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The quest for golden bananas: investigating carotenoid regulation in a Fe'i group *Musa* cultivar

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Abstract

The regulation of carotenoid biosynthesis in a high carotenoid accumulating Fe'i group *Musa* cultivar, 'Asupina', has been examined and compared to that of a low carotenoid accumulating cultivar, 'Cavendish', to understand the molecular basis underlying carotenogenesis during banana fruit development. Comparisons in the accumulation of carotenoid species, expression of isoprenoid genes and product sequestration are reported. Key differences between the cultivars include greater *carotenoid cleavage dioxygenase 4* (*CCD4*) expression in 'Cavendish' and the conversion of amyloplasts to chromoplasts during fruit ripening in 'Asupina'. Chromoplast development coincided with a reduction in dry matter content and fruit firmness. Chromoplasts were not observed in 'Cavendish' fruits. Such information should provide important insights for future developments in the biofortification and breeding of banana.

Keyword index

Musa, Secondary metabolism, Carotenoids, Banana, Fruit development, Pro vitamin A, Biofortification.

INTRODUCTION: Carotenoids are C40 isoprenoid compounds and are responsible for the majority of red to yellow colours of flowers and fruits. Crops plants are a major source of pro-vitamin A carotenoids (pVAC) which are converted into vitamin A in the liver. Vitamin A is important for growth and development and is essential for vision. [1]. Globally, banana is a major crop and the staple food for more than 400 million people primarily in humid tropic and subtropical regions [2]. It has been ranked fourth after rice, wheat and maize in terms of importance as a food crop [3]. In regions where banana constitutes the principle food source such as Uganda, where over 70% of the population depend on it [4], vitamin A deficiency (VAD) is widespread. The World Health Organisation (WHO) estimates that >250 million children aged 1- 4 years in 122 countries in Africa and South-East Asia, are at the risk of VAD[5]. Consequently, an estimated 250,000 to 500,000 affected children become permanently blind every year, half of them dying within 12 months of losing their sight. The introduction or promotion of micronutrient-rich banana varieties could have a significant long-term beneficial impact on the health of populations in these regions [6]. Biofortification can combat micronutrient deficiency in large populations by increasing the micronutrient content of staple food crops. Currently, there are established research programs for the biofortification of banana to combat VAD using both conventional breeding practices and genetic modification strategies. Conventional breeding is a major challenge for bananas because the majority of cultivars are sterile triploids; however, both approaches are limited by a lack of understanding regarding the regulatory mechanisms underlying natural variation in carotenoid accumulation between cultivars.

Most banana varieties are derived from *Musa. acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) with a wide diversity of diploid, triploid, and tetraploid cultivars [6]. The A genome is present in East African Highland Bananas (AAA) and the commercial cultivars ‘Cavendish’ and ‘Lady Finger’ (AAA). A and B genomes are present in Silk, Pome and plantain bananas (AAB). The genotypes of a small number of polynesian Fe’i bananas (*Musa* × *troglodytarum L*) are believed to contain a T-genome [7]. ‘Asupina’ a Fe’i banana from Papua New Guinea, is thought to have an ATT genome [8]. There is

considerable variation in the carotenoid content of different banana (*Musa*) cultivars [9]. For example, 'Cavendish', and 'Lady Finger' bananas contain relatively low levels of β-carotene (~2.7 - 3.4 μg g^{-1} dry weight; DW) [9], whilst other less widely consumed cultivars such as the Fe'i cultivars 'Asupina' and 'Uht en yap' which have characteristically orange fruit pulp, accumulate much greater amounts (~72 - 83 μg g^{-1}) [9,10].

Carotenoids are biosynthesised in nearly all types of plastids and are derived from the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [11]. The first step in the MEP pathway is regulated by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and is rate limiting [12,13]. The first committed step in carotenoid biosynthesis is the condensation of two molecules of geranyl geranyl diphosphate (GGPP) by phytoene synthase (PSY) which forms phytoene (Figure 1) this is also rate-limiting [14-17]. Phytoene undergoes a series of desaturation and isomerization reactions to produce lycopene. The cyclization of lycopene ends, catalysed by two lycopene cyclises, ε-lycopene cyclase (LCYE) and β-lycopene cyclase (LCYB), produces α- and β-carotene. Cyclic carotenes can be hydroxylated and oxygenated by a series of enzymes to generate xanthophylls, e.g. lutein, zeaxanthin, violaxanthin, and neoxanthin [1,11]. The regulation of carotenoid biosynthesis is multifaceted and is co-ordinated with other processes including flower and fruit development [11,18]. The accumulation of specific carotenoids is transcriptionally regulated by the expression of associated isoprenoid and carotenoid specific biosynthesis genes, for example by increased or reduced expression of up or downstream genes or at catalytic branch points [11,19,20]. However, transcriptional regulation is not the primary regulatory mechanism affecting total or individual carotenoid accumulation levels in all cases as the sink strength of the storage mechanism used to sequester carotenoids and also carotenoid turnover can strongly influence carotenoid accumulation.

Carotenoid levels in the pulp of mature fruits from a number of different banana cultivars have been reported [6,9,10,21,22]. However, very little data is available regarding how carotenoid

accumulation is regulated during fruit development. Such information could be critical in the development of future pVAC biofortification and breeding strategies for banana. In this study, we examined the changes in carotenoid accumulation in two different banana cultivars, a low accumulating cultivar; 'Cavendish' (AAA), and a high accumulating Fe'i cultivar; 'Asupina' (ATT). Carotenogenesis was studied across fruit development from bunch emergence through to the full-ripe (FR) stage. Isoprenoid gene expression and correlations with carotenoid accumulation patterns were investigated to reveal transcriptional regulation mechanisms. The expression patterns of a carotenoid cleavage deoxygenase (*CCD*) gene and changes in plastid structure were also examined in each cultivar to investigate regulatory mechanisms associated with carotenoid degradation or sequestration, respectively.

MATERIALS AND METHODS

Plant material. 'Asupina' (AAT) and 'Cavendish' (AAA) banana plants were grown in at the South Johnstone Research Station, Queensland Department of Agriculture and Fisheries (DAFF), Australia. Each cultivar was represented by 3 independent plants. Following bunch emergence, three fruits were collected every 3 weeks from the top, middle and bottom of each plant. Developmental stages (S) are represented by weeks post bunch emergence. S3 was the earliest fruit stage collected, 3 weeks after bunch emergence. 'Cavendish' fruits were collected from S3 to S9, plus mature full-green (FG) and FR stages. 'Asupina' fruits were collected from S3 to S24, plus mature FG and FR stages. Each fruit was peeled and diced. Pulp from the top, middle and bottom fruit of each independent plant at each developmental stage was pooled, flash frozen in liquid nitrogen, freeze dried and stored at -80 ºC. Dry matter content (DMC) was determined by weighing triplicate samples pre and post freeze-drying.

Analysis of carotenoids. Carotenoid extractions were made from 50 or 200 mg samples of freeze dried 'Asupina' or 'Cavendish' pulp respectively, prepared in triplicate for each biological replicate under low

light conditions. Freeze-dried samples were homogenised using a TissueLyser (Qiagen). A 2 ml aliquot of acetone was added to the powder. Samples were mixed by vortex and centrifuged for 5 min at 4000 x*g* and 4 ºC. Acetone extraction was repeated twice more and the supernatants combined and partitioned by the addition of 2 ml of petroleum ether: diethyl ether (2:1, v/v) and 6 ml of filter-sterilised 1% (w/v) sodium chloride. The phases were mixed, centrifuged, and the organic phase transferred retained. Samples were dried under vacuum, stored at −20 °C and analysed by HPLC-PDA within 7 days of extraction. Samples were resuspended in 1:1 (v/v) methanol: *tert*-methyl butyl ether (TBME) and 10 µl subjected to quantitative analysis using HPLC with a C30 3µm silica-based reverse-phase column (4.6 x 250 mm) coupled to a 5 µm (4.0 x 23 mm) C30 guard cartridge (YMC, Kyoto, Japan). An Agilent 1200 series HPLC system was used (G1311A pump, G1322A degasser, G1329A auto sampler injector, G1316A column oven and G1315D PDA detector). The mobile phases were solvent A (1:1 (v/v) methanol: TBME) and solvent B (5:1:1 (v/v/v) methanol: TBME: distilled water).The elution gradient started with 57% (v/v) Solvent A from which a linear gradient to 100% Solvent A by 23 min was performed. A conditioning phase of 6 min was used to return the column to the initial concentration of A and B. Column temperature was maintained at 24 °C and detection performed continuously from 190 to 700 nm with the online PDA detector. Carotenoids were identified by comparison of spectral properties and retention times with authentic standards and reference spectra [23-25]. For quantitative analyses, 200 mg α-tocopherol acetate (Sigma-Aldrich) was added to each sample as an internal standard prior to extraction. All peaks were normalised relative to the internal standard to correct for extraction and injection variability. An authentic external standard, β-carotene (Sigma-Aldrich), run separately, was used to quantify carotenoid amounts. All carotenoid peaks were integrated at 450nm and underwent a second normalisation to correct for their individual extinction coefficients relative to β-carotene [24].

Isolation of pulp expressed gene sequences for RT-qPCR primer design. Total RNA was extracted as detailed in Valderrama-Cháirez et al., [26]. Polyadenylated mRNA was purified from DNase-treated total RNA (100 – 500 µg) using a GenEluteTM mRNA Miniprep Kit (Sigma-Aldrich) as directed by the

manufacturer. cDNA was prepared from mRNA from five different stages of fruit development of 'Cavendish' and 'Asupina' using a Trancriptor First Strand cDNA Synthesis Kit v.06 (Roche). cDNA samples were used as templates to amplify fragments of *DXS*, *GPPS*, *LCYB* and *CCD4* by PCR with degenerate primers designed in conserved coding regions aligned from multiple sequences of each gene retrieved from GenBank, and *Musa* specific sequences retrieved from the published *Musa malaccensis* genome [27]. All primers used are detailed in Supporting Table 1. Amplified fragments were ligated into pGEM-T Easy vectors (Promega), sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) and confirmed to have high homology with similar gene accessions in GenBank by BLAST analysis. Where degenerate primers were not successful for PCR amplification, partial gene fragments were used to design *Musa* specific primers which enabled consensus sequences to be amplified by overlapping partial coding sequences obtained through 5' and 3' rapid amplification of cDNA ends (RACE). Both 5´ and 3´ RACE reactions were carried out using a Second Generation 5´/ 3´ RACE Kit (Roche) as directed by the manufacturer. Asupina *CCD4* could not be obtained by RACE. The final Asupina *CCD4* sequence was amplified by PCR with *Musa* specific *CCD4* primers from Asupina gDNA. Isolated sequences have been deposited in GenBank, their respective accession numbers are detailed in supporting Table 2.

Sequence analysis. Nucleic acid and protein sequences were analysed using Geneious [28] version 7.1.8 (http://www.geneious.com) and protein sequence annotations were obtained using the PANTHER [29] gene ontology and Phobius [30] protein prediction database plugins.

RNA extraction and real-time RT-qPCR. RNA was extracted as above. Total RNA (1µg) was treated with DNase I using an RQ1 RNase-free DNase Kit (Promega), as directed by the manufacturer. RNA was reverse transcribed using a Trancriptor First Strand cDNA Synthesis Kit v.06 (Roche). cDNA was diluted 1:5 (v:v) in Rnase free water prior to real-time qPCR analysis. Per 20µl reaction, 5 µl of cDNA was added to 1 X GoTaq® qPCR Master Mix (Promega) premixed with primers at a final concentration of 0.2 µM.

159 Reactions were performed using a Rotor-Gene Q real-time PCR System (Qiagen). Reaction conditions
160 were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 5 s
161 and 82 °C for 5 s. Relative expression levels were calculated using the 2–ΔCT method [31,32] and
162 normalised Ct data obtained from target GOIs with Ct values from *Cyclophilin* (*CYP*) and *Ubiquitin 2*
163 (*UBQ2*) genes as internal controls. The expression stability of *CYP* and *UBQ2* the appropriate number of
164 reference genes required for RT-qPCR analysis for each cultivar during fruit development was confirmed
165 prior to their use as internal controls. Expression stability of the internal controls was assessed using the
166 statistical algorithms geNorm and NormFinder. *CYP* and *UBQ2* were found to be the more stable than
167 *RPS4*, *RPS2*, *CAC*, *RAN*, *ACT* and *DNAJ* under our experimental conditions. All gene specific primers were
168 designed using Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).
169 'Cavendish' *DXS* primers were designed against the 'Asupina' *DXS* coding sequence isolated in this study
170 and the respective PCR product confirmed by sequencing. All remaining qPCR primers for target genes
171 were designed in conserved regions of respective coding sequences isolated from both 'Cavendish' and
172 'Asupina' pulp. Primers used for RT-qPCR analysis are detailed in Supporting Table 3. Melt curve analysis
173 and sequencing of RT-qPCR amplification products verified primer specificity. All samples were taken
174 from three biological replicates and analysed in triplicate.

175 **Microscopy.** Six additional fruits were collected from each cultivar for microscopy. Three FG fruits of
176 both 'Asupina' and 'Cavendish' were kept at room temperature until the FR stage was reached.
177 Approximately 1 cm^3 sections were taken from the middle cross section of each fruit and fixed in 3%
178 paraformaldehyde at 4 °C overnight. Fixed samples were transferred to 70% (v/v) ethanol and freehand
179 sections (20 – 50 µm) were cut using double-edged blades, overlaid with cover slips and examined with
180 a Nikon Eclipse Ni light microscope (Nikon).

181 **Statistical analysis.** Student t-tests were used to calculate significant differences between pair-wise
182 comparisons. Significance was determined when t-tests returned a P-value ≤ 0.05. Correlations between

gene expression and carotenoid accumulation were determined by Pearson correlation (r) analysis using GraphPad Prism. R2 values were considered significant when a two-tailed P-value was ≤ 0.05.

RESULTS

Carotenoid content increases during fruit development. Fruits were collected at 3 weekly intervals following post bunch emergence. 'Asupina' fruits took an average of 30 weeks to reach FR maturity whereas 'Cavendish' fruits reached this stage within 15 weeks. The fruits of both cultivars increased in size throughout development with immature fruits emerging with green peel and white pulp. The peel and pulp of 'Cavendish' fruits gradually changed to yellow through fruit development whereas 'Asupina' changed to deep orange (Figure 2). The colour changes in both cultivars were concurrent with increased carotenoid accumulation during fruit development as determined by HPLC analysis. Lutein, α-carotene and β-carotene were detected in the pulp of both cultivars but the percentage composition and quantity of total carotenoids varied between the two. Lutein was the predominant carotenoid species in early stages of development in both cultivars and remained so until fruits reached FR in 'Cavendish'. Conversely in mature fruits of 'Asupina' β-carotene was the predominant carotenoid species accounting for 70.6% of total carotenoids by the FR stage (Figure 3, Supporting Table 4). 'Asupina' accumulated higher amounts of total carotenoids than 'Cavendish' throughout fruit development but the difference was more pronounced between the two cultivars during the transition to mature FG and FR fruit stages. During this phase, the accumulation of total carotenoids increased exponentially in 'Asupina' but not in 'Cavendish' (Figure 3) correlating with the transition in 'Asupina' from fruit with green peel and pale pulp to fruit with deeply orange pigmented peel and pulp. This also correlated with the switch in the predominant carotenoid species detected in the pulp of 'Asupina' from lutein to β-carotene. Quantification of total carotenoids showed that the FR fruits of 'Cavendish' contained approximately 14 μg g^{-1} DW total carotenoids, whereas FR 'Asupina' fruits accumulated 18-fold more total carotenoids,

207 containing approximately 257 μg g^{-1} DW. FR 'Asupina' fruits contained 79-fold more β-carotene and 13-
208 fold more α-carotene than FR 'Cavendish' fruits (Figure 3, Supporting Table 4).

209 **Biosynthetic gene expression varies between cultivars during fruit development.** Carotenoid
210 accumulation is the net result of compound biosynthesis, turnover and sequestration [11]. To
211 investigate if carotenoid accumulation patterns observed during fruit development in the two cultivars
212 correlated with changes in the expression of a range of genes involved in isoprenoid biosynthesis the
213 transcript level of five selected genes (*DXS, GGPS, PSY1, PSY2a*, and *LCYB*) were analysed by RT-qPCR.
214 Banana *PSY1* and *PSY2a* genes had previously been isolated [33]. Pulp expressed *DXS*, *GPPS* and *LCYB*
215 gene sequences were isolated for this study prior to RT-qPCR primer design and analysis. The GenBank
216 accession numbers for isolated sequences are included in supporting Table 2**.** The expression profiles of
217 each gene varied both throughout fruit development and between the two cultivars but they did not
218 correlate the with accumulation profiles of total carotenoids. A spike in the expression profiles of both
219 *GGPS* and *LCYB* was observed at the FR stage in 'Cavendish fruits (Figure 4), but this did not correlate
220 with an increase in total carotenoids or a change in the ratio of α to β-carotenoids between FG and FR
221 stages. In 'Asupina', the expression profiles of each gene generally decreased throughout development
222 with the exception of *GGPS* which increased up to the FG stage before decreasing at FR (Figure 4), this
223 did not correlate with the accumulation of total carotenoids or any individual carotenoid species.
224 Interestingly, the only significant positive correlation between carotenoid-specific gene expression and
225 the accumulation of individual or total carotenoids in either cultivar, as identified by Pearson correlation
226 (r) analysis, was observed between lutein and the expression of *PSY2a* and only in 'Asupina' (p = 0.023)
227 (Figure 4). Both the expression of *PSY2a* and the accumulation of lutein decreased in the pulp of mature
228 'Asupina' fruits (FG-FR) coinciding with the previously observed switch in the predominant carotenoid
229 ratio from lutein to β-carotene. The exponential increases in β-carotene observed in the 'Asupina' pulp
230 during the transition to fruit maturity (FG and FR) did not correlate with an increase in *LCYB* expression;
231 *LCYB* expression actually decreased in these stages (Figure 4). In combination, these results suggest that

in ‘Asupina’ pulp the α-carotene branch of the carotenoid pathway (Figure 1) appears to be transcriptionally regulated by the expression of *PSY2a*, whilst the β-branch of the pathway is likely influenced by an alternative regulation mechanism, considerably so in the latter stages of fruit development.

***CCD4* expression in higher in ‘Cavendish’ than in ‘Asupina’.** Possible carotenoid turnover was investigated by isolating pulp expressed *CCD4* coding sequences from each cultivar to enable comparison of transcript abundance**.** The GenBank accession numbers for isolated sequences are included in supporting Table 2**.** Transcript levels were analysed during fruit development by RT-qPCR to determine whether carotenoid accumulation patterns observed in either cultivar correlated with changes in *CCD4* expression. The expression of *CCD4* was higher during fruit development in ‘Cavendish’ than in ‘Asupina’ (Figure 5). Furthermore, *CCD4* had a significant negative correlation across fruit development with β-carotene accumulation in ‘Cavendish’ fruits (P = 0.027), but not in ‘Asupina’. Notably, S6 ‘Cavendish’ fruits accumulated the lowest levels of β-carotene (Figure 3, Supporting Table 4 B) and had the highest expression level of *CCD4.*

Amyloplasts convert to chromoplasts upon fruit ripening in Fe’i bananas. Steady-state levels of carotenoids are affected by the rates of biosynthesis, degradation and sequestration. In plants, carotenoids are both synthesised and sequestered in plastids. Microscopic analysis was performed to investigate carotenoid accumulation in plastids within the pulp of FG and FR ‘Asupina’ and ‘Cavendish’ fruits. ‘Asupina’ contained more non-pigmented plastids of varying sizes compared to ‘Cavendish’ at the FG stage (Figure 6). Furthermore, in ‘Asupina’, a transition from non-pigmented plastids into orange-yellow pigmented plastids consistent in appearance with chromoplasts was observed (Figure 6). This transition was not observed in ‘Cavendish’ fruits (Figure 6).

Dry matter content of mature fruits is significantly different between cultivars. Due to the variations in plastid type, size and abundance observed microscopically in the mature fruits of the two cultivars, dry

matter content (DMC) of fruit pulp was assessed (Supporting Figure 1). 'Cavendish' had a significantly higher DMC (22.4%, FG; 23% FR) than 'Asupina' (19.4%, FG; 16.8% FR) at both FG (P = 0.013) and FR (P = 0.006) which manifests as firmer fruit. For the transition from FG to FR, there was virtually no change in DMC in Cavendish. However, a 13% difference in DMC was observed between the FG and FR stages of 'Asupina' which correlated with a dramatic reduction in fruit firmness and an increase in β-carotene.

DISCUSSION: Carotenoid accumulation occurs predominantly in the final stages of 'Cavendish and 'Asupina' fruit development and correlates with pulp colour intensity. Previously, little was known about carotenoid biosynthesis during banana fruit development or the underlying regulatory mechanisms accounting for variations in carotenoid content between high and low accumulating cultivars. A clear increase in pulp colour intensity was observed between the transition to FR banana fruits of both the low carotenoid accumulating cultivar 'Cavendish' (*Musa* sp., AAA group) and high accumulating Fe'i cultivar 'Asupina'. However, the change was vastly more prominent for 'Asupina' which appeared orange by the FR stage in contrast to cream to yellow 'Cavendish' fruits. The increase in colour intensity in both cultivars and the higher pigmentation of 'Asupina' fruits relative to 'Cavendish' correlated with enhanced carotenoid content. Quantification of total carotenoids revealed that ripened 'Asupina' fruits accumulate ~18-fold more carotenoids that 'Cavendish' principally due to higher levels (79-fold) of β-carotene. The composition of the carotenoid species present varied through fruit development between the two cultivars. Lutein accumulated predominantly in early stages of 'Asupina' development through to fruit maturity after which β-carotene accumulation increased exponentially becoming the predominant carotenoid (~70%) in FR fruit. Conversely, lutein remained the predominant carotenoid throughout 'Cavendish' fruit development.

Carotenoid accumulation during banana fruit development is under complex regulation and varies between cultivars. The regulation of carotenoid biosynthesis is highly complex and multifaceted with

dynamic changes in composition occurring throughout a plant's life cycle in response to developmental cues during germination, photomorphogenesis, photosynthesis, fruit development and in response to external environmental stimuli [11,18]. There are reports describing correlations in carotenoid gene expression during fruit ripening coinciding with changes in carotenoid content [1,19,34-40] but this had not been investigated in banana. Both the first step in the MEP pathway, (regulated by DXS) and the first committed step in the carotenoid pathway (the condensation of GGPP to form phytoene by PSY) are rate limiting reactions in the biosynthesis of carotenoids [12-17]. Over-expression of *DXS* enhanced total carotenoid levels in Arabidopsis (*Arabidopsis thaliana*) seedlings by >12% whilst RNAi mediated silencing of *DXS* reduced total carotenoids by 13-15% [12,13]. *DXS* expression is developmentally regulated during fruit ripening in tomato, correlating with an increase in both *PSY* mRNA transcript abundance and carotenoid accumulation [41]. Multiple isozymes of PSY have been isolated from many species including banana [33,42,43], their activity appears redundant but their expression is tissue specific and often show unique responses to environmental stimuli. For example, in tomato *PSY1* encodes a fruit and flower specific isoform responsible for carotenogenesis in chromoplasts, whilst *PSY2* is predominantly expressed in green tissues [42]. Conversely, in banana, *PSY1* has been demonstrated to be primarily expressed in leaves, while *PSY2* is primarily expressed in fruits [33]. *PSY* expression is also regulated by ABA, high light, salt, drought, temperature, photoperiod, developmental cues and post-transcriptional feedback [18]. Previously, we have demonstrated that 'Asupina' PSY enzymes have around twice the enzymatic activity of corresponding 'Cavendish' PSYs [33], suggesting that differences in PSY activity may contribute to the differences in 'Asupina' and 'Cavendish' fruit pVAC content. However, we have since expressed 'Asupina' PSY enzymes in 'Cavendish' bananas and although this approach increased the pVAC content of the fruit, they were significantly lower than the levels in 'Asupina' (data unpublished).

In the current study, transcript abundance of *DXS, GGPS, PSY1*, *PSY2a* and *LCYB* was measured throughout fruit development in the two cultivars and expression patterns analysed for correlations

with accumulation patterns of individual carotenoid species or total carotenoid levels. The only significant positive correlation observed between transcript abundance and the accumulation of carotenoids was between lutein and the expression of *PSY2a,* and only so in 'Asupina'. Both lutein and *PSY2a* transcript abundance decreased during fruit development indicating that in 'Asupina' fruit *PSY2a* expression influences carotenoid biosynthesis in early stages of fruit development. The most significant changes in carotenoid levels both in and between 'Asupina' and 'Cavendish' fruits however, are observed in late FG-FR stages of fruit development. In combination, these results suggest that transcript abundance of *DXS, GGPS, PSY1*, *PSY2a* or *LCYB* does not regulate total carotenoid levels in mature fruit of either cultivar. Gene isolation and expression analyses could be extended to additional isoprenoid genes and subsequent transcript analyses. However, these initial results suggest a post-transcriptional regulatory mechanism may be responsible for the differences in total pVAC levels between "Asupina and 'Cavendish' fruit, rather than the transcriptional regulation of isoprenoid genes.

Recently, it has been reported that PSY and wild-type ORANGE (OR_{WT}) family proteins in *Arabidopsis* interact with each other in plastids [44]. Changes in OR_{WT} expression exerted minimal effect on PSY transcript abundance but overexpression of OR_{WT} significantly increased the amount of enzymatically active PSY, suggesting that OR_{WT} proteins serve as major posttranscriptional regulators of PSY [44]. OR_{WT} genes have not been isolated from any banana and their role on carotenogenesis in banana has not been investigated. It's possible that posttranscriptional regulation of PSY has a greater influence on carotenoid accumulation in banana than transcriptional regulation. If so, increased levels of enzymatically active PSY in 'Asupina', coupled with the greater activity of 'Asupina' PSYs relative to 'Cavendish' [33], may in concert contribute to the differences in pVACs between the cultivars.

Carotenoid accumulation is also affected by turnover. Carotenoids are cleaved forming apocarotenoids by a group of CCD enzymes and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) [11]. CCDs act by selective oxidative cleavage of a double bond within the polyene chain that forms the backbone

of all carotenoids. Apocarotenoid products include the phytohormones abscisic acid and strigolactones plus a wide range of flavour and aroma compounds [45]. NCEDs cleave the 11, 12 (11', 12') double bonds of 9-cis-violaxanthin or 9-cis-neoxanthin, catalysing the first step in abscisic acid biosynthesis [45].The expression of CCDs which catabolise enzymatic turnover of carotenoids can also affect the ratio of specific carotenoids in a given system as they inversely regulate carotenoid accumulation. Among the small CCD family, two classes (CCD1 and CCD4) have been highly associated with carotenoid turnover. CCD1 has been found to contribute to apocarotenoid volatiles in the flowers and fruit of many plant species [46-48], whilst CCD4 has demonstrated a more dramatic influence on carotenoid accumulation. For example, high *CCD4* expression is responsible for white-fleshed peach (*Prunus persica*) cultivars [49] and white petal variants of chrysanthemum (*Chrysanthemum morifolium* Ramat.) [50]. Down-regulation of *CCD4* elevates carotenoid levels in chrysanthemum producing flowers with yellow petals [50,51]. Increased carotenoid catabolism associated with an increase in the expression of *CCD* genes has also been demonstrated to be partially responsible for a pale yellow mutant of ponkan (*Citrus reticulata* Blanco) [52].

In the present study, lutein accumulated in early stages of 'Asupina' development, followed by an exponential increase in β-carotene accumulation in late (FG-FR) stages of development. This indicates that during early stages of fruit development either the α-carotene branch of the carotenoid biosynthetic pathway leading to the synthesis of lutein is favoured or that products biosynthesised via the β-carotene branch of the carotenoid pathway are catabolised at a higher rate in early stages of fruit development. Subsequently, a regulatory change occurs resulting in a switch in the predominant carotenoid species present in the pulp from lutein to β-carotene in mature fruit. Conversely, in 'Cavendish' fruit it appears that the α-carotene branch of the biosynthetic pathway is favoured throughout development or products biosynthesised via the β-carotene branch are catabolised at a higher rate, thus lutein remains the predominant carotenoid present in the pulp from bunch emergence through to ripened fruit maturity. However, in 'Asupina' despite the accumulation of lutein correlating

with the expression of *PSY2a* in early stages of fruit development as previously discussed, the exponential increase in β-carotene observed in the pulp during the final stages of fruit maturity did not correlate with changes in *PSY2a* expression, an increase in *LCYB* expression or a decrease in *CCD4* expression. Rather, *LCYB* transcript abundance decreased in these stages. In 'Cavendish' though, *CCD4* expression was negatively correlated throughout fruit development with the accumulation of β-carotene suggesting that β-carotene is a substrate for the 'Cavendish' CCD4. Previously, CCD4 mediated turnover of β-carotene has been reported in peach [53], chrysanthemum [54] and Arabidopsis [55].

These results suggest that *CCD4* expression may contribute to the low carotenoid status of 'Cavendish' fruit. However, neither carotenoid catabolism nor an increase in flux through the β-carotene branch of the carotenoid biosynthesis pathway due to elevated *LCYB* expression appear to be the regulatory mechanism underlying the exponential increase in β-carotene observed in the final stages of 'Asupina' fruit maturity.

High levels of carotenoids in Fe'i bananas correlate with the transition of amyloplasts to chromoplasts and a reduction in dry matter content. In the present study, more undifferentiated plastids were observed in the pulp of FG 'Asupina' fruit than in 'Cavendish'. In 'Asupina', non-coloured plastids transitioned into orange chromoplasts between the FG and FR stage. Interestingly, no carotenoid crystals were observed 'Asupina" fruits, the chromoplasts observed appeared globular in appearance. The physical form of carotenoid deposition in plants has an impact on their liberation efficiency from food matrices, and a better liberation and bio-accessibility of carotenoids from non-crystalline chromoplasts has been hypothesized [56].

The strength of sink used to sequester carotenoids such as plastid type and compartment size can strongly influence carotenoid accumulation. This is well demonstrated in high-pigment (*hp*) mutants of tomato (*Solanum lycopersicum*) and an orange (*Or*) curd mutant of cauliflower (*Brassica oleracea* botrytis) [57-60]. Both *hp-1* and *hp-2* tomato mutants accumulate high levels of lycopene in fruits

associated with increases in plastid size and number. However, in *hp-1* fruits, this does not correlated with an increase in transcript abundance of carotenoid biosynthesis genes but with elevated levels of chromoplast-specific carotenoid-associated protein (CHRC) which enhances carotenoid sequestration and stability [61]. Further, the cauliflower *Or* mutant, which appears orange due to a high β-carotene accumulation, is not associated with changes in isoprenoid gene expression but with the development of chromoplasts, not observed in the wild-type [62]. It has been suggested that carotenoid sequestration in chromoplasts may prevent carotenoid end products from overloading plastid membranes, the site of carotenoid biosynthesis, avoiding negative feedback to the biosynthetic pathway by the end product thus enabling high carotenoid deposition [63]. Recently, a homolog to the cauliflower *Or* gene (*CmOr*) was identified in orange fleshed melon (*Cucumis melo*) [64]. The transition of amyloplasts to chromoplasts in 'Asupina' may also be due to a homolog of the cauliflower *Or* gene, however, the potential role of *Or* in banana fruit remains to be investigated. The isolation of an *Or* homolog in banana could be of great importance for future pVAC biofortification strategies, particularly so for the genetic modification of low carotenoid cultivars.

'Asupina' fruit had a significantly lower DMC than 'Cavendish' at the FG stage becoming further pronounced at the FR stage. This was concurrent with a loss in fruit firmness and the observation of chromoplasts in 'Asupina'. These results are significant as if these traits are linked then high carotenoid cultivars such as the Fe'i group 'Asupina' may not be ideal for biofortification breeding programmes, as although they have high carotenoid levels, their use could result in the development of cultivars with reduced fruit dry matter content and firmness. Texture is viewed by consumers as one of the most important attributes in determining a banana or plantain preferred for cooking [65]. Thus any changes in fruit texture or a loss in firmness are unlikely to be acceptable to consumers.

Taken together, our results suggest that *CCD4* expression may contribute to the low carotenoid status of 'Cavendish' fruit. However, the primary difference accounting for the variation between

'Asupina' and the low carotenoid accumulating triploid (AAA) 'Cavendish' bananas is likely due to enhanced storage sink strength in 'Asupina' due to the formation of chromoplasts in mature fruit. It is possible that this is due to a homolog of the cauliflower *Or* gene within 'Asupina' that is yet to be identified. These insights should prove important in the future for the development of banana PVA biofortification strategies.

ABBREVIATIONS USED

CCD. carotenoid cleavage dioxygenase

CHRC. carotenoid-associated protein

CYP. Cyclophilin

DAFF. Department of Agriculture and Fisheries

DMC. dry matter content

DXS. 1-deoxy-D-xylulose-5-phosphate synthase

FG. full-green

FR. full-ripe

GGPP. geranylgeranyl diphosphate

GGPS. geranygeranyl diphosphaste synthase

LCYB. β-lycopene cyclase

LCYE. ε-lycopene cyclase

MEP. 2-C-methyl-D-erythritol 4-phosphate

NCED. 9-cis-epoxycarotenoid dioxygenase

Or. orange gene

PSY. phytoene synthase

pVAC. pro-vitamin A carotenoids

UBQ .Ubiquitin

VAD. vitamin A deficiency

WHO. World Health Organisation.

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417

418 **SUPPORTING INFORMATION**

419 Supporting Table 1: Primers used for isoprenoid gene isolation.

420 Supporting Table 2: GenBank accession numbers for isolated isoprenoid genes.

421 Supporting Table 3: Primers used for RT-qPCR analyses.

422 Supporting Table 4: Carotenoid content of developing 'Asupina' and 'Cavendish' fruits.

423 Supporting Figure 1: Dry matter content of mature stage banana pulp tissue.

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FIGURE CAPTIONS

Figure 1: Schematic representation of the carotenoid biosynthesis pathway in plants

The structures of the predominant carotenoids that accumulate in 'Cavendish' and 'Asupina' bananas are illustrated. Enzymes are indicated by the following annotation: PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO; ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotene isomerase; LCYE, lycopene ε-cyclase; LCYB, lycopene β-cyclase; β-OH, carotene β-hydroxylase; ε-OH, ε-hydroxylase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase.

Figure 2: Representative photographs of low and high carotenoid accumulating banana fruits during the final stages of development

C, 'Cavendish'; A, 'Asupina'; FG, full-green fruit; FR, full-ripe fruit. Stages (S) 12 and 24 represent the final stages of fruit development prior to the FG stage for 'Cavendish' and 'Asupina' respectively, with the number corresponding to weeks post bunch emergence.

Figure 3: Carotenoid content of developing 'Asupina' (A) and 'Cavendish' (B) banana fruits

Total carotenoid contents were calculated as the sum of each carotenoid, as determined by HPLC-PDA analysis. Values are the average of nine measurements comprised of three biological replicates analysed in triplicate ± SEM.

Figure 4: Expression of isoprenoid metabolic genes in developing 'Asupina' (A) and 'Cavendish' (B) banana fruits.

Relative expression levels of genes associated with carotenoid biosynthesis in developing banana fruits as determined by RTqPCR. Values are the average of three biological replicates analysed in triplicate ± SEM.

Figure 5: *CCD4* expression in developing 'Asupina' (A) and 'Cavendish' (B) banana fruits.

Relative expression level of *CCD4* in developing banana fruits as determined by RTqPCR. Values are the average of three biological replicates analysed in triplicate ± SEM.

Figure 6: Representative microscopic images of mature stage banana pulp cells

C, 'Cavendish'; A, 'Asupina'; FG, full-green fruit; FR, full-ripe fruit. Scale bars are 100µm.

FIGURE GRAPHICS

Figure 1

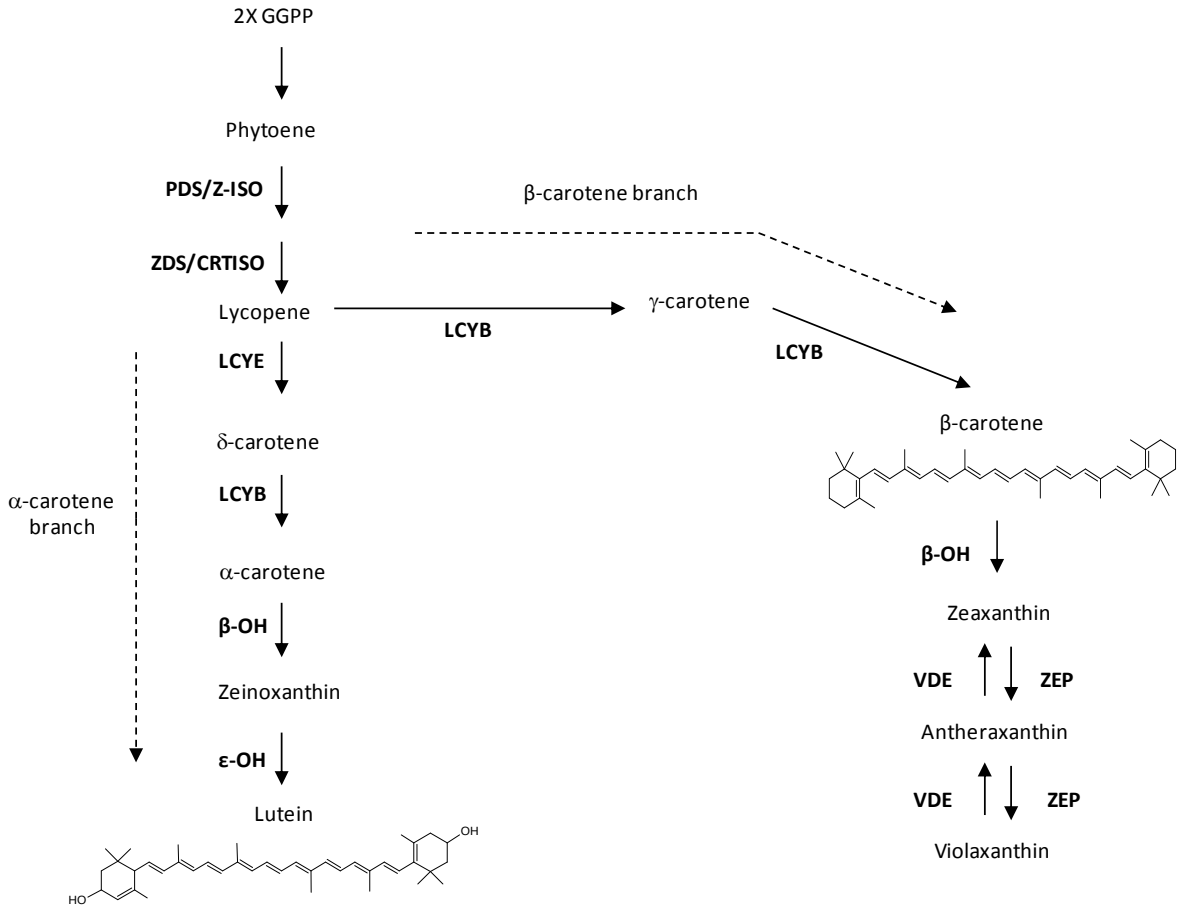


Figure 2

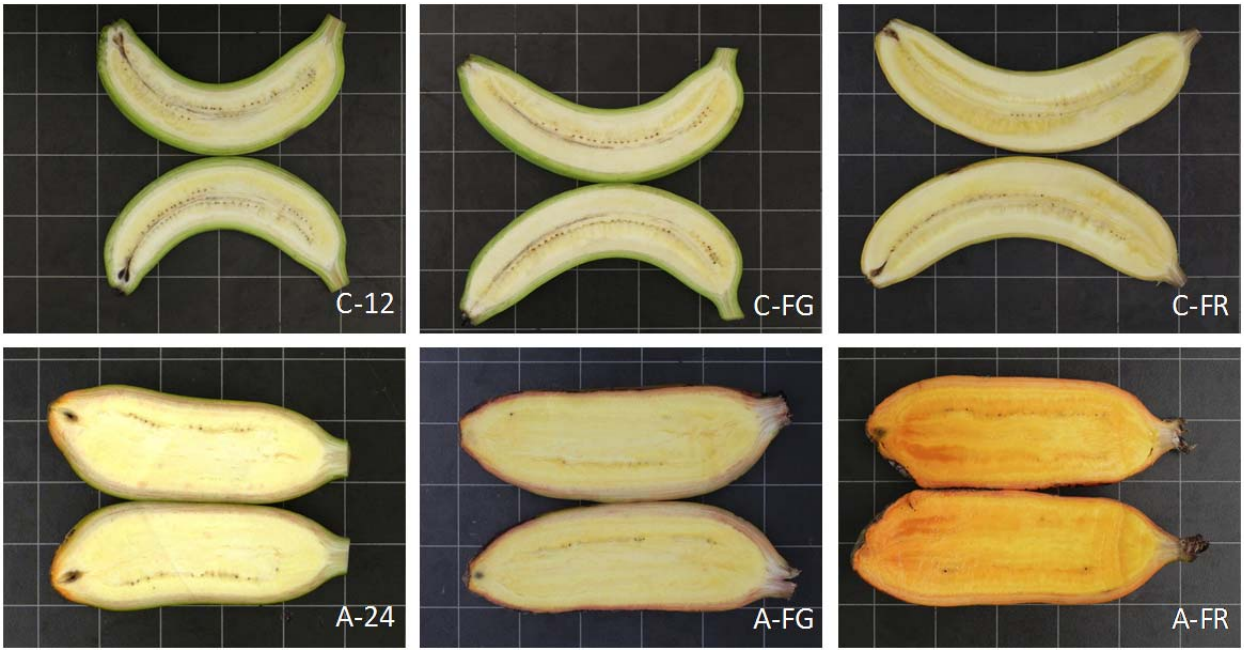


Figure 3

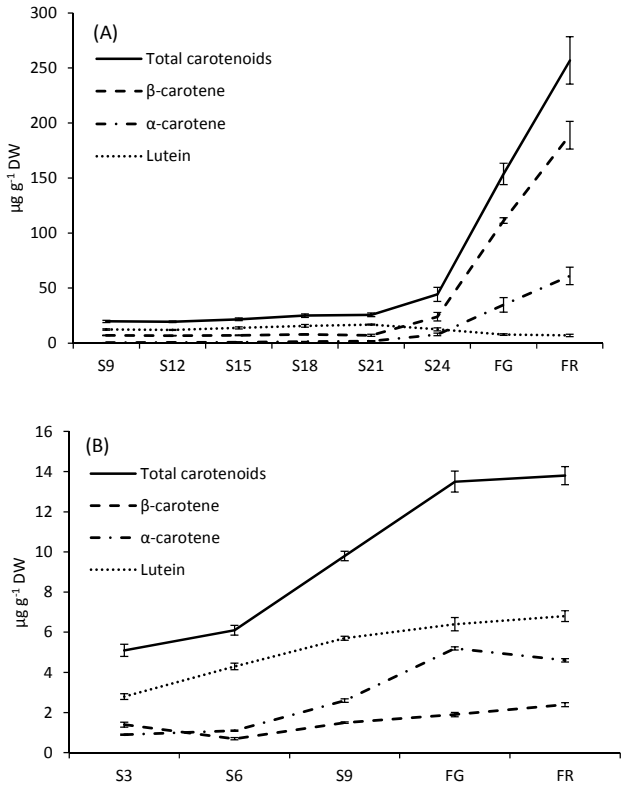

(A)
Total carotenoids
β-carotene
α-carotene
Lutein
µg g-1 DW
300
250
200
150
100
50
0
S9
S12
S15
S18
S21
S24
FG
FR
(B)
Total carotenoids
β-carotene
α-carotene
Lutein
µg g-1 DW
16
14
12
10
8
6
4
2
0
S3
S6
S9
FG
FR

Figure 4

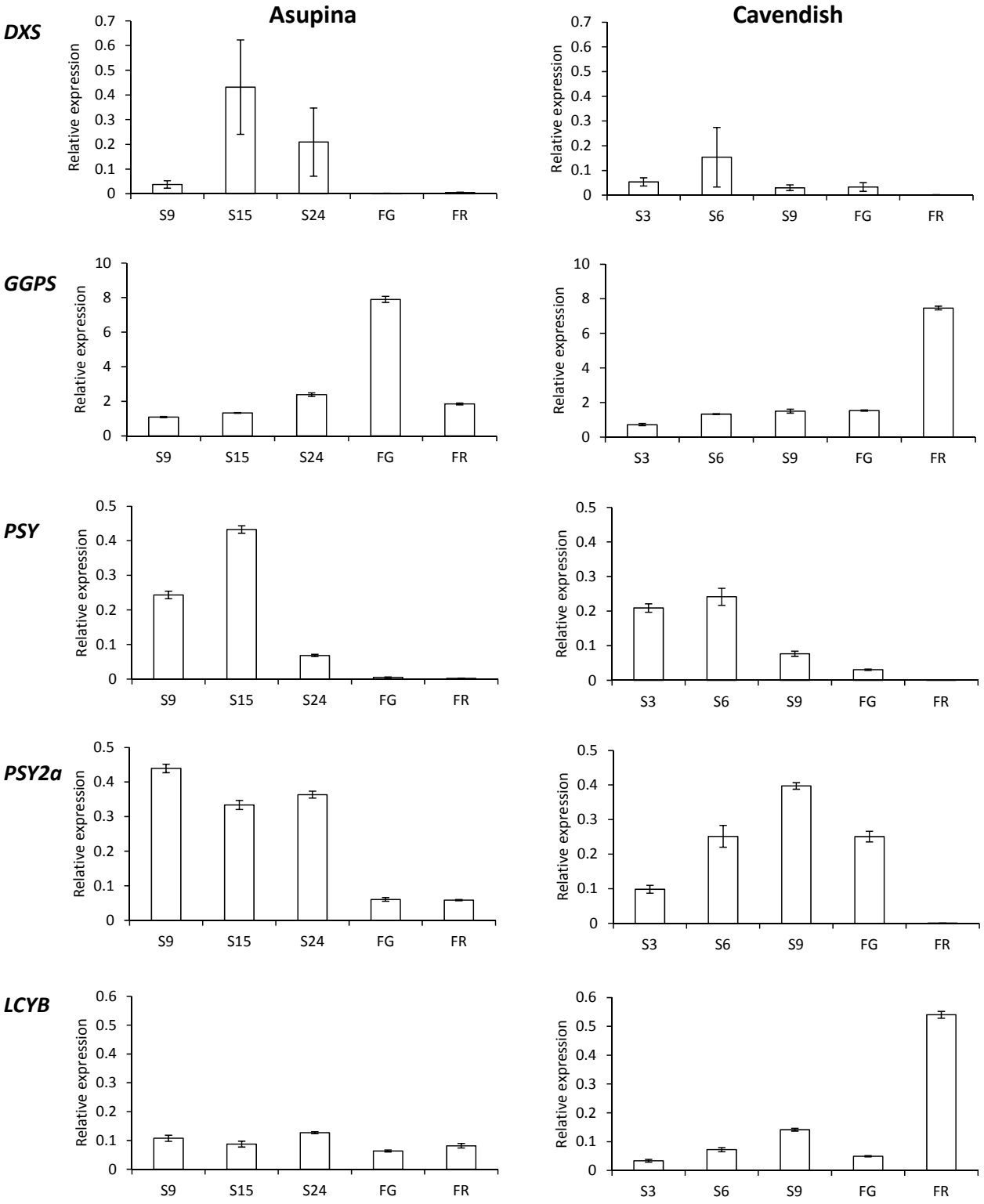

Asupina
Cavendish
DXS
GGPS
PSY
PSY2a
LCYB
Relative expression
S9
S15
S24
FG
FR
S3
S6

Figure 5

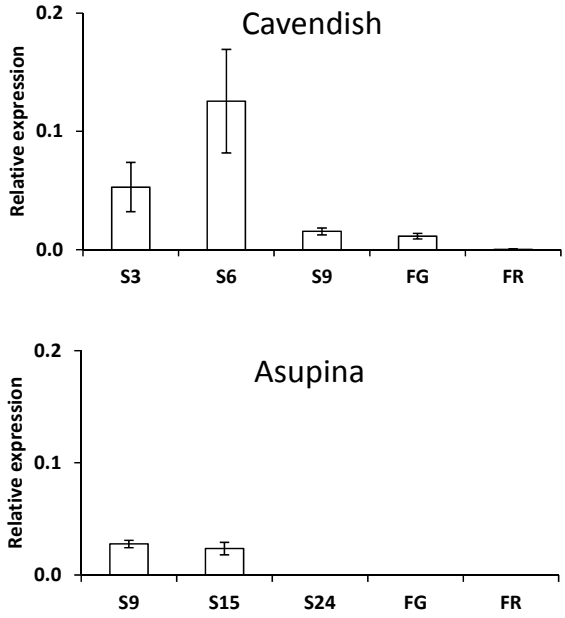
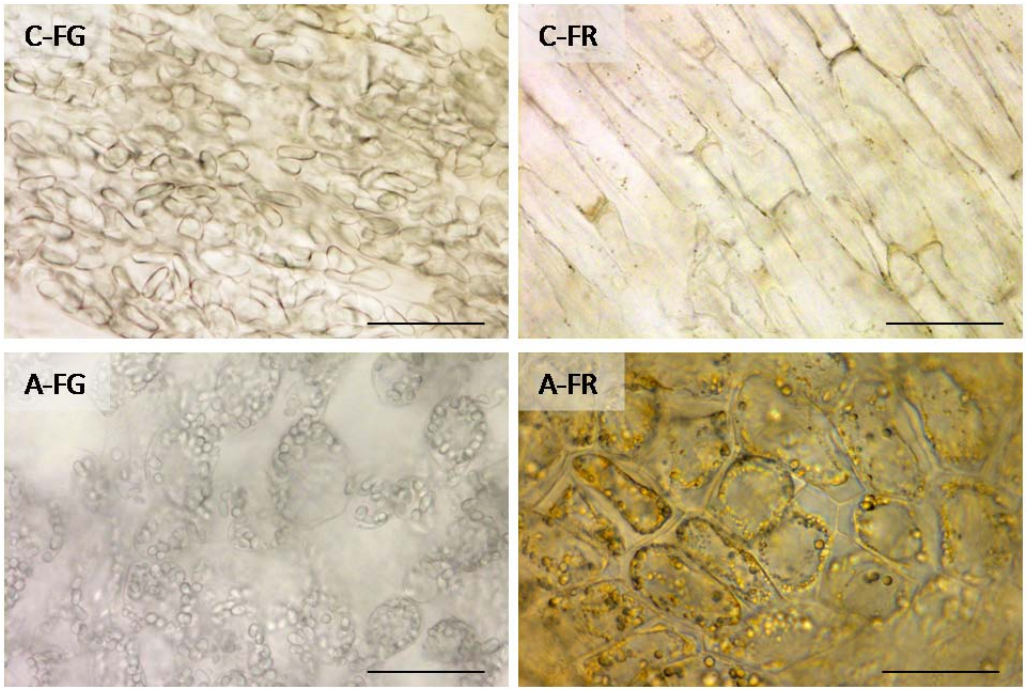

Cavendish
Relative expression
0.2
0.1
0.0
S3
S6
S9
FG
FR
Asupina
Relative expression
0.2
0.1
0.0
S9
S15
S24
FG
FR

Figure 6



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