positive for the presence of residual Agrobacterium.

CFT-1 contained 28 plants of the 'Sukali Ndizi' cultivar with 16 putative *Psy1B73* and 12 putative *Apsy2a* transgenic lines. Out of these 28 plants, only 5/16 *Psy1B73* and 5/12 *Apsy2a* lines were confirmed to have the transgene by PCR.

CFT-2 contained 34 putative 'Sukali Ndizi' lines (8 x *Psy1Q60* and 26 x *Apsy2a*) and 9 putative EAHB hybrid M9 lines (4 x *Psy1Q60* and 5 x *Apsy2a*). Out of the 34 'Sukali Ndizi' lines, 4/8 *Psy1Q60* and 8/26 *Apsy2a* tested positive for the transgene of interest while 6 of the 9 EAHB hybrid M9 lines tested positive for the transgene of interest (4/4 *Psy1Q60* and 2/5 *Apsy2a*).

4.3.3. Field trial selection of events for biochemical and expression analysis

The aim of this part of the study was to analyse the levels of carotenoid accumulation in the fruits of all transgenic lines present in the CFTs to enable the selection of only the elite lines for further studies. For logistical purposes, it was difficult to analyse all transgenic lines at various stages of development and across successive crop generations. Therefore, fruit from the "mother plant" of all 28 transgenic lines planted in both CFTs was initially just sampled at the full ripe (FR) and full green (FG) stages, and analysed using high-performance liquid chromatography (HPLC) (Table 4.2). The PVA carotenoid concentration of transgenic 'Sukali Ndizi' lines in both CFTs ranged from

Table 4.2. HPLC analysis - Carotenoid content in the fruit pulp of transgenic 'Sukali Ndizi' (SN) and EAHB hybrid M9

CFT-1 plants			
Line ID	**Cultivar**	**Transgene**	**β-CE (µg/g DW)**
Wild-type	SN	-	0.5
NU211-12-11	SN	*Psy1B73*	2.8
NU211-12-16	SN	*Psy1B73*	0.7
NU211-12-22	SN	*Psy1B73*	0.2
NU211-12-23	SN	*Psy1B73*	0.5
NU211-12-39	SN	*Psy1B73*	6.8
NU211-13-203	SN	*APsy2a*	0.7
NU211-13-202	SN	*APsy2a*	0.7
NU211-13-27	SN	*APsy2a*	0.3
NU211-13-16	SN	*APsy2a*	0.2
NU211-13-36	SN	*APsy2a*	0.2
CFT-2 plants			
Line ID	**Cultivar**	**Transgene**	**β-CE (µg/g DW)**
Wild-type	SN	-	1.4
NU223-16-49	SN	*Psy1Q60*	1.5
NU223-16-46	SN	*Psy1Q60*	0.9
NU223-16-42	SN	*Psy1Q60*	1.2
NU223-16-05	SN	*Psy1Q60*	2.0
NU223-13-10	SN	*APsy2a*	1.9
NU223-13-14	SN	*APsy2a*	1.8
NU223-13-19	SN	*APsy2a*	1.2
NU223-13-46	SN	*APsy2a*	1.2
NU223-13-51	SN	*APsy2a*	1.1
NU223-13-63	SN	*APsy2a*	1.7
NU223-13-86	SN	*APsy2a*	0.6
NU223-13-33	SN	*APsy2a*	1.5
Wild-type	M9	-	5.8
NU233-16-04	M9	*Psy1Q60*	11.1
NU233-16-06	M9	*Psy1Q60*	10.3
NU233-16-13	M9	*Psy1Q60*	5.6
NU233-16-43	M9	*Psy1Q60*	10.0
NU233-13-03	M9	*APsy2a*	7.3
NU233-13-10	M9	*APsy2a*	33.1

Red denotes lines that were selected for further analysis

0.2-6.8 µg/g DW while the levels in the non-GM controls ranged from 0.5-1.4 µg/g DW. With the EAHB M9 transgenic plants, the PVA carotenoid concentration ranged from 5.6-33.1 µg/g DW with the one non-GM control containing 5.8 µg/g DW. Based on these results, it was decided that only lines with a PVA carotenoid concentration greater than 2 µg/g DW (for 'Sukali Ndizi') and 5 µg/g DW (for EAHB hybrid M9) would be used for further HPLC characterisation and qPCR expression profiling. As such, a total of 8 transgenic lines were subjected to further analyses. These included 2 x *Psy1B73* 'Sukali Ndizi' lines (NU211-12-11 and NU211-12-39) and 6 x EAHB hybrid M9 lines (4 x *Psy1Q60* lines (NU233-16-04, NU233-16-06, NU233-16-13 and NU233-16-43) and 2 x *APsy2a* lines (NU233-13-03, NU233-13-10)).

4.3.4. Further analysis of transgenic 'Sukali Ndizi' lines

From the 2 transgenic 'Sukali Ndizi' lines selected, a total of 72 samples were collected (Table 4.3). As previously described in Chapter 2.1.5.1, composite samples (1 fruit from the top, middle and bottom of the bunch) were collected at the full green (FG) and full ripe (FR) stages from the "mother plant" as well as from 3 successive crop generations (R1, R2 and R3). For the first ratoon crop (R1), samples were also collected at 4 different bunch development stages designated S6, S9, S12, and S15 representing 6, 9, 12 and 15 weeks after bunching, respectively. Samples from non-GM 'Sukali Ndizi' plants were also collected as controls. The levels of transgene expression and PVA carotenoid accumulation were then assessed in the selected lines for the samples collected.

4.3.4.1 Transgene expression in transgenic 'Sukali Ndizi' lines

The level of expression of *Psy1B73* in 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) was initially assessed by qPCR across 6 stages of fruit development (S6, S9, S12, S15, FG and FR) and the data normalised and expressed relative to the *Psy1B73* expression at the S6 stage of P1-11 (Figure 4.3). The levels of *Psy1B73* transcripts in line P1-11 increased more than 4-fold during the early stages of development from S6 to S9 followed by a reduction of more than 4-fold during the

Table 4.3. Sampling schedule for the transgenic 'Sukali Ndizi' lines

MP		**R1**		**R2**		**R3**	
Type of samples	Number of samples	Type of sample	Number of samples	Type of sample	Number of samples	Type of sample	Number of samples
FG	3	S6	3	FG	3	FG	3
FR	3	S9	3	FR	3	FR	3
Total	**6**	S12	3	**Total**	**6**	**Total**	**6**
		S15	3	**Grand total:** 36 samples/line X 2 lines= **72**			
		FG	3				
		FR	3				
		Total	**18**				

following 3 week period. The levels of *Psy1B73* transcripts decreased further at the full green stage (FG) and after ripening (FR). In line P1-39, however, except for an initial small decrease in expression from S6 to S9, the expression of *Psy1B73* remained constant at about 2-fold the initial level all the way through to FR (Figure 4.3).

The levels of *Psy1B73* expression were also monitored in the 'Sukali Ndizi' lines P1-11 and P1-39 for 3 years across 3 more successive generations as shown in Figure 4.4. These analyses were done on fruit samples taken at the FG stage. P1-11 showed a 2-fold and 3-fold increase in *Psy1B73* transcripts in the second (R2) and third ratoon (R3), respectively, compared to the mother plant (MP) despite an initial 50% drop in the first ratoon (R1). The levels of transcripts in the MP of line P1-39 were almost 4-fold higher than in the MP of line P1-11. An initial increase in expression was seen in R1 followed by a decrease in the following generations, R2 and R3.

4.3.4.2. Carotenoid accumulation in transgenic 'Sukali Ndizi' lines

After examining transgene expression in the fruit of 'Sukali Ndizi' lines P1-11 and P1-39, PVA accumulation was analysed in the same tissues by reversed phase HPLC. This analysis was done (i) across 4 generation of plants (MP, R1, R2 and R3) at the FG fruit stage only and (ii) at fruit development stages S6, S9, S12, S15, FG and FR for the R1 generation only.

When fruit from the R1 generation was analysed at fruit development stages S6, S9, S12, S15, FG and FR, the level of β-carotene equivalents (β-CE) in the wild-type (NT) was very low, ranging from 0.5 µg/g DW at S12 to a maximum of 0.9 µg/g DW at FG (Figure 4.5 and Table 4.4). The accumulation of PVA carotenoids was subsequently found to decrease to 0.4 µg/g DW at FR. In the transgenic line P1-11, the level of β-CE started at 5.5 µg/g DW at S6 followed by a slight drop at S9 and down to its lowest level at S12 (0.9 µg/g DW). The β-CE levels then increased to 4.8 and 4.1 µg/g DW at FG and FR, respectively. Fruit samples obtained from line P1-39 at S6 were found to contain

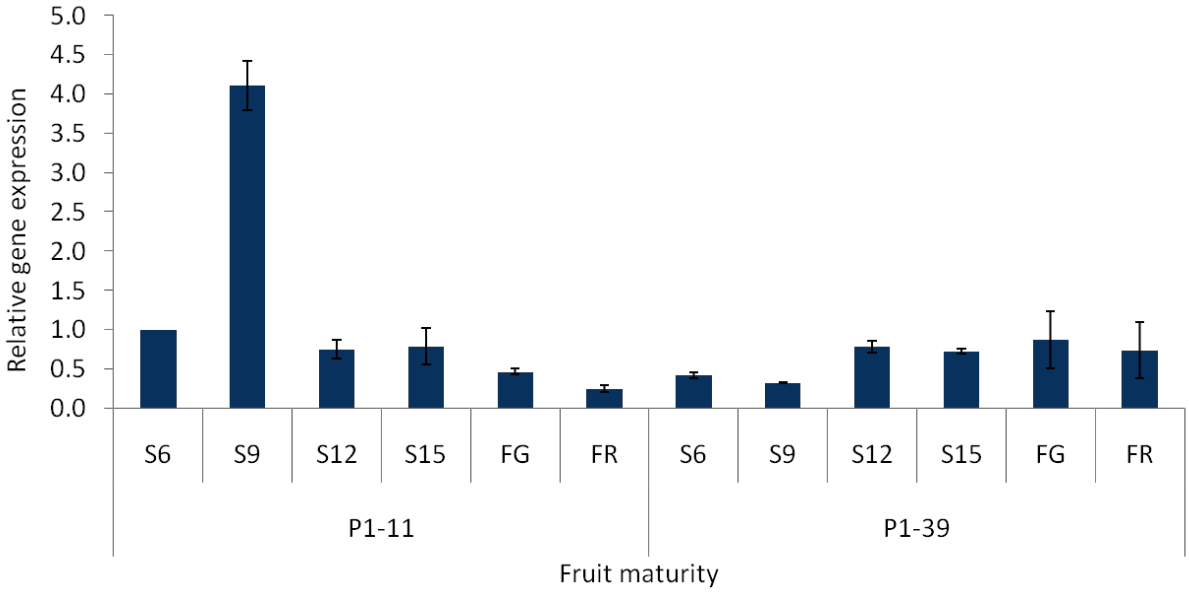

Figure 4.3. Relative expression of the *Psy1B*73 transgene in the fruit pulp of transgenic 'Sukali Ndizi' lines at different stages of fruit development - RT-qPCR analysis. The expression of *Psy1B73* in transgenic 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) is shown at S6, S9, S12, and S15 representing 6, 9, 12 and 15 weeks after flowering respectively, FG, full green mature fruit and FR, full ripe fruit. Values are relative to the mean expression value of P1-11 at S6 stage, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 technical replicates.

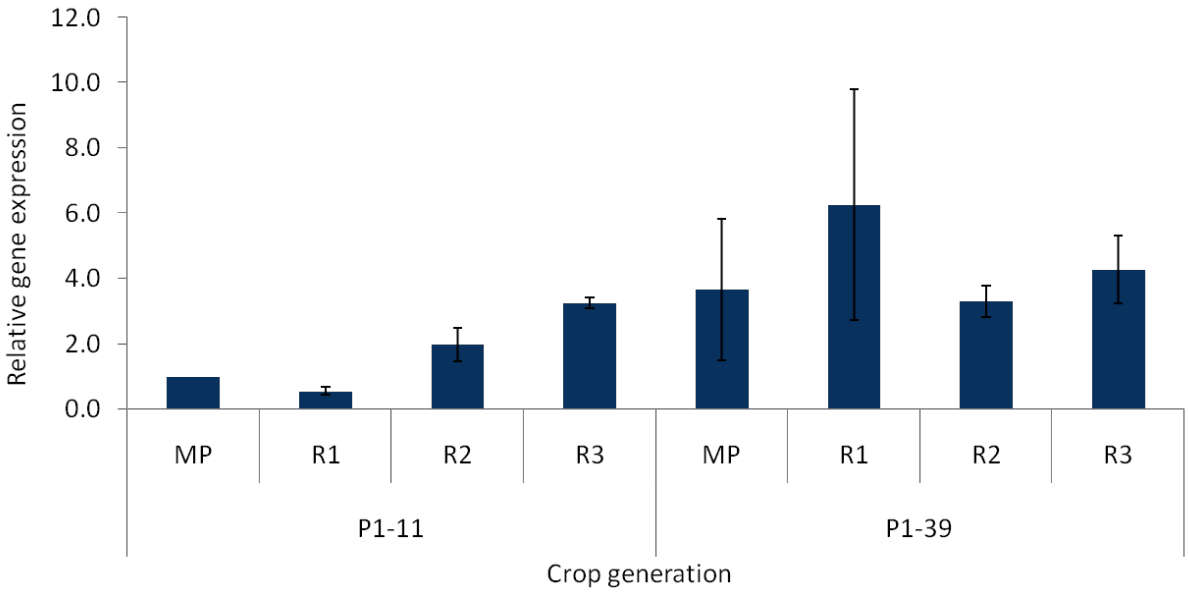

Figure 4.4. Relative expression of the *Psy1B73* transgene in full-green fruit pulp of transgenic 'Sukali Ndizi' lines from different crop generations - RT-qPCR analysis. The expression of *Psy1B73* in transgenic 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) is shown in the mother plant (MP), R1, ratoon 1, R2, ratoon 2 and R3, ratoon 3. Values are relative to the mean expression value of P1-11 mother plant (MP), which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 technical replicates.

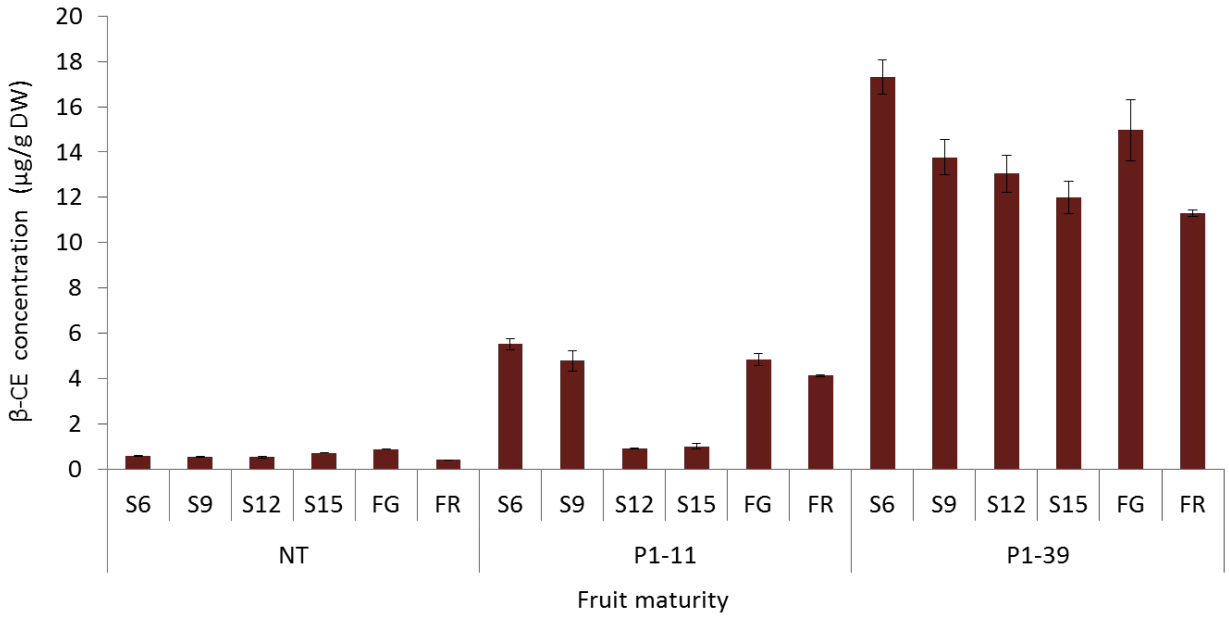

Figure 4.5. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic Psy1B73 'Sukali Ndizi' lines at different stages of fruit development in the second generation (R1) crop. Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) are shown at S6, S9, S12, and S15 representing 6, 9, 12 and 15 weeks after flowering, respectively, FG, full green mature fruit, FR, full ripe fruit and NT, non-transgenic control. Values are means ± SD, n= 3 technical replicates.

Table 4.4. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *Psy1B73* 'Sukali Ndizi' at different stages of fruit development in the second generation R1 crop

Transgenic Line*	Fruit maturity	Carotenoid (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
NT	S6	0.2	0.0	0.4	0.4	0.6
	S9	0.4	0.0	0.4	0.3	0.5
	S12	1.0	0.2	0.3	0.2	0.5
	S15	2.2	0.5	0.3	0.4	0.7
	FG	3.7	0.8	0.3	0.3	0.9
	FR	5.1	0.3	0.2	0.1	0.4
P1-11	S6	8.5	1.1	4.6	0.6	5.5
	S9	10.2	1.8	3.4	1.0	4.8
	S12	4.8	0.8	0.4	0.3	0.9
	S15	3.2	0.2	0.6	0.6	1.0
	FG	6.7	1.6	3.6	1.0	4.8
	FR	8.9	0.9	3.2	1.0	4.1
P1-39	S6	0.7	0.0	14.3	6.0	17.3
	S9	0.8	0.2	11.5	4.4	13.8
	S12	2.0	0.9	10.8	3.5	13.0
	S15	4.9	1.9	9.5	3.1	12.0
	FG	0.8	0.1	13.5	2.9	15.0
	FR	0.9	0.2	10.2	2.1	11.3

*Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) are shown at S6, S9, S12, and S15 representing 6, 9, 12 and 15 weeks after flowering respectively. FG, full green mature fruit; FR, full ripe fruit and NT, non-transgenic control. Values are means of n= 3 technical replicates.

17.3 µg/g DW of β-CE. These levels remained steady throughout bunch development to reached 15 µg/g DW at FG (Figure 4.5 and Table 4.4).

In addition to examining β-CE content, the amount of each individual carotenoid in samples from the non-GM control and the transgenic 'Sukali Ndizi' lines (P1-11 and P1-39) were also calculated as a percentage of the total amount of carotenoids. This was done at all of the various stages of fruit development and in the R1 crop only (Figure 4.6). In the wild-type control (NT) and P1-11, lutein constituted at least 50% of all carotenoids measured with the exception of the S6 and S9 stages in the non-GM control. In the non-GM control, the increase in lutein during fruit development correlated with a gradual drop in β-carotene content and an increase in α-carotene. In lines P1-11 and P1-39, a similar trend was seen whereby an increase in the proportion of lutein was correlated with an increase in α-carotene and a decrease in overall β-carotene. However, in line P1-39, the overall proportions of the main PVA carotenoid, β-carotene, were roughly between 65 to 95% depending on the developmental stage of the fruit (Table 4.4). Enhanced carotenoid accumulation in the fruit pulp of the transgenic line P1-39 was associated with a change in fruit coloration. Figure 4.7 shows representative pictures of longitudinal and cross sections of fruits from the non-transgenic wild-type and transgenic *Psy1B73* line P1-39 with enhanced orange colour. In addition, chromatograms in Figure 4.7 show representative PVA HPLC profiles demonstrating a shift in the type of carotenoids being accumulated from proportionally more non-PVA carotenoids (lutein) in the wild-type to proportionally more PVA carotenoid (β-carotene) in line P1-39.

Analysis of PVA accumulation in fruit was also done across 4 generations (MP, R1, R2 and R3) of the non-GM plant and two Psy1B73 transgenic 'Sukali Ndizi' lines at the FG fruit stage only (Figure 4.8 and Table 4.5). The levels of PVA carotenoids in the non-GM control showed little intergenerational fluctuation and remained low at around 1 µg/g DW β-CE (Table 4.5). In both transgenic lines, however, a clear increase in PVA levels

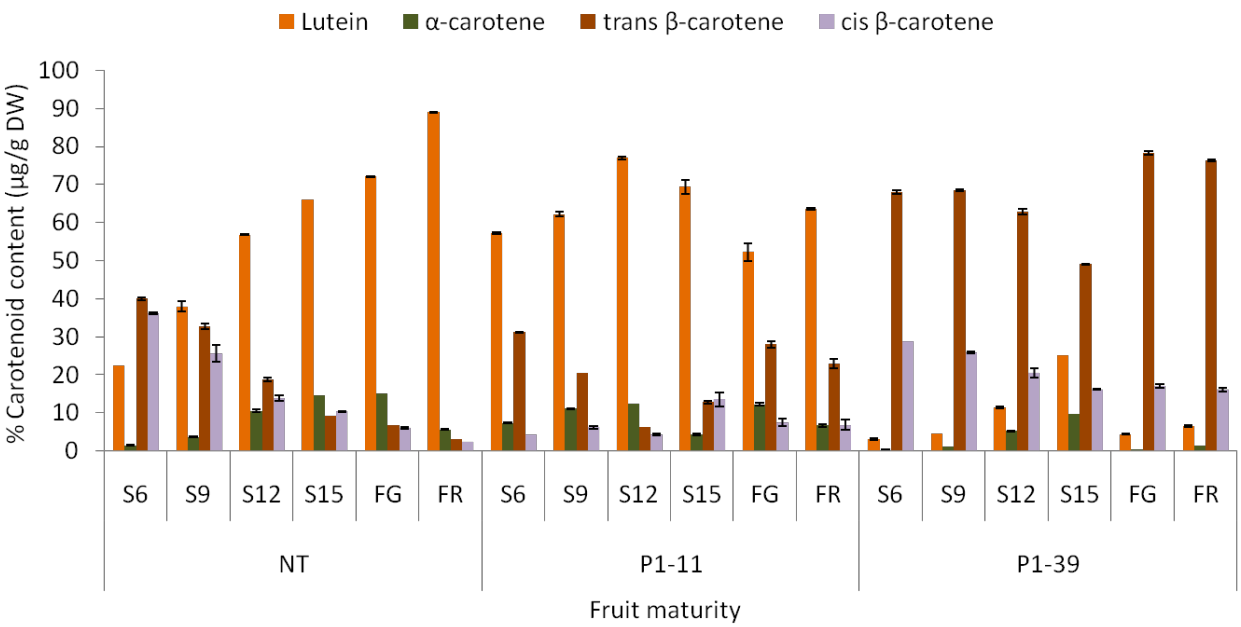

Figure 4.6. Percentage of respective carotenoids in the fruit pulp of transgenic Psy1B73 'Sukali Ndizi' lines at different stages of fruit development in the second generation R1 crop. Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) are shown at S6, S9, S12, and S15 representing 6, 9, 12 and 15 weeks after flowering, respectively, FG, full green mature fruit, FR, full ripe fruit and NT, non-transgenic control. Values are means of n= 3 technical replicates.

Figure 4.7. Longitudinal sections, cross sections and chromatograms showing representative PVA HPLC profiles of wild-type and *Psy1B*73 transgenic 'Sukali Ndizi' line NU211-12-39 (P1-39) at full green. Peaks were identified using a Multi Wavelength Detector at maximum wavelength (λmax= 450nm: Peak 1 - lutein; peak 2 - α-carotene; peak 3 - *trans*-β-carotene and peak 4 - *cis*-β-carotene.

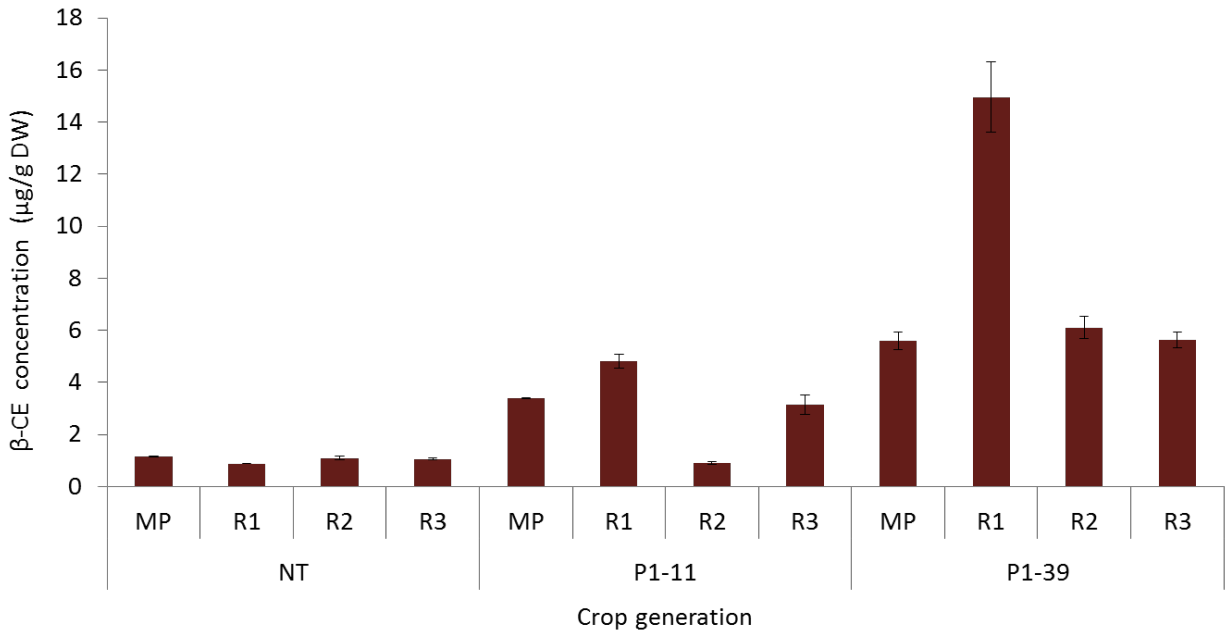

Figure 4.8. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *Psy1B73* 'Sukali Ndizi' lines at different crop generations. Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) are shown, MP, mother plant, R1, ratoon 1, R2, ratoon 2 and R3, ratoon 3. NT, non-transgenic control. Data from the mature green fruit, FG. Values are means ± SD, n= 3 technical replicates.

Table 4.5. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *Psy1B73* 'Sukali Ndizi' lines from different crop generations

Transgenic Line*	Crop Generation	Carotenoid (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
NT	MP	3.8	0.8	0.4	0.3	0.9
	R1	4.0	1.0	0.4	0.4	1.2
	R2	4.9	0.8	0.6	0.0	1.1
	R3	3.8	0.8	0.6	0.1	1.0
P1-11	MP	6.4	1.5	2.3	0.7	3.4
	R1	6.7	1.6	3.6	1.0	4.8
	R2	4.7	0.8	0.4	0.3	0.9
	R3	8.0	3.3	1.2	0.6	3.2
P1-39	MP	8.0	2.1	3.6	2.0	5.6
	R1	0.8	0.1	13.5	2.9	15.0
	R2	6.7	1.9	4.1	2.1	6.1
	R3	8.0	2.1	3.6	2.1	5.7

*Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-39 (P1-39) and NU211-12-11 (P1-11) are shown. MP, mother plant, R1, ratoon 1, R2, ratoon 2 and R3, ratoon 3. NT, non-transgenic control. Data from the mature green fruit, FG. Values are means of n= 3 technical replicates.

occurred during the transition from MP to the first ratoon R1, even though it is more pronounced and the levels were much higher in P1-39. From R2, the PVA levels tended to drop back to those levels observed in the first generation MP.

The amount of each individual carotenoid was also calculated as a percentage of the total amount of carotenoids (Figure 4.9). The distribution of individual carotenoids at the FG stage across 4 consecutive generations was consistent with that previously observed during fruit development in R1. Indeed, lutein always constituted around 50% of total carotenoids and any reduction in the amount of lutein was associated with an increase in the amount of β-carotene. The only exception to these observations was in R1 of line P1-39 for which β-carotene was clearly the major constituent, however the normal trend was re-established in that line in the successive two generations, R2 and R3 (Figure 4.9).

4.3.5. Further analysis of transgenic EAHB hybrid M9

Samples from the 6 transgenic EAHB hybrid M9 lines were obtained from the "mother crop" at bunch development stages S3, S6, S10 and FG, giving a total of 72 samples collected. Samples from non-genetically modified EAHB hybrid M9 plants were also collected as controls. The levels of transgene expression and PVA carotenoids accumulation were then assessed in the selected lines for the samples collected.

4.3.5.1. Transgene expression in transgenic EAHB hybrid M9

Initially, the relative level of transgene expression of *Psy1Q60* in the mother plant of EAHB hybrid M9 lines NU233-16-04 (P1-4), NU233-16-06 (P1-6), NU233-16-13 (P1-13) and NU233-16-43 (P1-43) was assessed by qPCR across 4 stages of fruit development (S3, S6, S10 and FG) and the data normalised and expressed relative to the *Psy1Q60* expression at the S3 stage of line P1-4 (Figure 4.10 and 4.11). Although a similar pattern of transgene expression was seen in lines P1-4 and P1-6, the level of *Psy1Q60* expression in line P1-6 was roughly twice as high at all developmental stages than line

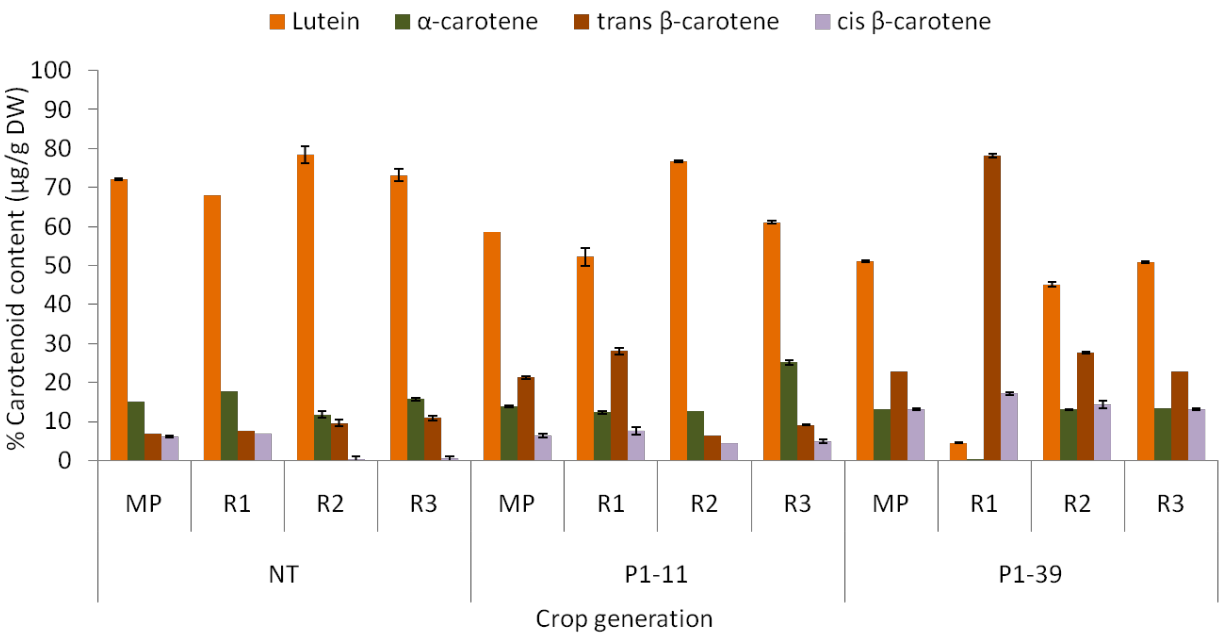

Figure 4.9. Percentage of respective carotenoids in the fruit pulp of transgenic *Psy1B73* 'Sukali Ndizi' lines at different crop generations. Transgenic *Psy1B73* 'Sukali Ndizi' lines NU211-12-11 (P1-11) and NU211-12-39 (P1-39) are shown, MP, mother plant, R1, ratoon 1, R2, ratoon 2 and R3, ratoon 3. NT, non-transgenic control. Data from the mature green fruit, FG. Values are means of n= 3 technical replicates.

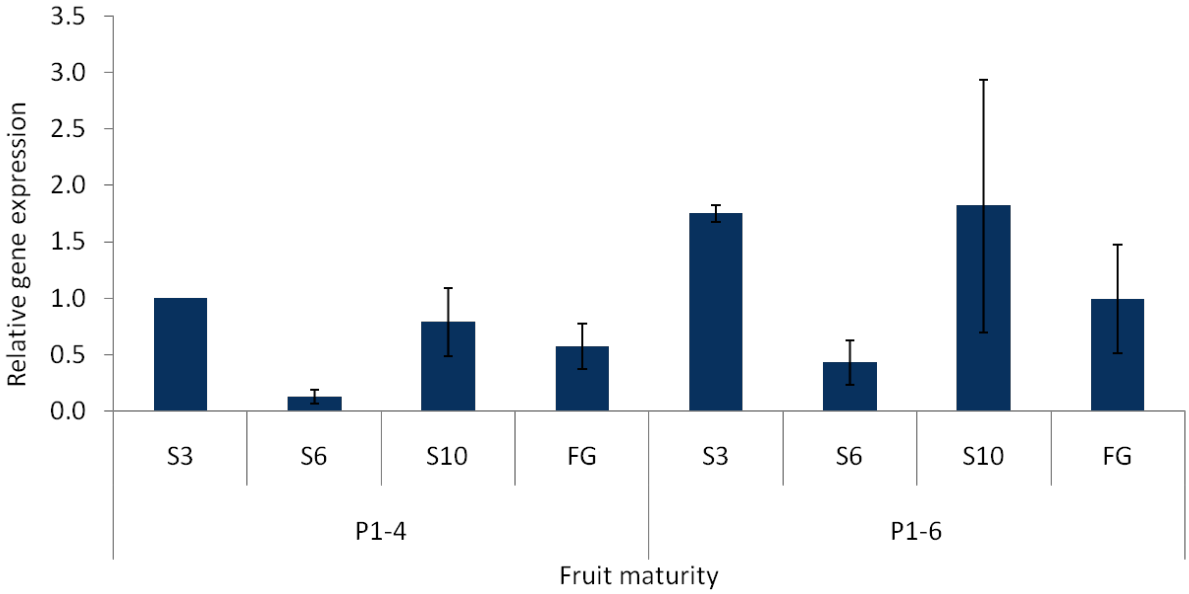

Figure 4.10. Relative expression of the *Psy1Q60* transgene in the fruit pulp of transgenic EAHB hybrid M9 lines at different stages of fruit development - RT-qPCR analysis. The mean expression of the *Psy1Q60* transgene in transgenic EAHB hybrid M9 lines NU233-16-4 (P1-4) and NU233-16-6 (P1-6) is shown at S3, S6 and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit. Values are relative to the mean expression value of P1-4 at S3 stage, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 technical replicates.

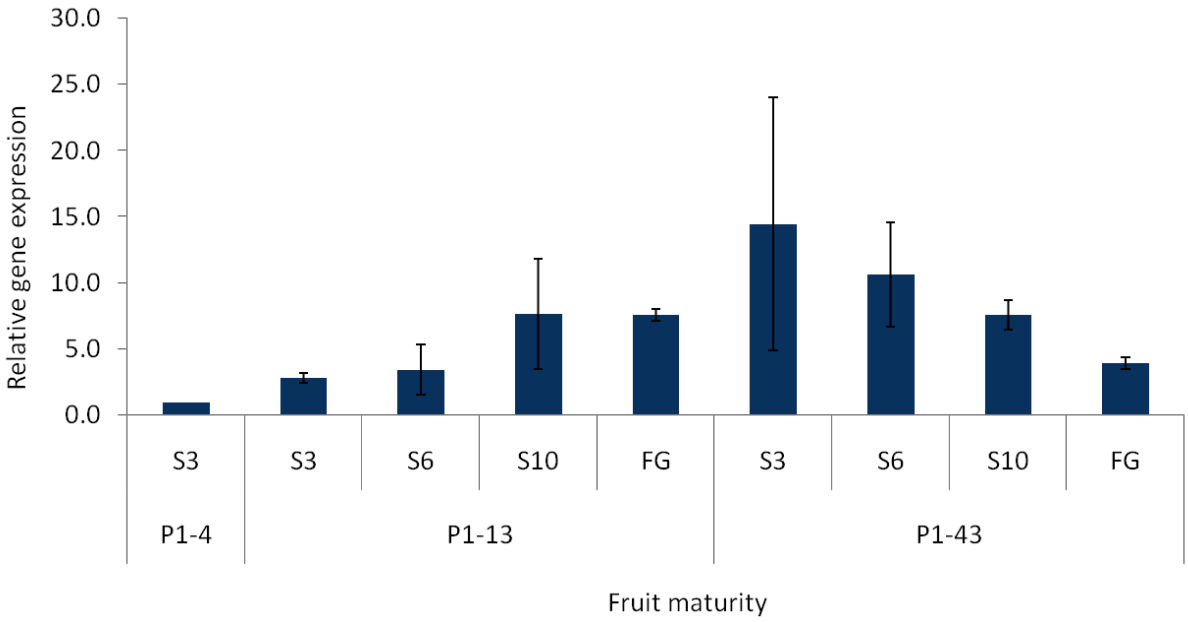

Figure 4.11. Relative expression of the *Psy1Q60* transgene in the fruit pulp of transgenic EAHB hybrid M9 lines at different stages of fruit development - RT-qPCR analysis. The expression of the *Psy1Q60* transgene in transgenic EAHB hybrid M9 lines NU233-16-13 (P1-13) and NU233-16-43 (P1-43) is shown at S3, S6 and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit. Values are relative to the mean expression value of P1-4 at S3 stage, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 technical replicates.

P1-4. In both these lines, a 4-fold reduction in *Psy1Q60* expression was seen from S3 to S6 followed by an increase to S10 and final decrease in transcript abundance at FG. In line P1-13, a constant increase in *Psy1Q60* expression was seen across fruit development from S3 to FG where the levels were 8-fold over the levels of P1-4 at S3. The opposite trend was seen in line P1-43 where the levels of expression started at just below 15-fold over the levels of P1-4 at S3 and constantly dropped to about 3-fold at FG.

Similarly, the level of *APsy2a* expression was analysed in 2 selected transgenic EAHB hybrid M9 lines NU233-13-03 (P2-03) and NU233-13-10 (P2-10) by qPCR, the data normalised and expressed relative to the *APsy2a* expression at the S3 stage of line P2-3 (Figure 4.12). Similar to the expression of *Psy1Q60* in lines P1-4 and P1-6, an initial reduction in expression was seen from S3 to S6 followed by an increase through to FG.

4.3.5.2. Carotenoid accumulation in transgenic EAHB hybrid M9

Similarly to the transgenic 'Sukali Ndizi' lines P1-11 and P1-39, the levels of PVA accumulation were analysed in all 6 transgenic EAHB hybrid M9 lines at each fruit development stage (S3, S6, S10 and FG) for the mother plant (MP) generation only. For all M9 lines tested, including the NT control line, the levels of β-CE progressively increased during fruit development (Figure 4.13 and Table 4.6). The non-transgenic NT line reached a maximum of 5.8 µg/g DW β-CE at FG while *Psy1Q60* transgenic lines P1-4, P1-6, P1-13 and P1-43 reached a maximum of 11.1, 10.3, 5.6 and 10.0 µg/g DW β-CE at FG, respectively (Table 4.6). Figure 4.14 shows the proportions of respective carotenoid measured in *Psy1Q60* lines and, as previously observed in the 'Sukali Ndizi' cultivar, the levels of the non-PVA carotenoid lutein were the highest (in proportion) at the early stages of fruit development (S3 and S6). As the fruit matured, the increase in β-CE was associated with a drop in the level of lutein in favour of the PVA carotenoids, α- and β-carotene. Indeed, at the later stages of fruit development (FG) in the wild-type and *Psy1Q60* lines of the M9 cultivar, α- and β-carotene represented a higher proportion

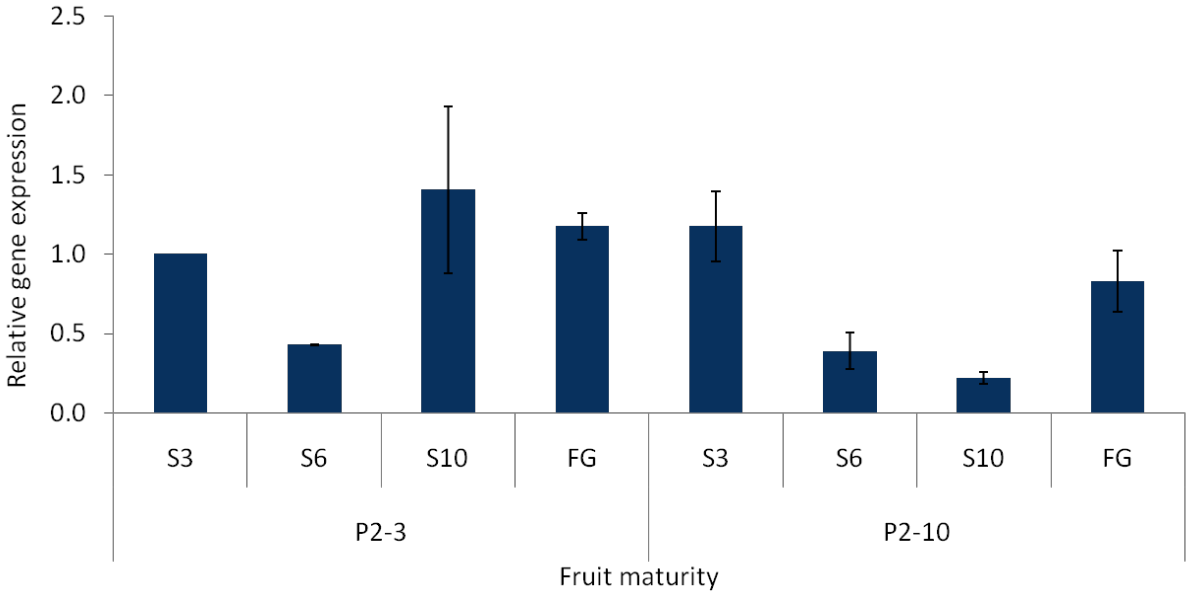

Figure 4.12. Relative expression of the *APsy2a* transgene in the fruit pulp of transgenic EAHB hybrid M9 lines at different stages of fruit development - RT-qPCR analysis. The expression of the *APsy2a* transgene in transgenic EAHB hybrid M9 lines NU233-13-3 (P2-3) and NU233-13-10 (P2-10) is shown at S3, S6 and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit. Values are relative to the mean expression value of P2-3 at S3 stage, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 technical replicates.

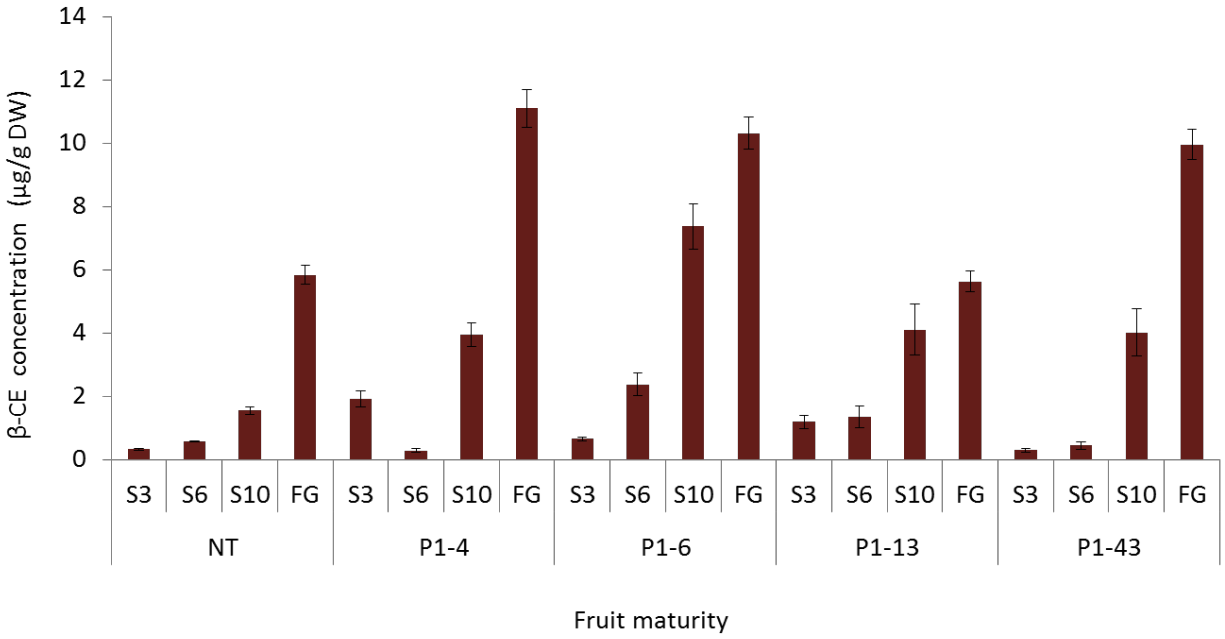

Figure 4.13. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *Psy1Q60* hybrid M9 lines at different stages of fruit development. Transgenic *Psy1Q*60 hybrid M9 lines NU233-16-4 (P1-4), NU233-16-6 (P1-6), NU233-16-13 (P1-13) and NU233-16-43 (P1-43) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means ± SD, n= 3 technical replicates.

Table 4.6. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *Psy1Q60* EAHB hybrid M9 lines at different stages of fruit development

Transgenic Line*	Fruit maturity	Carotenoid (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
NT	S3	1.0	0.2	0.2	0.1	0.3
	S6	1.5	0.5	0.3	0.2	0.6
	S10	1.6	1.5	0.6	0.5	1.6
	FG	1.4	4.6	2.8	1.5	5.8
P1-4	S3	1.7	0.4	1.3	0.8	1.9
	S6	1.2	0.3	0.1	0.0	0.3
	S10	2.0	3.2	1.7	1.2	3.9
	FG	2.3	9.4	5.4	2.0	11.1
P1-6	S3	1.2	0.3	0.4	0.0	0.7
	S6	1.9	0.5	1.6	1.0	2.4
	S10	2.2	6.4	3.4	1.5	7.4
	FG	2.6	9.1	4.8	1.9	10.3
P1-13	S3	3.2	0.6	0.6	0.5	1.2
	S6	1.8	0.4	0.8	0.7	1.3
	S10	2.6	2.8	2.2	1.1	4.1
	FG	2.7	3.8	3.0	1.5	5.6
P1-43	S3	0.7	0.2	0.2	0.0	0.3
	S6	0.6	0.3	0.3	0.0	0.4
	S10	2.1	2.7	2.1	1.1	4.0
	FG	2.5	8.3	4.8	1.9	10.0

*Transgenic *Psy1Q*60 (P1) EAHB hybrid M9 lines NU233-16-4 (P1-4), NU233-16-6 (P1-6), NU233-16-13 (P1-13) and NU233-16-43 (P1-43) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means of n= 3 technical replicates.

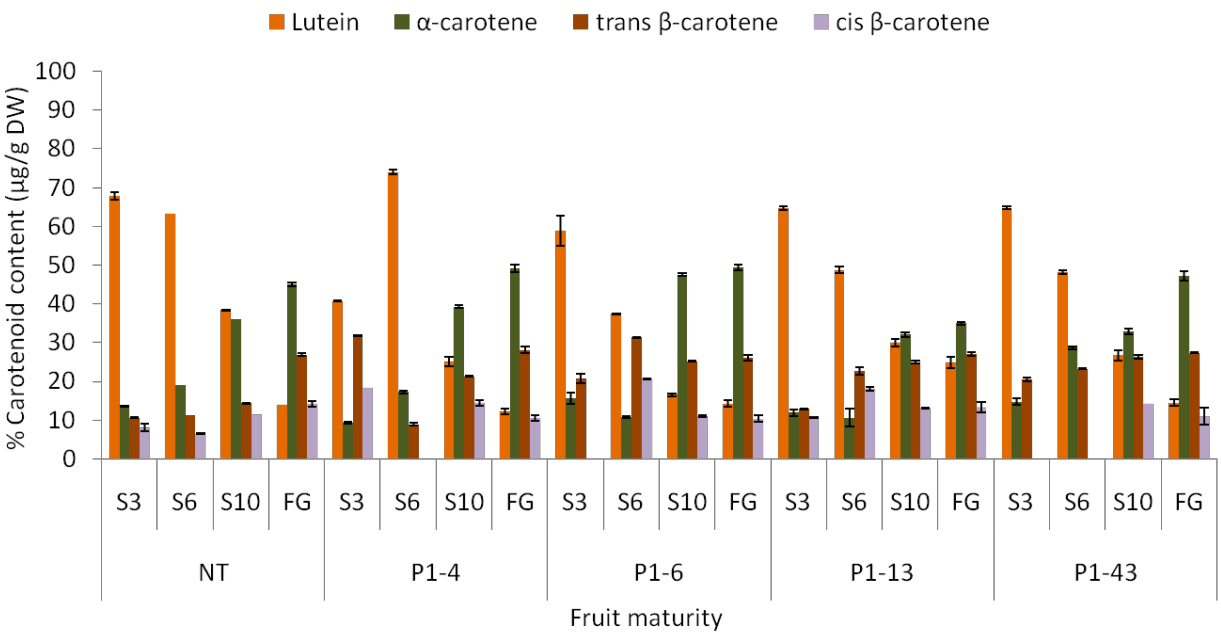

Figure 4.14. Percentage of respective carotenoids in the fruit pulp of transgenic *Psy1Q60* EAHB hybrid M9 lines at different stages of fruit development. Transgenic *Psy1Q60* EAHB hybrid M9 lines NU233-16-4 (P1-4), NU233-16-6 (P1-6), NU233-16-13 (P1-13) and NU233-16-43 (P1-43) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means of n= 3 technical replicates.

(30-50%) of all carotenoids (Table 4.6). Interestingly, this observation was different from *Psy1B73* lines of the 'Sukali Ndizi' cultivar, where β-carotene was the major constituent.

The levels of β-CE and the proportion of carotenoids in transgenic M9 lines P2-3 and P2-10 expressing *Apsy2a* were also analysed (Figure 4.15, 4.16 and Table 4.7). Similar to the M9 *Psy1Q60* lines, the β-CE levels increased constantly during fruit development to 7.3 µg/g DW β-CE at FG in line P2-3 and 33.1 µg/g DW β-CE in line P2-10. In both lines, lutein was a major constituent at S3 and S6 with more than 70% of all carotenoids measured. This trend reversed from S10 onwards with an increased accumulation of α- and β-carotene at just below 40% and just above 50% respectively in both lines.

4.3.6. Phenotype and agronomic data analysis

The agronomic performance of all 8 transgenic banana lines (2 'Sukali Ndizi' and 6 EAHB hybrid M9 lines) as well as non-transgenic control lines was evaluated under field conditions. The fruit maturation times (flowering to fruit maturity) of the 2 *Psy1B73* expressing 'Sukali Ndizi' lines were in the range of 18-19 weeks, which is similar to the non-transgenic control plants (Table 4.8). Except for line P1-39 being slightly taller, all other agronomic characteristics measured were similar to the control plants. However, all 'Sukali Ndizi' transgenic lines expressing *Psy1B73* or *Psy1Q60* under the control of the expansin 1 promoter displayed a characteristic "Golden leaf" phenotype. In these lines, golden and yellow streaks on the three inner young leaves were observed during the early stages of plant development (Figure 4.17-A). This characteristic leaf coloration was mainly observed during the rainy seasons with the leaves reverting to a normal green colour during the dry/sunny seasons. The petioles of these plants also displayed a distinctive yellow/orange colour, as did some parts of the fruits and stalk at the full green stage (Figures 4.17-C and 4.18-A and B).

In comparison to the non-transgenic M9 control plants, transgenic M9 line P2-10 showed differences in most of the agronomic traits examined in this study. Differences

Figure 4.15. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *APsy2a* EAHB hybrid M9 lines at different stages of fruit development. Transgenic *APsy2a* EAHB hybrid M9 lines NU233-13-10 (P2-10) and NU233-13-3 (P2-3) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means ± SD, n= 3 technical replicates.

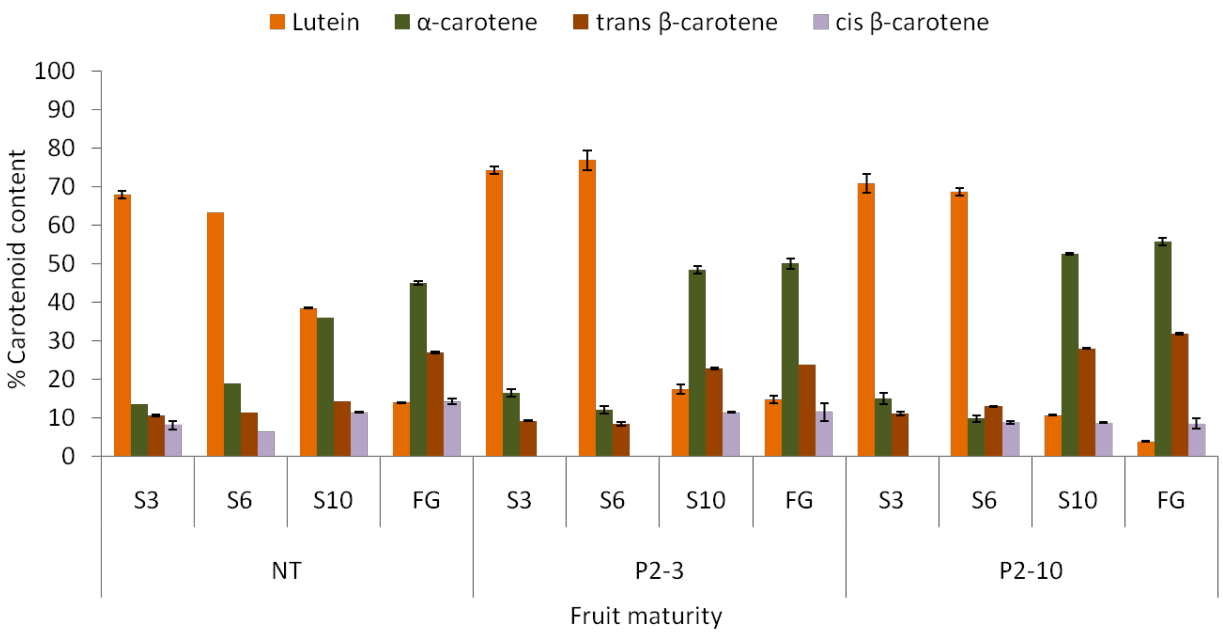

Figure 4.16. Percentage of respective carotenoids in the fruit pulp of transgenic *APsy2a* EAHB hybrid M9 lines at different stages of fruit development. Transgenic *APsy*2*a* EAHB hybrid M9 lines NU233-13-10 (P2-10) and NU233-13-3 (P2-3) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means ± SD, n= 3 technical replicates.

Table 4.7. HPLC analysis - Carotenoid concentration in the fruit pulp of transgenic *APsy2a* EAHB hybrid M9 lines at different stages of fruit development

Transgenic Line*	Fruit maturity	Carotenoid (µg/g DW)				
		Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
NT	S3	1.0	0.2	0.2	0.1	0.3
	S6	1.5	0.5	0.3	0.2	0.6
	S10	1.6	1.5	0.6	0.5	1.6
	FG	1.4	4.6	2.8	1.5	5.8
P2-3	S3	1.4	0.3	0.2	0.0	0.3
	S6	4.6	0.7	0.5	0.0	0.9
	S10	1.6	4.6	2.1	1.1	5.0
	FG	2.0	6.7	3.2	1.5	7.3
P2-10	S3	2.4	0.5	0.4	0.0	0.7
	S6	3.6	0.5	0.7	0.5	1.2
	S10	2.6	13.0	6.9	2.2	14.5
	FG	2.0	28.9	16.5	4.4	33.1

*Transgenic *APsy2a* (P2) EAHB hybrid M9 lines NU233-13-3 (P2-3) and NU233-13-10 (P2-10) are shown at S3, S6, and S10 representing 3, 6 and 10 weeks after flowering respectively, FG, full green mature fruit and NT, non-transgenic control. Values are means of n= 3 technical replicates.

Table 4.8. Phenotypic and agronomic characteristic for selected CFT1 'Sukali Ndizi' lines

Transgenic line	Plant HEIGHT (cm)	Bunch WEIGHT (kg)	Bunch HEIGHT (cm)	Bunch Circumf. (cm)	Leaf Color	*Pulp Color	Fruit maturity duration (weeks)
P1-11	307	10	56	73	GREEN	CREAMY ORANGE	19
P1-39	347	8	81	90	GREEN	CREAMY ORANGE	18
Control plants (n= 4)	307	11	74	84	GREEN	CREAMY WHITE	19

*The fruit pulp of transgenic *Psy1B*73 lines had an orange colour characteristic to β-carotene rich food while fruit from non-transgenic control plants had a white creamy colour.

Figure 4.17. Phenotypic observations of a 'Sukali Ndizi' transgenic line expressing *Psy1B*73 under the control of the expansin 1 promoter. A, first fully expended "Golden leaf"; B, emergence of the bunch; C, fruits at full green stage (FG) and D, sucker formation at the base of the plant. The young leaves, fruit stalk and sucker of transgenic *Psy1B*73 lines showed the characteristic golden/orange colour, which eventually turned green at flowering stage.

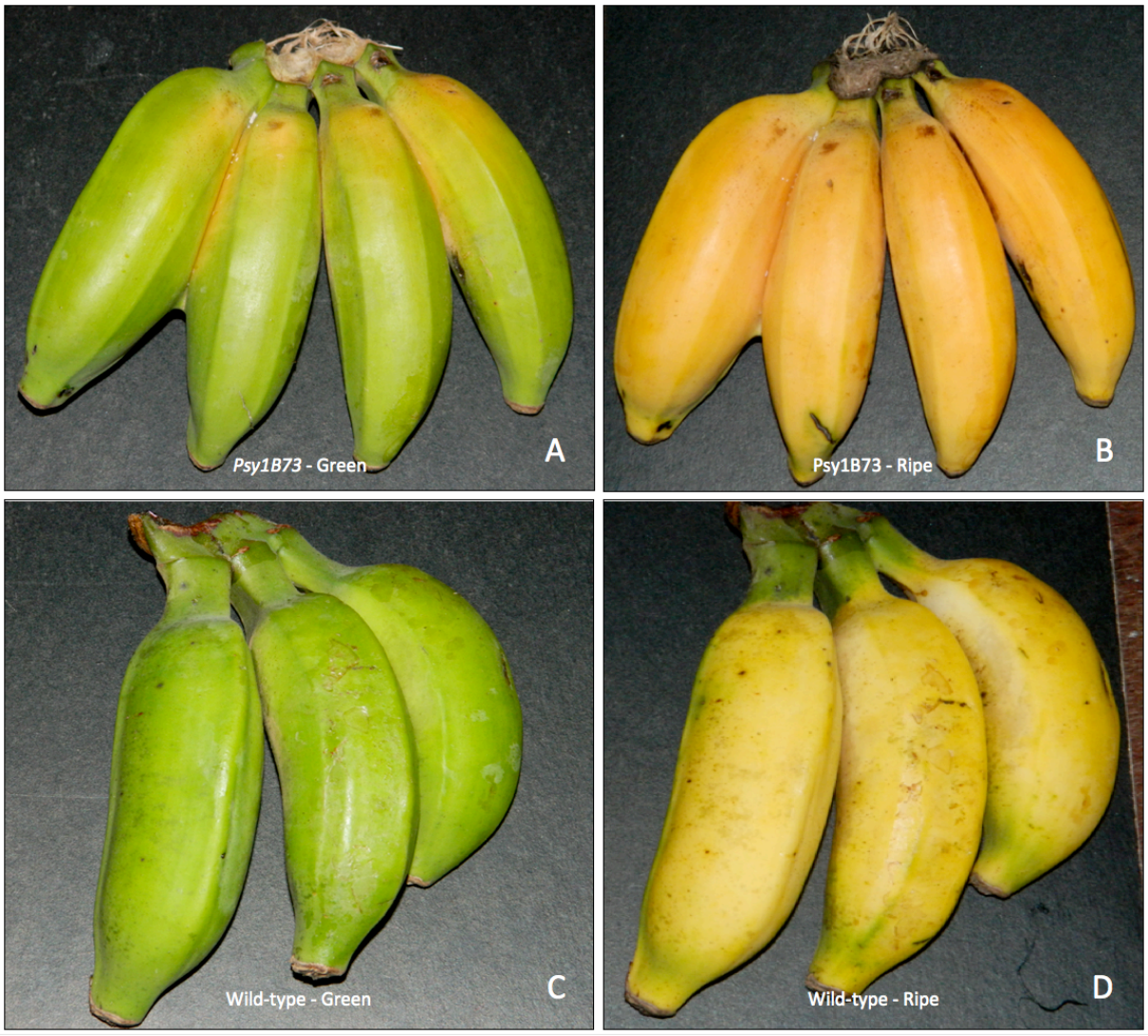

Figure 4.18. Phenotypic observations of mature green (FG) and ripe (FR) fruits of a 'Sukali Ndizi' non-transgenic control and transgenic line expressing *Psy1B*73 under the control of the expansin 1 promoter. The fruit pulp of transgenic *Psy1B*73 lines had an orange colour characteristic to β-carotene rich food both at full green and full ripe compared to the yellow colour of the non-transgenic control line at full ripe.

were observed for full green bunch weight as well as fruit maturation time, which were 10.5 kg and 14 weeks respectively for transgenic line P2-10 compared to 20.5 kg and 16 weeks for control plants (Table 4.9). In contrast, the agronomic traits of the other transgenic M9 line were similar to those of the non-transgenic control counterparts. Table 4.10 summarises the β-CE levels and bunch weights for all the lines generated during this project and shows what might be a negative correlation between carotenoid content and a yield in the generated events.

Table 4.9. Phenotypic and agronomic characteristic for selected CFT2 EAHB hybrid M9 lines

Transgenic line	Plant HEIGHT (cm)	Bunch WEIGHT (kg)	Bunch HEIGHT (cm)	Bunch Circumf. (cm)	Leaf Color	Pulp Color	Fruit maturity duration (weeks)
P1-4	341	16	70	117	GREEN	CREAM	15
P1-6	321	18	65	119	GREEN	CREAM	16
P1-13	322	10	57	105	GREEN	CREAM	15
P1-43	375	18	62	119	GREEN	CREAM	15.5
P2-3	329	20	67	120	GREEN	CREAM	15
P2-10	257	10.5	56	112	GREEN	CREAM ORANGE	14
Control plants (n= 2)	309.5	20.5	67	125	GREEN	CREAM	16

Table 4.10. Summary of β-CE levels to bunch weight relationship

Transgenic line	Cultivar	β-CE at FG (µg/g DW)	PVA change (fold)	Bunch weight (kg)	Bunch weight reduction (%)
P1-11	'Sukali Ndizi'	4.8	5.3	10	9.1
P1-39	'Sukali Ndizi'	15	16.7	8	27.3
Control line	'Sukali Ndizi'	0.9	1.0	11	0.0
P1-4	EAHB hybrid M9	11.1	1.9	16	22.0
P1-6	EAHB hybrid M9	10.3	1.8	18	12.2
P1-13	EAHB hybrid M9	5.6	0.97	10	51.2
P1-43	EAHB hybrid M9	10	1.7	18	12.2
P2-3	EAHB hybrid M9	7.3	1.3	20	2.4
P2-10	EAHB hybrid M9	33.1	5.7	10.5	48.8
Control line	EAHB hybrid M9	5.8	1.0	20.5	0.0

4.4. Discussion

In this chapter, transgene expression and carotenoid accumulation in 'Sukali Ndizi' and EAHB hybrid M9 lines transformed with genes *Psy1B73*, *Psy1Q60* and *APsy2a* were investigated. In summary, several lines with increased carotenoid accumulation in mature green fruit samples were identified, and these lines contained β-CE concentrations of up to 15.0 and 33.1 µg/g DW in *Psy1B73* 'Sukali Ndizi' line P1-39 and *Apsy2a* M9 line P2-10, respectively. An interesting observation was that no direct correlation between the levels of transgene expression and PVA accumulation was seen in these lines. The effects of transgene expression and carotenoid accumulation on the agronomical performance of each line were also investigated and very high accumulation of PVA in *Apsy2a* line P2-10 was found to be associated with a reduction in bunch size of 48.8%.

Being the first committed step into carotenoid biosynthesis, phytoene synthase (PSY) has been the primary target of genetic engineering approaches aimed at increasing the level of carotenoids in plants. In crops such as rice, tobacco, tomato, and canola, the strategy of *Psy* over-expression has been used with great success to increase the accumulation of PVA carotenoids (Römer *et al.*, 2000; Ye *et al.*, 2000; Paine *et al.*, 2005). In the present study, FG fruit from the transgenic 'Sukali Ndizi' line P1-39 expressing the *Psy1B73* transgene was shown to have 16.7-fold more PVA compared to non-GM control fruit. The increase in PVA levels commenced early on during fruit development and was maintained through to maturity of the fruit. In contrast, only a 5.3-fold increase in PVA was seen at the FG stage of line P1-11. Interestingly, line P1-11 and P1-39 had similar low levels of transgene expression, which indicates a poor correlation between transgene expression levels and accumulation of PVA carotenoids in the fruit of the 'Sukali Ndizi' cultivar. These results are in agreement with an earlier study in *Arabidopsis thaliana* where it was observed that a small amount of *AtPSY* is sufficient to produce high carotenoid levels in photosynthetically active tissue (Maass *et al.* 2009). Therefore, like other model crops, high transgene expression level does not appear to be critical for

accumulating high levels of PVA in the *Psy1B73* 'Sukali Ndizi' lines. The differences observed in PVA accumulation of lines P1-11 and P1-39 could be due to other factors such as levels of *Psy1B*73 protein and its activity, transgene integration pattern in the plant genome, catabolic activity on carotenoids or environmental pressure. Regarding the latter, carotenoid content in the fruit is made to vary depending on the season of fruit development and harvest. Environmental influences on carotenogenesis and carotenoid catabolism in plants are common and well documented. For example, light has been shown to have a direct effect on carotenoid gene expression and carotenoid accumulation in carrots (Stange *et al.*, 2008; Fuentes *et al.*, 2012). Depending on the plant requirements, carotenoids can also be broken down to form other downstream products of the carotenoid pathway such as the hormone abscisic acid (ABA) and other regulators, which are required for the plant growth and development (Eroglu *et al.*, 2012). The rate and balance of carotenoid biosynthesis and breakdown may vary from plant to plant and tissue to tissue and could explain some of the differences seen in this study.

Analysis of the PVA levels in the 2 transgenic 'Sukali Ndizi' lines during 4 crop generations revealed a substantial increase in PVA accumulation from the mother crop (MP) to the first ratoon (R1). This trend was also observed, albeit at a lower level, in the non-transgenic control line. A possible explanation for this is that, following planting into the ground, tissue cultured banana plants require some time to recover from the stress of being *in-vitro* for extended periods of time. It is common for the first generation of tissue cultured plants to require a period of adaptation after planting during which time the plant is not at its optimal physiological potential (Rai *et al.*, 2007). Although the higher PVA levels seen in the R1 generation were not maintained in successive generations, the PVA levels were still higher than those of the non-transgenic control plants over the 4 generations. This indicates that the enhanced PVA trait in transgenic 'Sukali Ndizi' banana, although influenced by environmental variables and non-environmental factors, is heritable through successive generations and not prone to

gene silencing. Similar results were observed in rice where successive progenies accumulated more carotenoids than their respective parents (Datta *et al.*, 2007; Rai *et al.*, 2007). However, in a related study in tomato, though the progenies were observed to have inherited the improved carotenoid phenotype, the metabolite levels in T_1 and T_2 generations were less than those in the T_0 lines (Fraser *et al.*, 2002).

All EAHB hybrid M9 transgenic lines expressing *Psy1Q60* showed a consistent increase in PVA accumulation from S3 to FG. Similar to observations made in the 'Sukali Ndizi' cultivar, no correlation was observed between the pattern and level of transgene expression and the pattern and levels of PVA accumulation. For example, although line P1-4 had up to 14-fold lower levels of transgene expression than line P1-13, it accumulated up to 11.5 µg/g DW β-CE at FG compared to only 5.6 µg/g DW β-CE in line P1-13. Similarly, in M9 transgenic lines P2-3 and P2-10 expressing *Apsy2a,* no correlation between transgene expression levels and PVA accumulation was seen. Both lines had similar patterns and low level of transgene expression but line P2-10 was able to accumulate as high as 33.1 µg/g DW β-CE at FG compared to only 7.3 µg/g DW β-CE in line P2-3. Irrespective of the cultivar, 'Sukali Ndizi' or M9, *Psy1B73 and Psy1Q60* have shown to be useful transgenes for the generation of lines with enhanced PVA levels at harvest (FG). As far as reaching PVA biofortification target levels is concerned, the *Apsy2a* transgene seems to be a more promising candidate since, out of only 2 lines tested, one (P2-10) had accumulated levels of PVA beyond what would be required to meet 50% of the estimated average requirement (EAR) in Uganda. Indeed, line P2-10 can provide 33.1 µg/g DW of β-CE or an equivalent to around 800 µg/100g fresh weight (FW). Considering that (i) only 120µg retinol equivalents (RE) per day are required to reach 50% of the estimated average requirement (EAR) in children, (ii) a child would consume at least 200g fresh weight (FW) of processed banana fruit per day and (iii) the β-carotene to retinol conversion ratio is estimated at 6:1 for banana, then a child would consume from line P2-10 around 267µg retinol equivalents (RE). Even if only 50% of that

amount was retained during the cooking process that child would get more than 120μg retinol equivalents (RE) per day.

The expression of the three transgenes used in the present study was controlled by the banana expansin 1 promoter. Results from studies using the tomato expansin 1 (*LeExp1*) gene have shown the gene to be specifically expressed in ripening fruit and to be regulated by ethylene, a hormone known to coordinate and induce ripening in many species (Rose *et al.*, 1997). As such, it was expected that the expression of the *Psy1B73*, *Psy1Q60* and *APsy*2*a* transgenes in transgenic banana lines would increase from the early stages of fruit development through to ripening, with peak expression between FG and FR. Unexpectedly, this trend was not observed.

Phytoene synthase catalyses the first committed reaction in the carotenogenesis pathway. The enzyme activity is critical for carotenoid accumulation in plant tissue, however, carotenoid accumulation may be affected by other plant physiological changes such as carotenoid catabolism and competitive uptake of the primary carotenoid biosynthetic pathway substrate, geranylgeranyl diphosphate (GGPP), by other isoprenoid pathways (Maass *et al.*, 2009; Fuentes *et al.*, 2012; Ruiz-Sola *et al.*, 2014). For example, expression of carotenoid dioxygenase enzyme CCD4 was directly linked to the varying accumulation of carotenoids and carotenoid-derived volatiles in peach (*Prunus persica L.* Batsch.) fruit flesh (Brandi *et al.*, 2011). In transgenic 'Sukali Ndizi' and M9 lines, overexpression of the *Psy* gene driven by the expansin 1 promoter resulted in varying levels of carotenoid accumulation in the fruit pulp. This may have been as a result of varying levels of carotenoid breakdown by the dioxygenases to form downstream products such as abscisic acid in order to support other plant processes (Ruiz-Sola *et al.*, 2014). Another possibility is the inability of the banana fruit pulp tissue to regulate and maintain sufficient chromoplast formation meaning that newly formed carotenoid compounds do not have the supporting structure for storage and protection. Indeed, in related studies in cauliflower (*Brassica oleracea* var. botrytis) the "Orange"

(*Or*) gene capacity to increase carotenoid accumulation in the plant tissue revealed that its expression has direct effect on chromoplast differentiation which subsequently affect carotenoid accumulation in plant tissue (Li *et al.*, 2001; Li *et al.*, 2006; Lu *et al.*, 2006; Lopez *et al.*, 2008) through formation of carotenoid-sequestering structures such as chromoplasts (Lu *et al.*, 2006).

For all the transgenic 'Sukali Ndizi' and M9 lines tested, the proportion of each individual carotenoid was measured in the fruit at several developmental stages in the R1 generation. These analyses revealed that (i) in proportion, lutein was almost always the main carotenoid at the early stages of fruit development and (ii) the levels of β-CE were higher toward full maturity of the fruit, which was associated with a switch to lesser amount of lutein to benefit the accumulation of the PVA carotenoids α- and β-carotene. As described above, lutein was the main carotenoid present at the early stages but also throughout fruit development in the non-transgenic 'Sukali Ndizi' and as such β-CE levels remained low in that cultivar. In the non-transgenic M9 however, β-CE levels increased dramatically from S6 to FG because of a shift from high levels of lutein to high levels of the PVA carotenoids α- and β-carotene as described by observation (ii) above. This can be explained by the results from previous studies on enhancement of β-carotene accumulation in conventionally bred maize lines (Harjes *et al.*, 2008) and enhancement of carotenoids in lycopene β-cyclase (LCYB) over-expressed transgenic maize (Maneesha *et al.*, 2008). Results from these experiments suggested a natural variability in the intersecting lycopene cyclases, which alters the flux through the β, Ɛ branch of the pathway, hence the varying accumulation of β-carotene and lutein seen in the maize kernels. Interestingly, the levels of β-carotene (all trans- and cis-) were proportionally high from the early stages of development in 'Sukali Ndizi' transgenic line P1-39 (at R1). It is speculated that these results might be due to endogenous lycopene β-cyclase activity channeling lycopene into the production of more β-carotene very early on in this line.

In addition to gene expression analysis and analytical chemistry analysis of carotenoids, the transgenic lines were monitored for phenotypic abnormalities and agronomical characteristic. The first obvious observation made was that the high level of PVA accumulation in transgenic EAHB M9 line P2-10 was associated with altered agronomic characteristics such as fewer bananas on the bunch resulting in lower bunch weight and smaller plant stature. Physiological imbalance in the MEP pathway, at a step where condensation reactions on the C20 GGPP result in C40 carotenoids and other prenyllipids and diterpenes could explain changes in growth characteristics in plants that have the ability to accumulate high levels of PVA (DellaPenna and Pogson, 2006; Lu and Li, 2008). The banana cultivar, 'Asupina', from which the *Apsy2a* transgene was derived, and other naturally occurring Fe'i type banana that are rich in carotenoids, have a characteristic small bunch and long cycle time. The overexpression of the *Apsy2a* gene may have led to an extreme acceleration of the isoprenoid metabolite influx into the carotenoid branch resulting into limited influx of required C20 GGPP into the other pathways, including the gibberellin pathway that regulates plant growth (Lu and Li, 2008).

Further, a "golden leaf" phenotype in 'Sukali Ndizi' lines P1-11 and P1-39 was the most distinctive trait observed. Interestingly, this phenotype which is only characteristic to the Exp1-*Psy1B73* promoter-gene combination was also seen in related transgenic banana field trials conducted by QUT in Australia with both 'Cavendish' and 'Lady finger' banana cultivars (James Dale personal communication). In Uganda as well as in Australia, all new leaves emerged with a golden/yellow colour which was progressively replaced by a "normal" green colour as the leaves matured. A possible explanation would be that carotenoids are highly expressed very early on and before the leaf emerges from the plant. At that stage the pool of GGPP, the common precursor to carotenoids and chlorophylls, is highly depleted to produce carotenoid at the expenses of chlorophyll. However, as the leaf mature and under increased light intensity, the expansin promoter becomes less active and the production of carotenoids is reduced to

favor the production of chlorophyll (Anderson and Chow, 2002; Telfer, 2002). In addition, a previous study on sour cherry *(Prunus cerasus* L.) showed that mRNA from the *PcExp1* gene was detected in leaves, petioles, petals and in fruits throughout growth and ripening, with the highest expression in the fully ripe fruits (Karaaslan and Hrazdina, 2010). By association, it is possible that the Exp1 promoter from banana (MaExp1) is not as tightly regulated as previously anticipated and might 'leak' during vegetative tissue expansion allowing increased accumulation of carotenoids in newly emerging leaves.

The adoption of the expansin 1 fruit specific promoter in these experiments was intended to enhance PVA accumulation in the mature fruit while moderating possible biochemical disruptions caused by an overexpressed *Psy* gene, a strategy previously adopted in transgenic tomato (Fraser *et al.*, 2002). 'Sukali Ndizi' line P1-39, unlike the other events generated had high PVA accumulation from the early stages of fruit development indicating a divergent phenomenon from the transgenic tomato experiments of Rose *et al*., 1997. However, the obtained results from line P1-39 confirmed observations made in sour cherry where the *PcExp1* gene was found to be active at all stages of fruit development through to fruit ripening (Karaaslan and Hrazdina, 2010).

Because of the above mentioned observations, the possibility of using different promoters to tightly control the expression of the phythoene synthase transgene during the development of the banana fruit pulp need to be considered in future studies.

Chapter 5 - Phytoene synthase 2a gene expression and accumulation of pro-vitamin A carotenoids during fruit development in non-transgenic EAHB and Fe'i bananas

5.1. Introduction

In plants, the precursor to the carotenoid biosynthesis pathway is the C20-geranylgeranyl diphosphate (GGPP) (Eisenreich *et al.*, 2001; Bouvier *et al.*, 2005). GGPP is also the precursor to a number of other pathways responsible for the production of very important plant metabolites such as gibberellins, plastoquinones, terpenes, chlorophylls, tocopherols and phylloquinones. Consequently, the availability of GGPP can impact considerably on plant growth and development. The condensation of two GGPP molecules into 15-*cis*-phytoene by the enzyme phytoene synthase (PSY) is the first commited step in carotenoid biosynthesis. The PSY activity on GGPP is rate limiting (Santos *et al.*, 2005) as it provides an influx into the carotenoid pathway away from other competing pathways (Isaacson *et al.*, 2002; Park *et al.*, 2002).

As part of an ongoing effort toward PVA biofortification of EAHB at NARO, a *psy2a* (*Apsy2a*) gene was isolated from 'Asupina', a Fe'i type banana from which the fruit is naturally rich in PVA carotenoids. *Apsy2a* has been previously used in this study (Chapter 4) to generate EAHB hybrid M9 transgenic banana lines with elevated levels of PVA in the fruit. There is limited information, however, on the molecular basis of carotenoid accumulation in EAHB and Fe'i type bananas. Therefore, the purpose of this study was to investigate the role of the *Psy2a* gene in the carotenoid accumulation process in the fruit pulp of the EAHB 'Mpologoma' and the Fe'i type banana 'Asupina'.

5.2. Materials and Methods

5.2.1. Plant samples and material preparation

5.2.1.1. 'Mpologoma' samples

'Mpologoma' fruit samples included in this study were selected from 4 plants growing at the National Agricultural Research Laboratories (NARL), Banana Germplasm Centre, Uganda. 'Mpologoma' samples were collected on a weekly basis from the identified plants between the 6^{th} and the 12^{th} week after flowering and finally at FG and FR. For PVA analysis, fruit samples were taken from the selected hand at the top, middle and bottom positions of each bunch, with one finger sampled from each position. The fingers were peeled, the pulp diagonally cut into two sections and further sliced into smaller cross sections then mixed thoroughly to make a composite sample. All fruit samples were then freeze-dried under vacuum, manually crushed into a mortar and stored at -80^{o}C before carotenoid quantification.

5.2.1.2. 'Asupina' samples

A total of 3 'Asupina' samples were collected on a weekly basis from the identified plants between the 3^{rd} and the 24^{th} week after flowering through to FG and FR. For PVA analysis, fruit samples were taken from the selected hand at the top, middle and bottom positions of each bunch, with one finger sampled from each position. The fingers were peeled, the pulp diagonally cut into two sections and further sliced into smaller cross sections then mixed thoroughly to make a composite sample. All fruit samples were then freeze-dried under vacuum, manually crushed into a mortar and stored at -80^{o}C before carotenoid quantification.

5.2.2. Sample preparation, carotenoid extractions and analysis

Carotenoids were extracted from 200mg of freeze-dried fruit pulp and carotenoid quantification carried out using HPLC according to the procedure outlined in Chapter 2.4.

5.2.3. Total RNA extraction and RT-qPCR

RNA was extracted from 100mg of fresh 'Mpologoma' and 'Asupina' fruit tissue using an "in house" RNA protocol as described in Chapter 2.2.3.2. Approximately 1 μg of total RNA was treated with RNase-free DNase (Promega) (Chapter 2.2.3.2) and cDNA synthesis done according to Chapter 2.2.4. Relative transcript levels of the *Psy2a* gene in the fruit pulp were evaluated by reverse transcription quantitative real-time PCR (RT-qPCR, Chapter 2.3.1.3) using specific primers (Table 5.1). The transcript amounts were normalised to the average value of 2 reference genes (RS4 and UBQ) and the data calculated using the ΔΔCT method. Data were expressed relative to the expression at developmental stages S6 and S9 for 'Mpologoma' and 'Asupina', respectively.

5.2.4. Primer design

Primers for qPCR were designed using the online software Primer3 (http://www-genome.wi.mit.edu) to anneal at 58^{o}C and amplicon size was maintained below 200 bp as recommended for SYBR green I reactions (Roche) (Table 5.1). Reference genes used in the experiment, Ubiquitin (UBQ) and Ribosomal protein S (RPS4), were selected based on qPCR expression data analysis using geNorm v3 for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm). Primer specificity was validated by semi-quantitative RT-PCR and electrophoresis through a 2% agarose gel. The reference genes used with the 'Asupina' samples were Cyclophilin (CYP) and Ubiquitin (UBQ) for which all the information and data were kindly provided by Steven Buah.

5.3. Results

5.3.1. Gene expression and carotenoid accumulation in non-transgenic 'Mpologoma'

Using reverse transcription quantitative PCR (RT-qPCR), the expression levels of the endogenous *Psya2* gene was measured in the EAHB cultivar 'Mpologoma' during the development of the fruit. *Psya2* transcript levels were measured at bunch developmental stages from week 6 (S6) to week 12 (S12) and at full green (FG) stage and all expressed relative to the *Psy2a* expression at S6. The results showed a decrease

Table 5.1. Primer sequences for PCR and qPCR analysis

Primer	Target/location	Primer Sequence 5'→3'	Accession No.
APsy2a FWD/Rev	Asupina *Psy2a*	CGACGAACTCTATCTCTACTG CTGAGTATGTTGGTGAGTTGA	JX195659.1
RS4 FWD/REV	RS4 reference gene	TGAGAGTGGCTTGACCCTGA GTGACATTTAGTCGTCTGCTGG	HQ853247
UBQ FWD/REV	UBQ reference gene	GGCACCACAAACAACACAGG AGACGAGCAAGGCTTCCATT	HQ853254
CYP FWD/REV	CYP reference gene	ATAGCGGGTCCACCAAGAAG GGCTCCTGCTGACGATAATG	HQ853241

in *Psy2a* expression from S6 to S7 followed by an increase starting at S8 and peaking at S12 (at about 1.5-fold the initial levels) with minimal fluctuation in between (Figure 5.1).

Carotenoid accumulation levels were also recorded in all of the above-mentioned samples with the addition of a FR sample. The carotenoid levels were low in the early stages of fruit development (2.1±0.9 µg/g DW β-CE at S6) but these levels progressively increased to a maximum of 21.1±3.8 µg/g DW β-CE at FR (Figure 5.2 and Table 5.2). When the composition of carotenoids was analysed, the non-PVA carotenoid lutein was the primary carotenoid present at S6, followed by the PVA carotenoids α-carotene, trans-β-carotene and cis-β-carotene (Figure 5.3). During development of the fruit and ripening, a constant trend was seen whereby the amount of lutein progressively diminished to favour the increased accumulation of α- and trans-β-carotene and a slight reduction in cis-β-carotene providing the yellowish flesh color shown in Figure 5.4.

5.3.3. Gene expression and carotenoid accumulation in non-transgenic Asupina

The results provided in this section were generated and shared by another QUT PhD student, Mr Stephen Buah, in a collaborative effort to understand the major difference in PVA accumulation between EAHB cultivars and Asupina.

Similarly to the 'Mpologoma' cultivar, RT-qPCR was used to assess the expression levels of the endogenous *Psy2a* gene in Asupina during fruit development. *Psy2a* transcript levels were measured from week 6 to 24 post-flowering then at FG and full ripe FR stages and all expressed relative to the expression at week 6. No significant increases in *Psy2a* gene expression were observed from S9 to S24. Although a 1.3-fold increase in *Psy2a* gene expression was seen between FG and FR (Figure 5.5), this was not statistically significant.

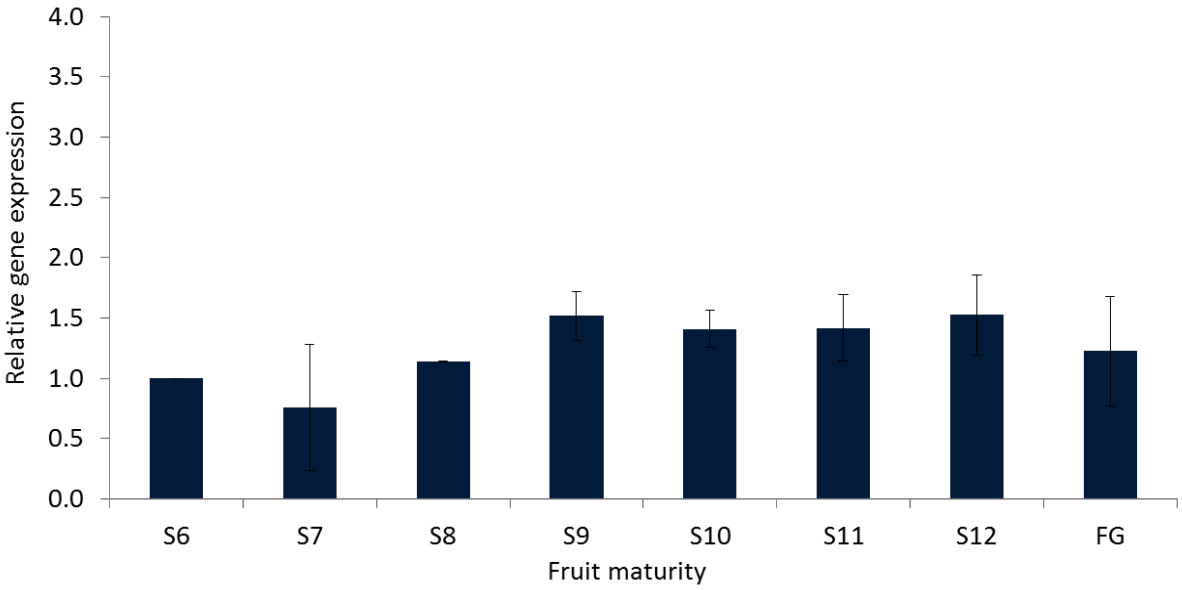

Figure 5.1. Relative expression of the endogenous *Psy2a* gene in the fruit pulp of non-transgenic 'Mpologoma' at different stages of fruit development - qPCR analysis. The expression of the endogenous *Psy2a* gene in the cultivar 'Mpologoma' is shown at S6, S7, S8, S9, S10, S11 and S12 representing 6, 7, 8, 9, 10, 11 and 12 weeks after flowering respectively, FG, full green mature fruit. Values are relative to the mean expression value at S6, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 biological replicates.

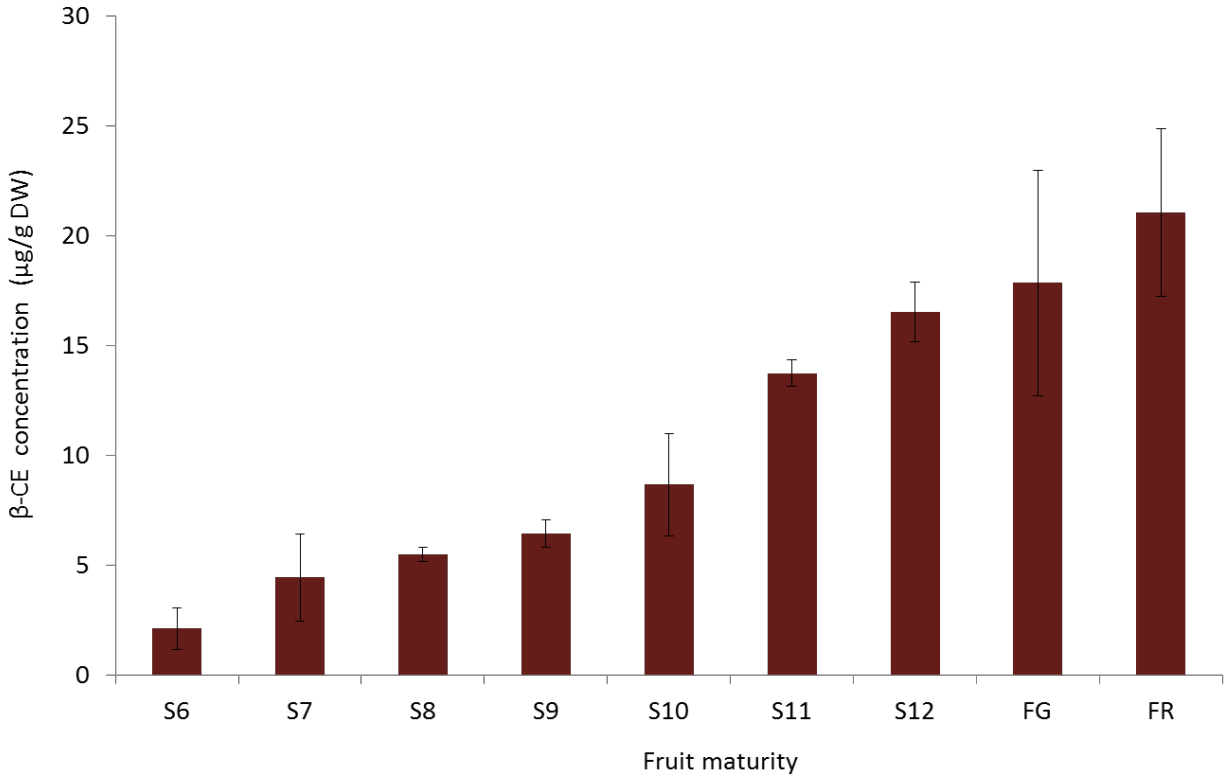

Figure 5.2. HPLC analysis - Carotenoid concentration in the fruit pulp of the non-transgenic cultivar 'Mpologoma' at different stages of fruit development. Non-transgenic 'Mpologoma' is shown at S6, S7, S8, S9, S10, S11, and S12 representing 6, 7, 8, 9, 10, 11 and 12 weeks after flowering, respectively, FG, full green mature fruit and FR, full ripe fruit. DW, dry weight. Values are means ± SD, n= 3 biological replicates.

Table 5.2. HPLC analysis - Carotenoid concentration in the fruit pulp of the non-transgenic cultivar 'Mpologoma' at different stages of fruit development

Fruit Maturity	Carotenoid concentration (µg/g DW)				
	Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
S6	1.7±0.2	1.3±0.7	1.1±0.5	0.7±0.1	2.1±0.9
S7	1.8±0.0	3.0±1.5	2.5±1.1	1.0±0.2	4.4±2.0
S8	1.9±0.3	3.9±0.4	3.1±0.1	0.9±0.1	5.5±0.3
S9	2.4±0.1	5.3±0.8	3.3±0.3	0.9±0.2	6.4±0.6
S10	2.4±1.0	6.9±2.2	4.6±1.2	1.3±0.4	8.7±2.3
S11	2.1±0.3	11.0±0.6	7.6±0.3	1.3±0.3	13.7±0.6
S12	2.4±0.1	13.0±1.3	9.1±0.6	1.9±0.3	16.5±1.4
FG	2.6±0.3	14.6±4.6	9.6±2.7	1.9±0.3	17.8±5.1
FR	3.0±0.6	16.9±2.9	11.4±2.5	2.3±0.2	21.1±3.8

Values are mean ± SD, n= 3. 'Mpologoma' fruit sampled at S6, S7, S8, S9, S10, S11, and S12 representing 6, 7, 8, 9, 10, 11, and 12 weeks after flowering, respectively; FG, full green mature fruit and FR, full ripe fruit. DW, dry weight. Values are means ± SD, n= 3 biological replicates.

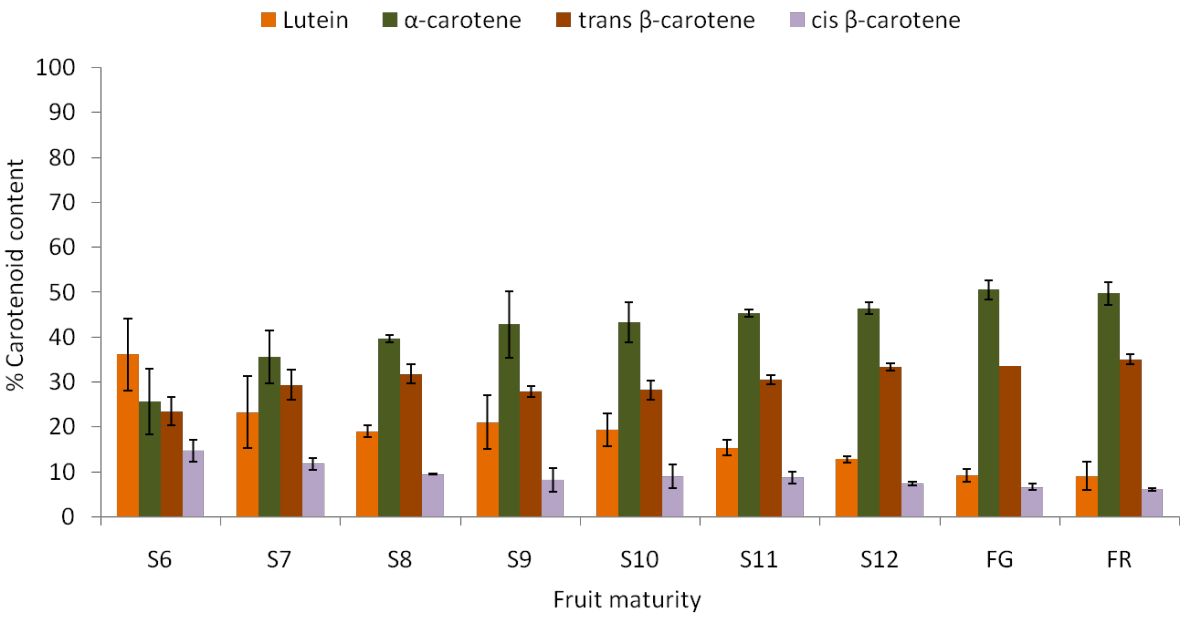

Figure 5.3. Percentage carotenoids in the fruit pulp of the non-transgenic cultivar 'Mpologoma' at different stages of fruit development. Non-transgenic 'Mpologoma' is shown at S6, S7, S8, S9, S10, S11, and S12 representing 6, 7, 8, 9, 10, 11 and 12 weeks after flowering, respectively; FG, full green mature fruit and FR, full ripe fruit. Values are means ± SD, n= 3 biological replicates.

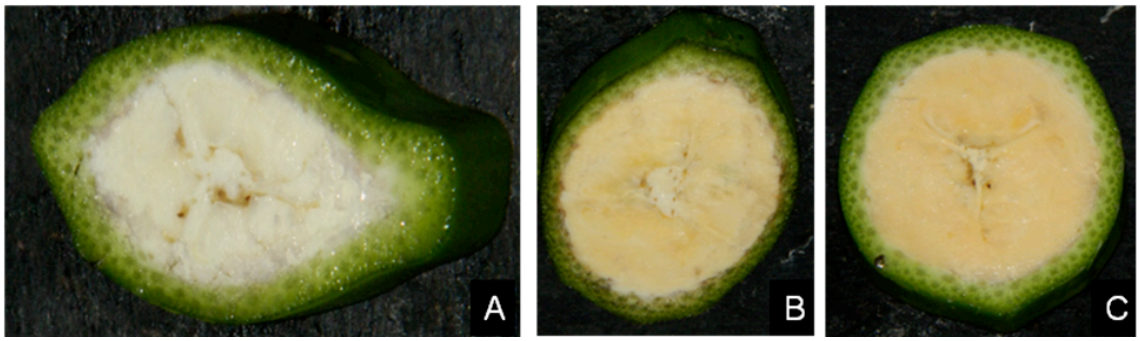

Figure 5.4. Cross section of the fruit from the cultivar 'Mpologoma' at different stages of fruit development. Fruit colour is shown at A, S6; B, S9 and C, S12, representing 6, 9 and 12 weeks after flowering. The fruit pulp at S9 had an orange colour characteristic to β-carotene rich food with increased intensity from S9 to S12.

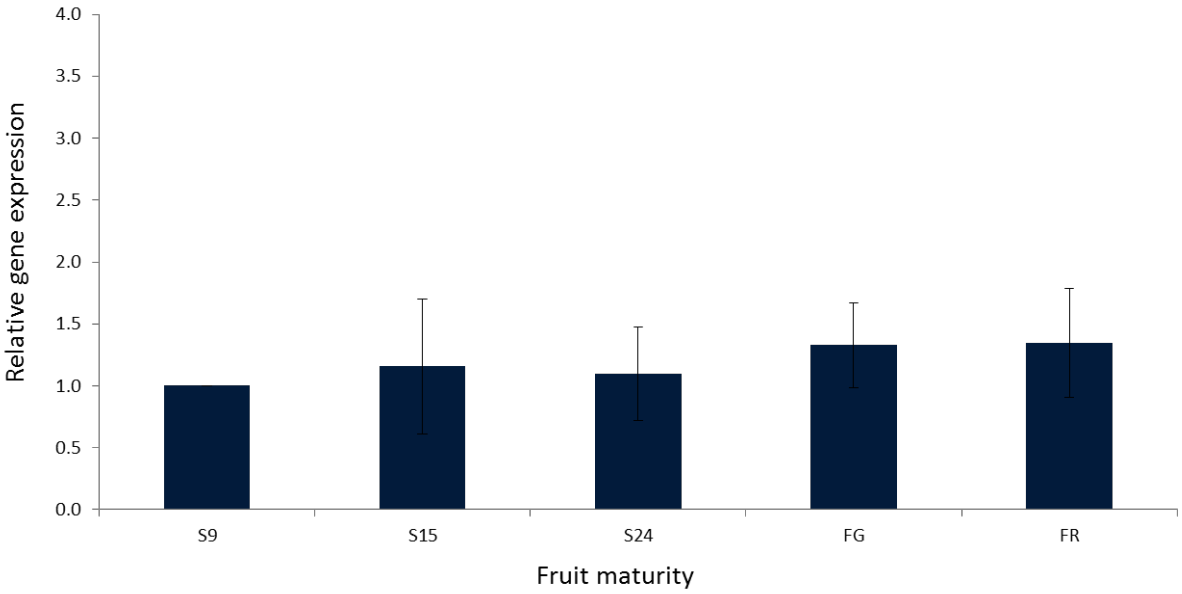

Figure 5.5. Relative expression of the endogenous *Psy2a* gene in the fruit pulp of the non-transgenic cultivar 'Asupina' at different stages of fruit development - qPCR analysis. The expression of the endogenous *Psy2a* gene in the cultivar 'Asupina' is shown at S9, S15 and S24 representing 9, 15 and 24 weeks after flowering respectively; FG, full green mature fruit and FR, full ripe fruit. Values are relative to the mean expression value at S9, which was arbitrarily set to a value of 1. Values are means ± SD, n= 3 biological replicates.

The levels of accumulated carotenoids in 'Asupina' remained consistently low from S3 up to S21 (Figure 5.6). However, during the 3 weeks between S21 and S24, the mean β-CE concentration had increased from 7.8±0.4 to 27.9±13.2 µg/g DW (Table 5.3). These levels increased by a further 4.7-fold in the subsequent 3 weeks to reach mean of 131.4±13.9 µg/g DW at FG and continued to increase during the ripening process to reach mean of 219.4±46.3 µg/g DW at FR. The composition of carotenoids in the fruit pulp of 'Asupina' at all stages of fruit development was also analysed (Figure 5.7). Up until week 21 of bunch development, lutein was found to be the main carotenoid accumulated in 'Asupina' and constituted more than 60% of total carotenoids. In addition, trans-β-carotene was consistently found to be the major PVA carotenoid accumulated in 'Asupina' ranging from around 15% at S6 to more than 70% at FR. An increase in mean β-CE concentration from 7.8±0.4 to 27.9±13.2 µg/g DW was seen between S21 and S24. This was associated with a decrease in the amount of lutein to around 30% at S24 and less than 5% at FG and FR, to favour the accumulation of α-carotene but more importantly, trans-β-carotene, in the final stages of ripening (Figure 5.7).

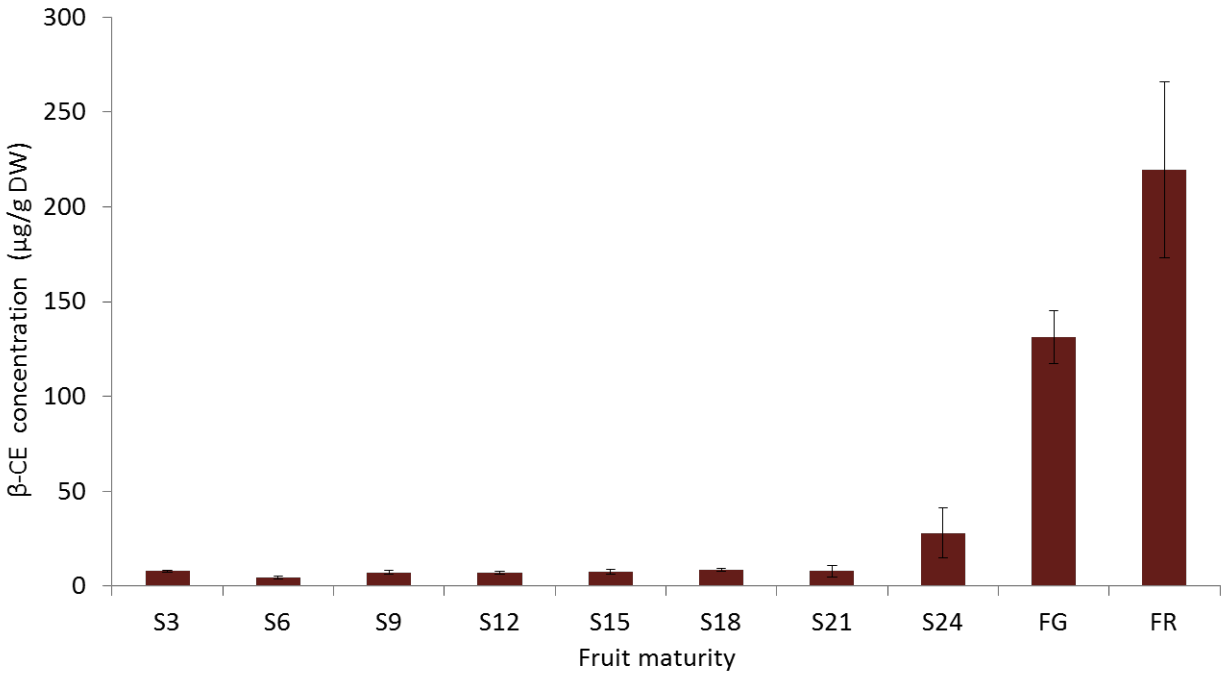

Figure 5.6. HPLC analysis - Carotenoid concentration in the fruit pulp of the non-transgenic cultivar 'Asupina' at different stages of fruit development. Non-transgenic 'Asupina' is shown at S3, S6, S9, S12, S15, S18, S21, and S24 representing 3, 6, 9, 12, 15, 18, 21, and 24 weeks after flowering, respectively; FG, full green mature fruit and FR, full ripe fruit. Values are means ± SD, n= 3 biological replicates.

Table 5.3. HPLC analysis - Carotenoid concentration in the fruit pulp of non-transgenic cultivar 'Asupina' at different stages of fruit development

Fruit Maturity	Carotenoid concentration (µg/g DW)				
	Lutein	*trans* α-carotene	*trans* β-carotene	*cis* β-carotene	β-CE
S3	11.8±0.4	0.5±0.0	6.6±0.2	1.0±0.1	7.8±0.4
S6	17.3±2.4	0.4±0.1	3.7±0.5	0.4±0.1	4.4±0.6
S9	12.4±2.1	0.4±0.2	6.0±0.9	0.9±0.1	7.1±1.0
S12	12.0±1.6	0.6±0.1	6.0±0.7	0.8±0.1	7.1±0.6
S15	13.8±2.7	0.8±0.2	6.2±0.8	0.8±0.3	7.4±1.2
S18	15.6±3.7	1.4±0.3	7.1±0.8	0.8±0.1	8.6±0.9
S21	16.8±1.4	1.8±0.6	5.9±2.3	1.1±0.5	7.9±3.1
S24	12.5±4.0	7.8±3.5	20.4±10.1	3.6±1.5	27.9±13.2
FG	7.7±2.0	34.7±19.8	109.1±6.6	4.9±0.9	131.4±13.9
FR	6.9±3.1	61±23.9	181.2±36.7	7.7±0.9	219.4±46.3

Values are mean ± SD, n= 3. 'Asupina' fruit sampled at S3, S6, S9, S12, S15, S18, S21 and S24 representing 3, 6, 9, 12, 15, 18, 21, and 24 week after flowering, respectively; FG, full green mature fruit and FR, full ripe fruit. DW, dry weight. Values are means ± SD, n= 3 biological replicates.

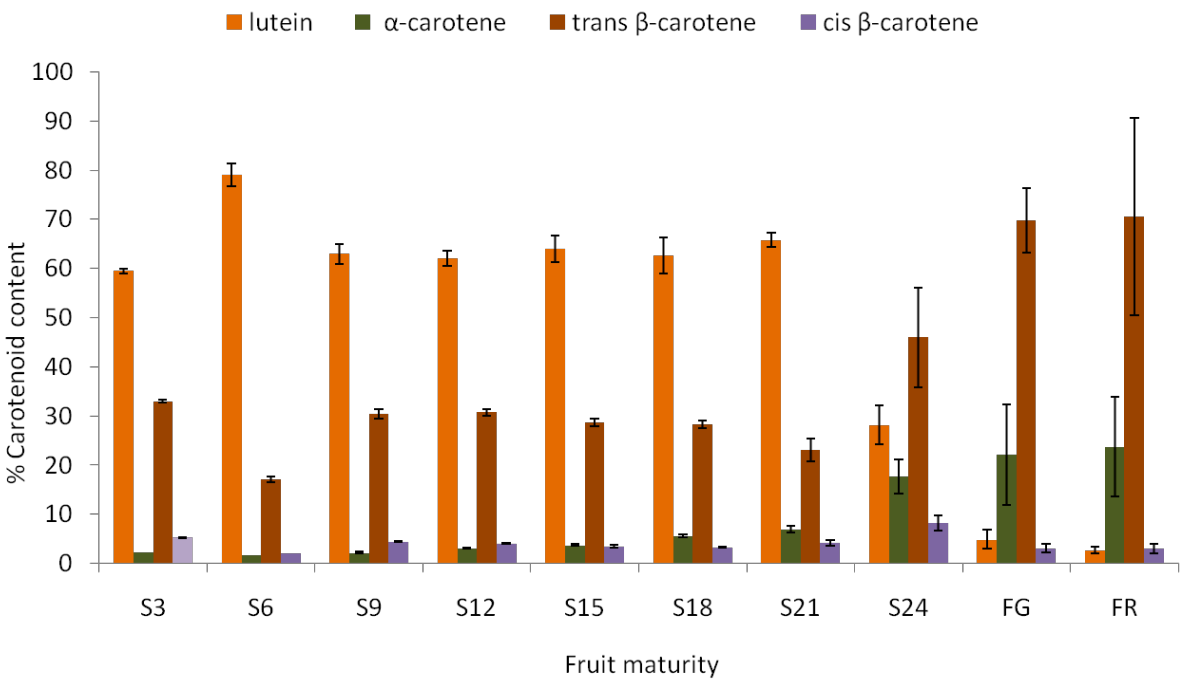

Figure 5.7. Percentage carotenoids in the fruit pulp of non-transgenic cultivar 'Asupina' at different stages of fruit development. Non-transgenic 'Asupina' is shown at S3, S6, S9, S12, S15, S18, S21, and S24 representing 3, 6, 9, 12, 15, 18, 21, and 24 weeks after flowering, respectively; FG, full green mature fruit and FR, full ripe fruit. Values are means ± SD, n= 3 biological replicates.

5.4. Discussion

The phytoene synthase 2a gene (*APsy2a*) from the Fe'i type banana 'Asupina' is currently being used as a transgene for the PVA biofortification of Ugandan EAHB varieties. The purpose of this study was to investigate the role of the *Psy2a* gene in carotenoid accumulation processes during fruit development of both the EAHB cultivar 'Mpologoma' and the Fe'i type banana 'Asupina'. As such, the levels of *Psy2a* expression were analysed in both cultivars and correlated with the accumulation levels of PVA carotenoids.

Key regulatory steps and regulation mechanisms controlling isoprenoid and carotenoid flux in plants have been extensively reviewed (Cazzonelli and Pogson, 2010). The results from this study showed that there is little variation in *Psy2a* transcript levels in both 'Mpologoma' and 'Asupina' cultivars across the fruit development cycle. However, the trend in carotenoid accumulation varied by ten times in order of magnitude between the two cultivars. The data presented in this Chapter provides no indication that the expression profile of this gene is responsible for the large increase in PVA carotenoid accumulation seen in 'Asupina' from week 21 and beyond.

The high levels of variation in PVA content across banana cultivars have been reported previously but only at the full green stage (Englberger *et al.*, 2003; Davey *et al.*, 2009). The results described in Chapter 3 provided an indication of the natural levels of variation in PVA content between different *Musa* cultivars and, more specifically, between different EAHB cultivars. In Chapter 5 it was noted that the levels of PVA at FG stage in 'Mpologoma' reached as high as 17.8±5.1 µg/g DW whereas a mean of only 9.9±6.9 µg/g DW was recorded for the same cultivar in Chapter 3. These levels of variation could be explained by the fact that the data generated in Chapter 3 was derived from 30 'Mpologoma' samples collected from 3 agricultural zones, 9 districts and several farms across Uganda whereas in Chapter 5 only 4 samples were collected from the research station garden at NARO. Therefore, even though the PVA values in

Chapter 3 ranged from 1.8 to 32.5 µg/g DW (data not shown) they averaged at 9.9±6.9 µg/g DW whereas Chapter 5 value for 'Mpologoma' were 14.2 and 21.5 µg/g DW (data not shown) and averaged at 17.8±5.1 µg/g DW.

The varying level of individual carotenoids in a given cultivar across the development of the fruit indicates an interchangeable occurrence at the branching level of the carotenoid biosynthesis pathway. The high levels of lutein detected during the early stages of fruit development in both 'Mpologoma' and 'Asupina' indicate an α-carotene favoured branching at the cyclase step with a subsequent high level of hydroxylation of PVA compounds into lutein by cytochrome P450 carotene hydroxylases. Similar results have been observed in 'Sukali Ndizi' at full green (Chapter 3) and also in maize, where enhanced activity of the cyclase enzyme resulted in accumulation of the non-PVA carotenoid, lutein (Vallabhaneni *et al.*, 2009; Yan *et al.*, 2010). This trend was reversed in both 'Mpologoma' and 'Asupina' as the fruit progressed to full maturity, a phenomenon which caused the observed increase in PVA carotenoids. In the cultivar 'Asupina', this reverse trend was significant with a rapid reduction of lutein coupled to a high accumulation of β-carotene in the fruit pulp. This suggests a β-carotene favoured cyclase branching with less hydroxylation activity downstream.

Chapter 6 - General discussion

In urban areas of the developing world and in most Western societies, where populations can afford more than one item in their diet, cases of micronutrient deficiency are lower than among populations dependent on a single staple food crop (van Poppel, 1996; Bouis, 2003). Uganda is no exception with rural poor communities relying heavily on micronutrient poor EAHB for sustenance.

Indeed, results from this study indicate that most banana cultivars grown in the Uganda, namely cooking type EAHB (AAA) and dessert bananas (AAB) contain low amounts of PVA carotenoids, with wide variations observed within data obtained from various agricultural zones. EAHB cultivars were found to accumulate mainly PVA carotenoids such as β- and α- carotene and also retained more than 80% of these metabolites after cooking. In addition, bioavailability studies carried out on Mongolian gerbils indicate that cooking as opposed to ripening enhances pro vitamin A (PVA) carotenoid bioavailability from bananas (Bresnahan *et al.*, 2012). This knowledge, combined with the fact that EAHBs are heavily consumed in Uganda, makes it the ideal target for biofortification in this country.

The work presented in this study combined with the abovementioned bioavailability study from Breshnahan *et al*., (2012) have enabled the determination of a β-carotene equivalents biofortification target level for the fruit of the EAHB transgenic line(s) that will be released to farmers in Uganda.. The target was calculated on the basis of delivering at least 50% of the Estimated Average Requirement (EAR) of vitamin A, which is equivalent to 120 μg/day for children under 60 months and 235 to 445 μg/day for lactating women, allowing the other fraction to be obtained from other components of the diet. The bioconversion ratio of β-carotene equivalent to vitamin A was also estimated at 6:1 from processed (cooked) banana flesh. The consumption of cooked bananas was estimated at 300 g/day for children and 500 g/day for women and the

conservative retention level of α-carotene and β-carotene after cooking was set at 70%. Using these parameters, it was calculated that, to achieve 50% of the vitamin A EAR, the GM bananas needed to contain at least 20 μg/g dry weight (DW) of β-carotene equivalents (β-CE).

The biofortification research conducted at QUT, Australia and at NARO, Uganda has provided a proof of concept that significant levels of PVA could be accumulated in a micronutrient poor 'Cavendish', 'Sukali Ndizi' or EAHB hybrid M9 using a transgenic approach and a single phytoene synthase transgene (James Dale personal communication). The project has now entered a product developmental phase in which two cultivars, the disease resistant EAHB hybrid M9 and the traditional EAHB 'Nakitembe' are being transformed with the phytoene synthase 2a (*Apsy2a*) gene from the Fe'i type banana 'Asupina' driven by either the constitutive maize polyubiquitin (Ubi) promoter or the fruit preferred banana 1-aminocyclopropane-1-carboxylate oxidase (ACO) promoter. The 800 generated transgenic lines will be initially assessed under confined field trial conditions at NARO. The selection of lead events will be based on two essential criteria, (i) a β-CE concentration in the mature green fruit pulp above 20 μg/g DW (the trait) and (ii) a minimum bunch weight of 20kg. Following initial assessment, the selected lines will undergo Southern blot analysis and only events containing a single copy of the introduced transgene will be considered. A full genome sequence analysis of these elite lines will reveal the integration sites. All lines with a clean insertion site (ie: outside a gene and with no new open reading frame created) will be advance to multi-location trials, toxicity studies, nutrition studies and deregulation with a scheduled product release in 2020 (James Dale personal communication).

A number of concerns and perception issues have been raised in Uganda over potential food safety aspects associated with allergenicity and toxicity from PVA biofortified bananas. These concerns can only be overcome through good science, intense, accurate and effective communication strategies to gain positive attitude and public acceptance

towards the product. Since 2006, the potential allergenicity of the maize Psy1 protein used in the generation of Golden Rice 2 has been ruled out by bioinformatics analysis using the AllergenOnline database of the University of Nebraska-Lincoln (Goodman and Wise, 2006). Results from these studies were considered at the inception of the banana project and a similar analysis done and repeated every year for the Apsy2a protein from 'Asupina'. Although there are successful biosafety legislation examples in Africa, progress has been slow, with only seven countries (Burkina Faso, Mali, Mauritius, South Africa, Sudan, Zimbabwe, Kenya) having developed functional national biosafety frameworks. Uganda, like most African countries, lacks biosafety legislation. The current biosafety regulatory system only allows up to confined field trial testing (Hansen *et al.*, 2003) and the raging debate on GM crops has sent mixed signals, hence influencing decision-making at policy level and slowing progress. The requirement for huge investments has been a key drawback in the progress towards breakthroughs in biotechnology and biosafety research and development in Africa. Indeed the cost of biotechnology research and regulatory compliance has been prohibitive and a major challenge in African countries, considering that most of them have minimal budget allocations for agricultural research and development (Hansen *et al.*, 2003).

However, given the prevailing circumstances in Africa and Uganda in particular, it is apparent that the adoption of new agricultural technologies, including biotechnologies, will play an important role in addressing food security and poverty challenges. To tap into the potential that biotechnology offers to agricultural productivity and food security, there is a need for renewed dedication by African governments towards biotechnology development. This can be achieved by developing capacity to negotiate access to intellectual property (IP), enact and operationalise IP and biosafety policies and guidelines that foster technological innovations. In addition, it is essential for African countries to understand the importance of minimizing the cost of regulations in order to maximize the benefits from biotechnology. Furthermore, there is a need for

sound stewardship that will ensure responsible and sustainable use of biotechnologies while minimizing any potential risks.

Unlike EAHBs, the dessert banana 'Sukali Ndizi' is a small and sweet banana that is eaten raw and ripe and is very popular as a snack especially amongst children in Uganda (Tushemereirwe *et al.*, 2006). Unfortunately, the current study indicates that 'Sukali Ndizi' not only accumulates low levels of carotenoids but these mainly consist of the non-PVA carotenoid lutein. In addition, this study has demonstrated a reduction in PVA carotenoid content in the transition from mature full green to full ripe fruit. This combined with the fact that ripening does not improve the retinol bioefficacy of bananas (Bresnahan *et al.*, 2012) suggests that 'Sukali Ndizi' is a poor target for biofortification in Uganda at the present stage.

Dessert banana, such as 'Cavendish', are the mainstream variety consumed in the Western world, which is largely supplied by large companies from Central and South America, the West Indies and West Africa. India is the biggest banana producer with close to 26 million metric tonnes a year and mainly produce dessert banana for local consumption. Sadly, India also has significant levels of clinical VAD (Stein *et al.*, 2008). Dessert banana such has 'Cavendish' could therefore become an attractive vehicle to combat VAD in India. As such, in the future we are looking at increasing the PVA levels in dessert banana to levels much higher than our current target to compensate for the lack of bioavailability from bananas that are eaten raw (Bresnahan *et al.*, 2012) and the possible losses in PVA carotenoid during the ripening process due to enzymatic or oxidative degradation.

Another cultivated variety of the genus *Musa* is the plantains, which are mainly consumed after cooking. This study also evaluated carotenoid accumulation in a plantain variety 'Gonja Nakatansese' at full green, full ripe and after boiling and steaming processing. The plantain consistently accumulated levels of PVA above 30 μg/g

DW β-CE and could therefore use to help alleviate VAD. Unfortunately, plantains are not as popular as EAHBs in Uganda as they do not hold the same biochemical characteristic allowing to make the traditional banana ("matooke") meal (Ajayi and Aneke, 2002; Dury *et al.*, 2002; Danso *et al.*, 2006; Tushemereirwe *et al.*, 2006; Ekunwe and Ajay, 2010; Honfo *et al.*, 2011). However, plantains are a major staple crop in West and Central Africa (Ivory Coast, Ghana, Cameroon and DR Congo), Central and South America and the Caribbean Islands. In West Africa, plantains are consumed between the green and yellow stages of ripeness (Yomeni *et al.*, 2004). They can be prepared through a number of methods, including boiling, steaming, mashing, baking, drying, and pounding into fufu (a popular West African staple made with boiled cassava, yams, or plantains that are pounded into a dough) (Akinyemi *et al.*, 2010; Ekunwe and Ajay, 2010) or processed into chips (Yomeni *et al.*, 2004; Adeniji *et al.*, 2010). Plantains, like most bananas, bear fruit all year round, which makes the crop an excellent food security crop particularly in developing countries with inadequate food storage, preservation and transportation technologies. Unfortunately, this study has also shown that despite its ability to accumulate high levels of PVA carotenoids at full green, the plantain 'Gonja Nakatansese' only retained about 50% of its PVA carotenoids after boiling and steaming while 30% of it was lost during the ripening process. In addition, traditional ways of cooking plantains most of the time involve roasting or deep-frying which, with regards to retaining the quantity and the integrity of the carotenoids, are not as good as steaming and boiling (Solange *et al.*, 2011). Due to their inherent high starch content, plantains naturally accumulate high levels of PVA carotenoids, however, populations relying on them as a staple still suffer from VAD (Solange *et al.*, 2011). This is potentially due to excessive carotenoid losses during the traditional cooking process (Yomeni *et al.*, 2004). Nevertheless, plantains remain an attractive target for biofortification in the future as they potentially hold the necessary metabolic pathways to allow extreme PVA accumulation.

Despite recent progress in our understanding of carotenogenesis in plants, the mechanisms that govern overall carotenoid accumulation in individual plant species remains largely unknown. In this study, a distinctive difference was observed in carotenoid accumulation between 'Asupina' and the EAHB cultivar 'Mpologoma' during fruit development through to full green and full ripe. Distinctive regulatory mechanisms appear to be involved in the control of carotenogenesis in the individual plant tissue cells beyond carotenoid biosynthesis and metabolism. Thus, the sequestration and stable storage of carotenoids in the tissue plastidial cells should be investigated to provide more insight on carotenoid deposition and their subsequent release from these cells.

In conclusion, this study has demonstrated the PVA biofortification of EAHB can be achieved successfully through the over-expression of *Psy* genes using a genetic engineering approach. More importantly, the events were generated in Uganda, by Ugandan scientists therefore creating strong foundations for the future of EAHB biofortification in this country and ultimately the potential benefit to Ugandan consumers.

Appendices

Appendix 1 - Generating a β-carotene standard curve using the HPLC

Step 1: Weigh 3.5mg of β-carotene standard compound into a cryovial and resuspend in 1mL of chloroform to make a stock solution.

Step 2: 15µL of the stock is diluted into 10mL of acetone to make a working solution.

Step 3: Reading the absorbance of the working solution in triplicate at 450nm on a spectrophotometer. The real concentration of the working solution made in step 2 is then calculated using the Lambert-Beer equation.

Example:

Lambert-Beer equation

E= εcd (E: extinction, ε: molar extinction coefficient, c: concentration and d: distance =1)

c= E/ε.

Table A 1.1. Calculated concentration of the β-carotene **standard (example)**

Absorbance of the working solution in triplicates		**Calculations**
Ab1	0.768	c= E/ ε
Ab2	0.772	c= 0.770/134000 mol/L
Ab3	0.771	Therefore values in ng/µL
Average	0.770	So C= (0.770/134000 mol/L) x 537 g/mol x 1000 = 3.087 ng/µL
Concentration (ng/µL)	3.087	

Step 4: Use the working solution to make a 8-10 series of samples of increasing β-carotene amount starting from zero into 2mL cryovials.

Step 5: Dry each cryovial under vaccum.

Step 6: Resuspended each sample in 200µL of solvent C [methanol:TBME (1:1, v/v)] before injecting 10µL into the HPLC for analysis

Table A 1.2. β-carotene standard concentrations run on the HPLC (example)

Code	Volume (µL) of stock per tube	ng/tube	Concentration (ng/µL) [resuspended in 200µL]	ng/10µL injected	AU (nm)
SD1	0.00	0.00	0.00	0.00	0.00
SD2	25.00	77.18	0.39	3.86	30.70
SD3	50.00	154.35	0.77	7.72	70.51
SD4	100.00	308.71	1.54	15.44	155.25
SD5	200.00	617.42	3.09	30.87	322.19
SD6	400.00	1234.83	6.17	61.74	747.29
SD7	500.00	1543.54	7.72	77.18	829.20
SD8	600.00	1852.25	9.26	92.61	1110.45
SD9	700.00	2160.96	10.80	108.05	1256.88
SD10	1000.00	3087.08	15.44	154.35	1854.12

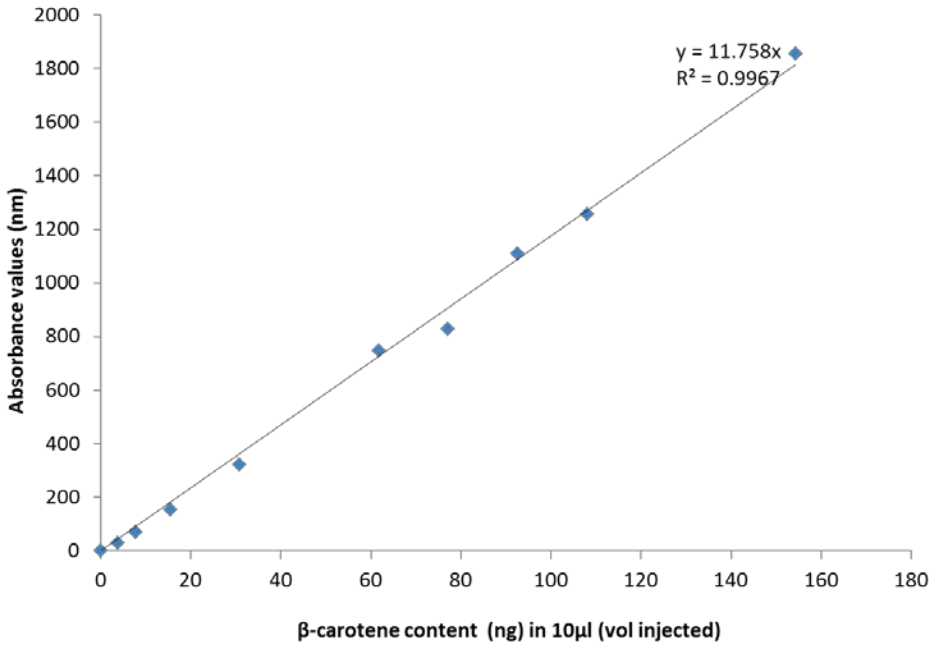

Figure A 1.1. β-carotene standard curve

Appendix 2 - General materials

A2.1 General Solutions

A2.1.1 Media and solutions for nucleic acid amplification

Agarose gel loading dye (6X): 0.25% (w/v) bromophenol blue, 50% TE, 50% glycerol

TAE Buffer (1X): 40mM Tris, 1mM EDTA, adjusted to pH7.6 with glacial acetic acid

GoTaq® green master mix (Promega)

Power SYBR ® Green PCR Master Mix (Applied Biosystems)

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen)

A2.1.2. Bacterial culture media, extraction and electrophoresis buffers

LB medium (1L): 10g Bacto-tryptone, 5g Yeast extract 10g NaCl, 15g Bacto agar, pH7.0

Yeast mannitol (YM) medium (1L): 10g Mannitol, 0.4g Yeast extract, 0.1g K_2HPO_4, 0.4g KH_2PO_4, 0.1g NaCl, 0.2g $MgSO_4.3H_2O$, 5g Bacto agar, pH6.8

2x YT media (1L): 16g tryptone, 10g yeast extract, 5g NaCl, adjusted pH to 7.0 before autoclaving

50x TAE electrophoresis buffer (1L): 242g Trizma base, 57.1mL Glacial acetic acid, 0.5M EDTA (pH8.0)

CTAB Buffer (DNA): 2% CTAB, 2M NaCl, 25mM EDTA (pH 8), 100mM Tris-HCl, (pH 8), 2% Polyvinylpyrrolidone (PVP 40)

TE buffer: 10mM Tris-HCl (pH8.0), 1mM EDTA

Loading dye (6X): 0.25% (w/v) bromophenol blue, 50% TE, 50% glycerol

Solution I: 50mM glucose, 10mM EDTA (pH 8.0), 25mM Tris-HCl

Solution II: 0.2 N NaOH, 1% SDS

Solution III: 60mL Potassium acetate solution, 11.5mL glacial acetic acid, 28.5mL H_2O

A2.1.3. Cell culture and regeneration media

MA2 cell suspension media

Compound	Concentration (g/L)
MS basal salts	4.3
MS vitamins	0.103
Biotin	0.001
2,4-D	0.001
Glutamine	0.99
Malt extract	0.1
Sucrose	30
pH5.3	

TMA1 media

Compound	Concentration (g/L)
MS Macro	1/10 strength
MS Micro	Full strength
MS vitamins	Full strength
Fe complex	Full strength
Biotin	0.001
Malt extract	0.1
Glutamine	0.1
Proline	0.23
Myo-inositol	0.1 (only in semi-solid co-culture media)
Citric acid	0.06 (only in semi-solid co-culture media)
Ascobic acid	0.06
PVP	10g in semi-solid co-culture media, 5g liquid medium
L- cysteine	0.4
IAA	0.001
NAA	0.001
2,4-D	0.004
Glucose	10
Sucrose	30 (in semi-solid co-culture media, 85.5g liquid media)
Gelrite	6 (in solid co-culture media)

MA3 Embryo development medium

Compound	Concentration (g/L)
SH Basal salts powder	3.2 (Cat #S6765 Sigma)
MS vitamins	Full strength
Biotin	0.001
Proline	0.23
Glutamine	0.1
Malt extract	0.1
Ascobic acid	0.06
PVP 10	10
Myo- inositol	0.1
Citric acid	0.06
L- cysteine	0.4
NAA	0.0002
Zeatin	0.00005
Kinetin	0.0001
2iP	0.0002
Sucrose	45
Lactose	10
Gelrite	2.4

pH5.3

MA4 Embryo germination media

Compound	Concentration (g/L)
MS Salts	Full strength
MS vitamins	Full strength
BAP	0.00005
IAA	0.00005
Sucrose	30
Phytagel	2.4

pH5.8

A2.1.4 Media and solutions for plant nucleic acid extraction

$CHCl_3$:IAA: chloroform and isoamyl alcohol at a ratio of 24:1

CTAB Buffer (DNA): 2% (w/v) CTAB, 1.4M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20mM EDTA, 100mM Tris-HCl, pH8.0, 2% polyvinylpyrrolidone (PVP 40)

CTAB Buffer (RNA): 150mM Tris base, 2% SDS, 1% 2-mercaptoethanol, 100mM EDTA (adjust pH to 7.5 with saturated boric acid), 2% polyvinylpyrrolidone (PVP40)

RNeasy Plant Mini Kit (Qiagen)

A2.1.5. Chemicals/solvents for HPLC analysis

HPLC chemicals and solvents (HPLC grade) were obtained from Fisher Scientific Co., Fair Lawn, NJ or Romil Ltd

All-trans-β-carotene standard was purchased from Sigma Chemical Co. (St. Louis, MO)

Methanol (MeOH) (HPLC grade)

Methyl tert-Butyl Ether (TBME) (HPLC grade)

Water (HPLC grade)

Acetone (HPLC grade)

Petroleum ether (HPLC grade)

Diethyl ether (HPLC grade)

Chloroform (HPLC grade)

α-tocopherol-acetate (internal standard)

Solvent C: Methanol/TBME (1/1; v/v)

Solvent D: Methanol/TBME/HPLC Water (5/1/1; v/v/v)

A2.1.6 Equipment

Vortex model G-560E, Scientific Industries INC

Centrifuge MIKRO 220R, Type 2205, HettichZentrifugen

NanoDrop® ND-2000 spectrophotometer, Thermo Scientific

High-performance liquid chromatography (HPLC), Agilent Technologies 1200 Series

HPLC column: Polymeric 5µm C30 stationary phase (4.6mm (i.d.) x 250mm) PrincetonSPHER C30 200A 5U: 250 x 4.6mm: Part No.: 250046-07574

Opaque amber glass HPLC vials (1.5 mL, 10 mm screw top vial, flat bottomed 12 x 32 mm)

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