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Gene Expression and Metabolite Profiling of Thirteen Nigerian Cassava Landraces to Elucidate Starch and Carotenoid Composition

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Abstract: The prevalence of vitamin A deficiency in sub-Saharan Africa necessitates effective approaches to improve provitamin A content of major staple crops. Cassava holds much promise for food security in sub-Saharan Africa, but a negative correlation between β-carotene, a provitamin A carotenoid, and dry matter content has been reported, which poses a challenge to cassava biofortification by conventional breeding. To identify suitable material for genetic transformation in tissue culture with the overall aim to increase β-carotene and maintain starch content as well as better understand carotenoid composition, root and leaf tissues from thirteen field-grown cassava landraces were analyzed for agronomic traits, carotenoid, chlorophyll, and starch content. The expression of five genes related to carotenoid biosynthesis were determined in selected landraces. Analysis revealed a weak negative correlation between starch and β-carotene content, whereas there was a strong positive correlation between root yield and many carotenoids including β-carotene. Carotenoid synthesis genes were expressed in both white and yellow cassava roots, but phytoene synthase 2 (*PSY2*), lycopene-ε-cyclase (*LCYε*), and β-carotenoid hydroxylase (*CHYβ*) expression were generally higher in yellow roots. This study identified lines with reasonably high content of starch and β-carotene that could be candidates for biofortification by further breeding or plant biotechnological means.

Keywords: carotenoid biosynthesis; liquid chromatography–mass spectrometry (LC–MS), provitamin A; biofortification

1. Introduction

Cassava (*Manihot esculenta* Crantz) is an important starchy root crop in the tropics. It is a common staple food in Africa, Asia, and Latin America [1], and particularly of high importance in sub-Saharan African countries. Cassava is cultivated for its tolerance to stress and ability to grow under drought conditions [2]. As a result, cassava serves as an important component for food security for low income farmers who rely on it as a major source of dietary energy. While cassava is rich in

starch and serves as a good energy source, it is extremely low in protein and important micronutrients, such as iron, zinc, and provitamin A carotenoids [3]. Therefore, a diet reliant on cassava predisposes one to malnutrition, especially provitamin A deficiency.

Carotenoids are C40 lipophilic isoprenoids synthesized in plants, algae, bacteria, and fungi [4]. Carotenoids play essential roles in photosynthesis and photoprotection and provide precursors for phytohormones abscisic acid (ABA) and strigolactones (SLs) [5]. They are also precursors of provitamin A, an essential component of human diet due to its roles in human health [6].

Carotenoids are synthesized in plants via the methylerythritol 4-phosphate (MEP) pathway, localized in the plastids [4] (Figure1). Carotenoid synthesis starts with the formation of the C5 prenyl phosphates; isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are formed when isomerized by isopentenyl diphosphate isomerase (IPI) [6]. IPP and DMAPP then undergo reaction condensation by geranylgeranyl diphosphate synthase (GGPPS) to form geranylgeranyl diphosphate (GGPP) [4]. Condensation of two molecules of GGPP by phytoene synthase (PSY) leads to the formation of phytoene, the first carotenoid product and known rate-limiting metabolite of carotenoid biosynthesis [4,5]. Plant tissues exhibiting low levels of carotenoid production are thought to have a low expression level of PSY; additionally, PSY expression driven to high levels has resulted in accumulation of β-carotene crystals in *Arabidopsis* and carrot [3]. An overexpression of PSY in nonphotosynthetic storage roots such as cassava (*Manihot esculenta*) and some nongreen plants like tomato fruits (*Solanum lycopersicum*), canola seeds (*Brassica napus*), and rice endosperm (*Oryza sativa*) has also resulted in an increased flux through the biosynthetic pathway, further confirming that high carotenoid levels depend on PSY expression [3,7]. Phytoene is converted into lycopene by a series of desaturation and isomerization reactions catalyzed by phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (ZISO), and carotenoid isomerase (CRTISO) [4–6]. Lycopene undergoes a cyclization reaction by lycopene ε-cyclase (LCYε) and/or lycopene β-cyclase (LCYβ) to produce α-carotene and β-carotene, representing the α- and β-branch of the pathway, respectively [5]. Subsequently, α-carotene and β-carotene undergo hydroxylation by β-carotenoid hydroxylases (CHYβ) to form lutein in the α-branch and zeaxanthin in the β-branch of the pathway [4–6]. Epoxidation and de-epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) form the xanthophyll cycle, a mechanism to protect plants against photodamage [5]. The pathway concludes by the conversion of violaxanthin into neoxanthin by neoxanthin synthase (NXS). Furthermore, oxidative cleavage of carotenoids by carotenoid cleavage dioxygenases (CCDs) such as 9-cis-epoxycarotenoid dioxygenase (NCED), produces apocarotenoids, phytohormones, signaling molecules and volatiles, thus maintaining a steady-state level of carotenoids and curbing excessive accumulation [5]. Figure 1 shows a simplified carotenoid pathway.

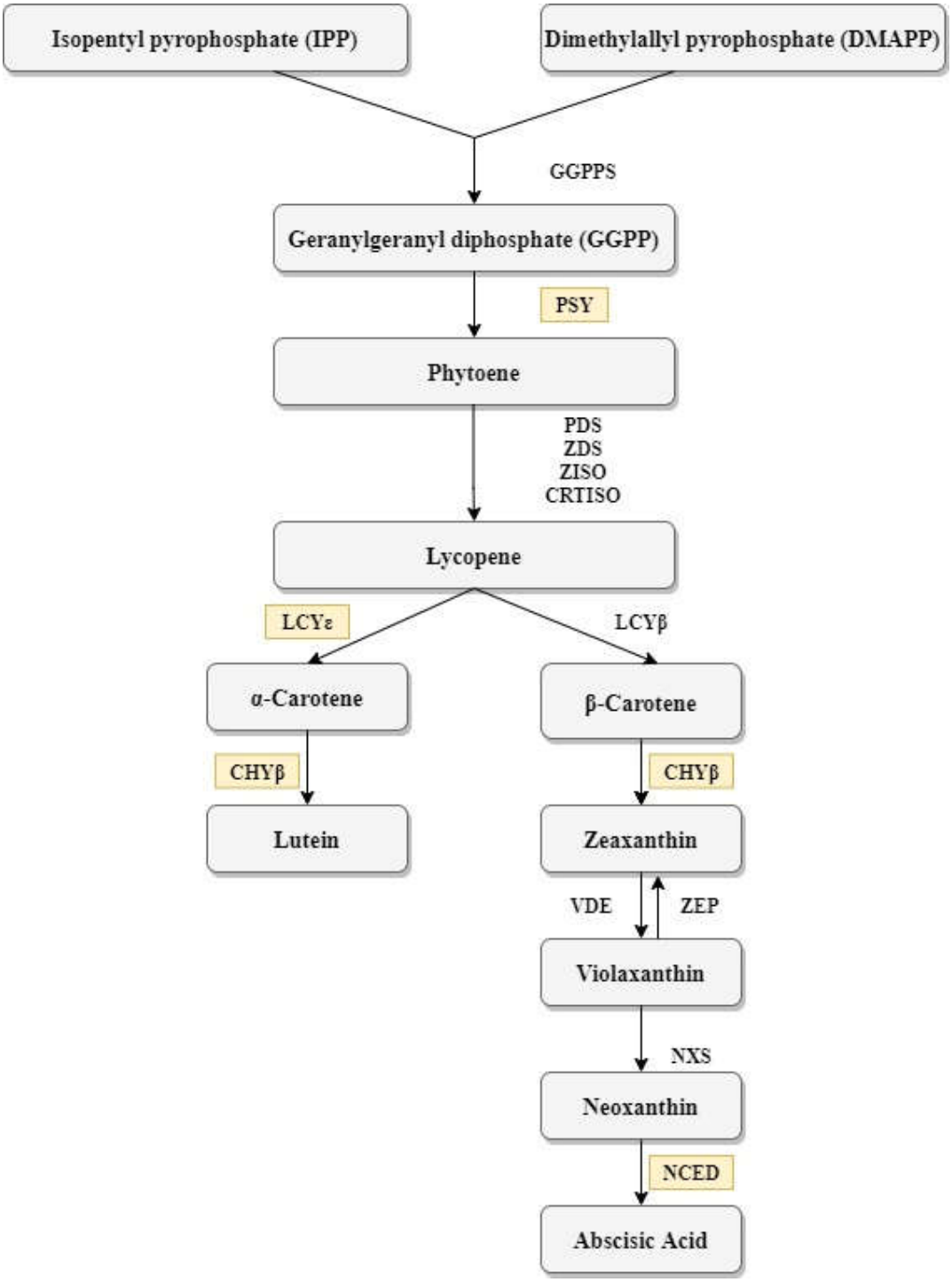

Figure 1. Schematic carotenoid biosynthesis pathway showing metabolites and genes involved in their synthesis. Genes measured by quantitative real-time polymerase reaction (qPCR) are marked. Geranylgenranyl diphosphosphate synthase (GGPPS), Phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (ZISO), carotenoid isomerase (CRTISO), lycopene ε-cyclase (LCYε), lycopene β-cyclase (LCYβ) β-carotenoid hydroxylases (CHYβ), zeaxanthin epoxidase (ZEP), violaxanthin de-epoxidase (VDE), 9-cis-expoxycarotenoid dioxygenase.

Food high in β-carotene is related to several health benefits because of its contribution to vitamin A in the body. Other carotenoids, such as β-cryptoxanthin and α-carotene can also be converted to vitamin A following cleavage by β-carotene oxygenase 1 (BCO1) [8]. Vitamin A maintains good

eyesight, keeps the skin and epithelial linings of the internal organs healthy, and maintains immunity against diseases [8]. It is also involved in growth, development, and reproduction [9]. Vitamin A deficiency (VAD) is a global health problem affecting mostly vulnerable groups, such as preschool children and pregnant women [10], and health consequences include a weakened immune system, poor eyesight, and eventual blindness. Globally, 1–2.5 million child deaths are attributed to nutritional deficiencies; an estimated 761,000 of these deaths are caused by vitamin A, iron, and zinc deficiency [11]. Due to these consequences, efforts are being made to improve the provitamin A carotenoid content of major staple crops such as cassava. These efforts include fortification, dietary diversification, supplementation, and biofortification of major staple crops such as rice and cassava. Of all these efforts, biofortification proves to be most sustainable [12].

Biofortification is the use of breeding or genetic engineering to enhance nutritional content of crops and subsequently improve human nutritional value [7]. Genetic engineering of cassava has the advantage in that conventional cassava breeding requires a lengthy process to breed for new traits, usually about 8–13 years [13]. Studies have reported a negative correlation between dry matter and carotenoid content due to genetic linkage in African cassava germplasm [2,13,14]. The observed negative correlation may pose a challenge to biofortification efforts for cassava.

Carotenoid content and expression of key transcript in carotenoid biosynthesis were studied previously in cassava landraces of storage roots with varying color. Analysis of 19 carotenoid types by high performance liquid chromatography with diode array detection (HPLC–DAD) detected a complex carotenoid composition as well as a significant association between lycopene β-cyclase (LYCβ) expression and total β-carotene content [15].

Our study compares the variations in carotenoid profiles of thirteen Nigerian cassava landraces as well as the expression levels of carotenoid biosynthesis genes in a select subset of these landraces. These landraces are screened in an effort to introduce more cassava lines to tissue culture, thereby widening the genetic base with desirable traits such as high dry matter, high starch and high micronutrient content, for further improvement by genetic transformation or gene editing. The aim of this study is also to better understand the relationship between agronomic traits, carotenoids, and expression of genes in the carotenoid biosynthesis pathway in cassava.

2. Materials and Methods

2.1. Plant Material

Thirteen cassava landraces were selected from the collection of the International Institute of Tropical Agriculture (IITA), Nigeria, based on variance in starch and β-carotenoid accumulation.

2.2. Field Trial and Layout

Trials were conducted over two seasons between July 2017 and August 2019, each lasting for 13 months. Trials for the two seasons were carried out at the same location at IITA Ibadan, Nigeria (N 7.490250,E 3.884143). Temperature and rainfall data were collected from the IITA weather station over the two-season period to monitor the potential impact of seasonal changes of weather conditions on detected trait variations. For the agronomic measurements, trials were laid out in a completely randomized block design with two replications. There were twenty plants per plot, each plot had four rows with five plants in each row ; two replications provided forty plants per genotype. Plants from both replication plots were harvested both seasons to confirm agronomic parameters and performance of all genotypes in the field.

Planting was done with a 1 m spacing between each plant and adjacent plots were separated by 1 m alleys. All the stakes used for planting were generated from fresh portions of mature stems. Hand weeding was done when needed. Since both field trials started in July (during the rainy season), plants received adequate water from rainfall during the initial growth stage, and there was no need for irrigation.

2.3. Sample Collection for Gene Expression and Metabolite Analyses

Source leaves and storage roots were collected for gene expression and metabolite analyses. Leaves and roots were immediately snap frozen in liquid nitrogen. Root samples were collected and prepared under a canopy tent to shield them from sunlight as much as possible. For molecular and metabolic analysis, three roots were randomly selected from the three plants harvested from the same plot to ensure homogeneity and minimize differences in soil characteristics. Selected roots were peeled, cleaned, and portions of the central part were chopped into small cubes and mixed together. The roots were then placed in aluminum foil and teabags, snap frozen in liquid nitrogen and stored at -80^{0}C.

2.4. Agronomic Data and Dry Matter Content (DMC)

For each trial, dry matter content (DMC) and fresh root weight (FRW) were measured at 4, 8, and 13 months after planting. Three plants per genotype were uprooted at random from the same plot at every sampling time to measure traits. iCheck Carotene by BioAnalyt (Teltow, Germany) a portable spectrophotometer, was used to measure total carotenoids. This tool determines the total carotenoid concentration based on absorbance at 450 and 525 nm [14]. At harvest, biomass from plants was used to estimate yield by weighing fresh roots and foliage separately. Harvest index (HI) was computed following the formula by Esuma et al. [2], for the calculation of harvest index: HI = FRW/(FRW + FSW), where HI is harvest index, FRW is fresh root weight, and FSW is fresh shoot weight. To measure dry matter content, roots were peeled, cleaned, and chopped. Three hundred grams of homogeneously mixed roots were collected into paper bags and oven dried at 60–80 °C for a minimum of 48 h. Samples were weighed after drying and the percentage DMC calculated as follows: DMC = (DSW/FSW) × 100 (where DSW is dry sample weight and FSW is fresh sample weight).

2.5. Metabolite Analysis

At eight months after planting, samples were collected for metabolite analysis. Thirty grams of root cubes were snap frozen and lyophilized prior to analysis. Lyophilized root samples were analyzed for starch content using a Total Starch Assay kit by Megazyme (Bray, Ireland). The standard protocol, which excludes D-glucose rinsing and measurement of the resistant starch of samples, was followed. Using a glucose standard, absorbance was measured at 510 nm and starch values were calculated using the weight and the absorbance value. Carotenoid content and composition were measured for the same samples by ultra-high performance liquid chromatography coupled with diode array detection and time-of-flight mass spectrometry (UHPLC–DAD–ToF–MS) at the Leibniz Institute of Vegetable and Ornamental Crops, Plant Quality and Food Security, Germany. Extraction and analysis of carotenoids and chlorophyll were conducted according to Frede et al. [16] using an Agilent Technologies 1290 Infinity II UHPLC (Agilent Technologies Sales and Services GmbH and Co. KG, Waldbronn, Germany) coupled to Agilent Technologies 6230 TOF LC/MS.

2.6. DNA and RNA Extraction and Amplification

Total nucleic acids (TNA) was extracted from cassava roots using a modified CTAB extraction protocol [16], followed by a clean-up step using a DNA clean and concentrator kit (ZYMO Research, Irvine, CA USA). Total RNA was extracted in biological and technical triplicates from TNA using RNA clean and concentrator with DNase I (ZYMO research, Irvine, CA USA) treatment to eliminate genomic DNA. The quantity of RNA was assessed using a NANODROP 8000 spectrophotometer (Thermo Scientific, Waltham, MA USA) and quality was further assessed by agarose gel electrophoresis. RNA was reverse-transcribed into cDNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA United States) according to manufacturer's instructions. The cDNA products were then used in quantitative real-time polymerase chain reaction (qPCR) experiment.

Using the Applied Biosystems Veriti Thermal Cycler, cDNA and gDNA samples were amplified with Phusion proofreading DNA polymerase (Thermo Scientific) and primers complementary to the

selected carotenoid genes, *CHYβ*, *LCYε*, and *NCED1* under the following thermal cycling conditions: 94 °C for 30 s (1 cycle), 94 °C for 10 s, 60 °C for 30 s, 72 °C for 1.45 min (35 cycles), 72 °C for 10 min. The qPCR products were run on 2% agarose gel with 1× Tris base, Acetic acid and EDTA (TAE) buffer (Sigma-Aldrich, St. Louis, MO, USA), at 100 V for 2 h to confirm PCR products. To confirm product sizes match those of the selected genes, a 100 bp DNA ladder (New England Biolabs) was used.

2.7. Primer Design and RT–qPCR Conditions

The expression levels of carotenoid genes, phytoene synthase 1 and 2 (*PSY1* and 2), β-carotenoid hydroxylase (*CHYβ*), lycopene-ε-cyclase (*LCYε*), and 9-cis-epoxycarotenoid dioxygenase (*NCED1*) were investigated in roots of selected genotypes by qPCR. Using a standard curve, four reference genes: Actin, protein phosphatase 2 (PP2A), ubiquitin (UBQ), and G/T binding protein (GTBP), were tested across all samples. Since Actin gave a higher and relatively stable expression than the other reference genes, it was selected as the reference gene for the qPCR experiment. The primers for RT–PCR were designed using Integrated DNA Technologies (IDT) primer design tool (PrimerQuest® Tool, Integrated DNA Technologies, Coralville, Iowa, USA), using the following parameters: annealing temperature of 60–65 °C, GC content of 40–60%, and amplicon size of 50–150 bp (Table S1). The qPCR was performed in three biological and three technical replicates in a Roche LightCycler 480 II Instrument (Roche Diagnostics, Indianapolis, IN, USA) using the Luna Universal qPCR Master Mix according to the manufacturer's instructions (New England Biolabs). Primer specificity was confirmed by the presence of a single melting peak in the dissociation curves. Each PCR reaction mix was prepared according to manufacturer's instructions. PCR cycling was performed as follows: 5 min at 94 °C followed by 40 rounds of 15 s at 94 °C, 10 s at 60 °C, 15 s at 72 °C, and finally 1 round of 35 s at 60 °C. Melting curve cycling consisted of: 15 s at 95 °C, 1 min at 60 °C, 30 s at 95° C, and 15 s at 60 °C. All primers used in the RT–qPCR reaction had an efficiency greater than 90%. The comparative ΔCt method was used to determine the standard curve Actin as reference gene for relative quantification. The relative expression data were calculated according to the Livak (2-ΔΔCt) method [17]. Primer sequences are included in Table S1.

2.8. Statistical Analysis

To distinguish groups of common genotypes, ANOVA tests were performed on β-carotene measurements from the UHPLC–DAD–ToF–MS analysis of the root tissues, percent starch measurements, and dry matter content from both 2018 and 2019 seasons using R. Tukey groupings were then generated using the agricolae library [18].

To further understand the results presented by UHPLC–DAD–ToF–MS analyses, principal component analysis was done to show the patterns of variation of metabolites. Principal component analyses (PCAs) were constructed for leaf and root data. For each test, data were first normalized in the following way: 1) for each sample, the total sum of all metabolites was found, 2) the average of all the samples' sums was calculated, 3) a scaling ratio for each sample was determined by dividing the average of all sums by each sample's total sum, and 4) all of the metabolites in each sample were multiplied by that sample's scaling ratio. This normalization method scaled measurements up if the total amount of metabolites found in the sample was less than average, and vice versa. These datasets were then input into the prcomp function in R. Visualizations were created using the ggbiplot library [19].

Spearman correlations were found for pairs of elements between datasets. Elements are defined as the following: various agronomic factors from the agronomic data, amount of metabolites from the metabolite data, and relative expression from the qPCR data. This determined if any elements correlated or anticorrelated across all genotypes. For each comparison, calculations were performed using only shared genotypes. Comparisons between agronomic factors and amount of metabolites were visualized in a correlation network using Cytoscape [20]. Only comparisons with a Spearman index greater than 0.5 and less than −0.5 were included in the network visualization.

3. Results

3.1. Starch, Dry Matter Content and Agronomic Data

In the cassava landraces analyzed, starch ranged from 50% to 80% dry weight. The genotype, white cassava Kaleso, had the highest starch content and IBA083565, yellow cassava, has the lowest starch content. The starch assay also showed that several genotypes with yellow storage roots have a high percentage of starch (Figure 2A,B). The agronomic field data can be found in Table S1.

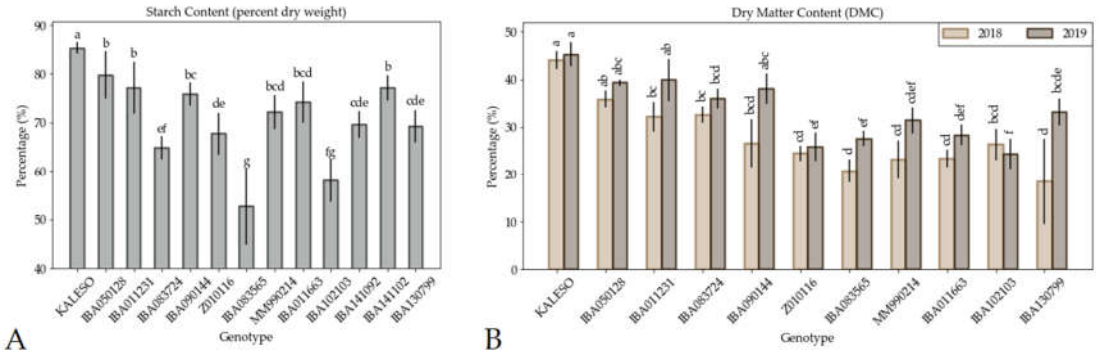

Figure 2. (**A**) Starch in dried 8-month-old root tissue from 13 cassava genotypes using starch enzymatic assay. (**B**) Dry matter content (DMC, %). Error bars represent the standard deviation of the mean from three biological replicates with three technical repetitions per genotype. Groupings generated from ANOVA and Tukey ad hoc tests are above each bar.

Dry matter content (DMC) was measured in grams dry weight and calculated as percentages. We observed that the white variety Kaleso had the highest DMC for both seasons, while IBA130799 had the lowest DMC in 2018 and IBA102103 had the lowest DMC in 2019. Comparing the two seasons, we observed a lower DMC in 2018 for all genotypes except IBA102103. Based on the meteorological data collected, the 2018 season was much drier compared to the 2019 season, which is likely to explain the variance in DMC between seasons. The dry matter content could not be measured for two of the cultivars, IBA141092 and IBA141102, in 2018 and are thus not included in Figure 2B. Our results show that starch and dry matter content are positively correlated (Figure 2).

3.2. Carotenoid Measurement by UHPLC–DAD–ToF–MS and iCheck Carotene

Based on root β-carotenoid content, genotypes were classified into low, intermediate/average, and high carotenoid content lines (Table 1). Analysis of leaf and storage root samples by UHPLC-DAD-ToF-MS identified chlorophyll A and B and nine carotenoid types in leaf tissues (Tables 2 and 3), and three carotenoid types in roots. Most carotenoids measured were below detection level in roots; conversely, in many cases, high levels were identified in leaves. Carotenoids detected in storage roots include all trans-β-carotene, 9-cis-β-carotene, and 15-cis-β-carotene. Total β-carotenoid content varied, with genotype IBA141092 having the highest content of all-trans β-carotene and its isomers in roots (Figure 3). It is important to note that β-carotenoids were also identified in cassava genotypes with white storage roots. Variation in root carotenoid component ranged from 0.3 to 36.1 μg g^{-1} dry weight for total β-carotenes and 0.2 to 22.7 μg g^{-1} dry weight for all-trans β-carotene, the highest component across all genotypes. While only three carotenoids were identified in root samples, nine carotenoids were identified in leaf samples, including β-carotenoids, and at much higher levels than in storage roots (Table 2).

Comparison between starch and β-carotenoids showed some cultivars that are high in β-carotenoids were also high in starch content (Figure 4). Pearson and Spearman correlations between the two traits showed a week negative correlation of −0.3 and −0.2, respectively.

iCheck Carotene by BioAnalyt, a portable spectrophotometer, was used to measure total carotenoids based on absorbance. This method identified the genotypes IBA141092, IBA141102, and IBA130799 as high carotenoid cassava genotypes. Of these three cultivars, IBA141092 had the highest

carotenoid content, while Kaleso, a cultivar with a white storage root, had a carotenoid content below detection level.

Both iCheck and the LC–MS analyses identified IBA141092 and Kaleso as having the highest and lowest carotene content, respectively. However, LC–MS analysis gave higher total carotene content compared to the iCheck analysis. It is important to note that the samples analyzed by LC–MS were younger than samples analyzed by iCHECK at harvest, with plants being 8 months and 13 months, respectively. HPLC methods have the advantage to quantify individual carotenoids and are reported to give higher carotenoid values than spectrophotometric methods [9,21].

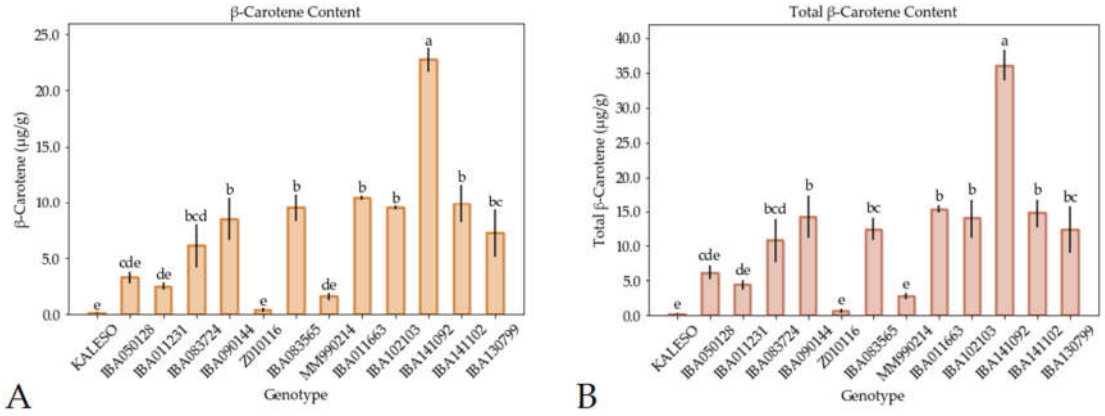

Figure 3. (A) All-trans β-carotene concentration in µg g^{-1} dry weight of cassava. (B) Total β-carotene in µg g^{-1} dry weight of cassava. Error bars represent the standard deviation from the mean. Groupings generated from ANOVA and Tukey adhoc tests are above each bar.

Table 1. Genotypes classification as low, intermediate, and high carotenoid content in storage roots based on the LC–MS analysis. Three plants per genotype were analyzed. W: white, PY: pale yellow, Y: yellow.

Genotype	Flesh Color	total ß-Carotene	Category
KALESO	W	0.3	low
IBA050128	PY	6.2	intermediate
IBA011231	PY	4.4	intermediate
IBA083724	Y	11.2	high
IBA090144	W	14.2	high
Z010116	W	0.6	low
IBA083565	Y	12.4	high
MM990214	W	2.7	low
IBA011663	Y	14.9	high
IBA102103	W	15	high
IBA141092	Y	36.1	high
IBA141102	Y	14.7	high
IBA130799	Y	12.4	high

Table 2. Carotenoid composition of leaf carotenoids (μg g^{-1} leaf dry weight). Three plants per genotype were analyzed. Variation between samples is given as SD.

Genotype	Lutein	β-Carotene	9-cis-β-Carotene	15-cis-β-Carotene	Zeaxanthin	Neoxanthin1	Neoxanthin2	Violaxanthin1	Violaxanthin2
KALESO	393 ± 135	229 ± 88	35 ± 16	5 ± 2	328 ± 27	70 ± 25	22 ± 7	55 ± 24	46 ± 17
IBA050128	529 ± 41	326 ± 256	48 ± 3	9 ± 0.8	481 ± 90	93 ± 12	27 ± 5	139 ± 41	82 ± 20
IBA011231	526 ± 71	299 ± 75	42 ± 10	7 ± 2	1849 ± 306	106 ± 16	17 ± 4	65 ± 29	34 ± 11
IBA083724	528 ± 38	289 ± 32	40 ± 5	7 ± 1	641 ± 82	87 ± 9	21 ± 4	75 ± 22	56 ± 7
IBA090144	570 ± 120	268 ± 62	33 ± 7	7 ± 2	702 ± 37	88 ± 19	22 ± 6	62 ± 33	33 ± 8
Z010116	582 ± 96	305 ± 50	45 ± 9	8 ± 2	523 ± 167	113 ± 22	26 ± 7	102 ± 41	61 ± 13
IBA083565	721 ± 29	395 ± 38	54 ± 9	10 ± 0.9	791 ± 131	116 ± 5	29 ± 5	123 ± 36	67 ± 22
MM990214	781 ± 188	402 ± 84	51 ± 7	12 ± 3	915 ± 462	137 ± 31	20 ± 4	71 ± 4	52 ± 7
IBA011663	575 ± 88	329 ± 61	44 ± 10	9 ± 1	993 ± 282	97 ± 14	19 ± 6	55 ± 13	40 ± 8
IBA102103	841 ± 36	500 ± 31	68 ± 2	15 ± 0.9	1478 ± 211	166 ± 10	27 ± 6	140 ± 36	94 ± 12
IBA141092	812 ± 74	405 ± 33	49 ± 2	11 ± 1	734 ± 34	125 ± 14	20 ± 2	86 ± 12	61 ± 10
IBA141102	722 ± 48	366 ± 31	45 ± 7	10 ± 1	1201 ± 135	118 ± 1	27 ± 5	87 ± 28	43 ± 7
IBA130799	716 ± 26	385 ± 87	48 ± 14	11 ± 4	1165 ± 315	119 ± 22	23 ± 1	93 ± 5	50 ± 15

Table 3. Chlorophyll A and B composition in leaf samples (μg g^{-1}). Three plants per genotype were analyzed.

Genotype	Chlorophyll A	Chlorophyll B	Chl A: Chl B
KALESO	8006 ± 2688	913 ± 314	8.77
IBA050128	9775 ± 397	1111 ± 106	8.8
IBA011231	9025 ± 1185	1173 ± 169	7.69
IBA083724	8453 ± 999	1096 ± 109	7.71
IBA090144	7856 ± 1689	1060 ± 265	7.41
Z010116	10,229 ± 1415	1321 ± 221	7.75
IBA083565	10,564 ± 802	1397 ± 97	7.56
MM990214	11,653 ± 2037	1609 ± 403	7.24
IBA011663	9087 ± 1861	1220 ± 267	7.45
IBA102103	13,231 ± 947	1841 ± 75	7.19
IBA141092	10,215 ± 812	1380 ± 84	7.4
IBA141102	10,281 ± 811	1525 ± 124	6.74
IBA130799	10,238 ± 2126	1406 ± 368	7.28

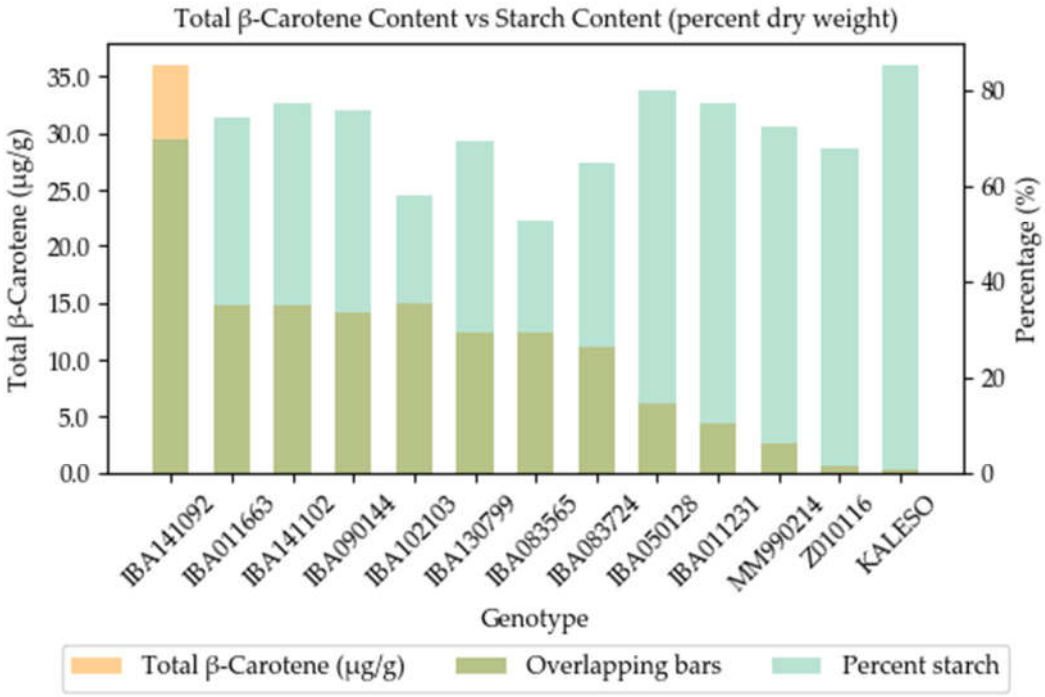

Figure 4. Comparison of total β-carotene and starch content for cassava genotypes.

3.3. Network Analysis and Correlations of Agronomic and Metabolite Data

PCA showed the opposite relation between zeaxanthin and violaxanthin, as can be expected in the xantophyll cycle (Figure S1). Similarly, there was an opposite relation between zeaxanthin and chlorophyll A. There was no clear clustering depending on the root color, based on the carotenoid and chlorophyll content in either leaves or roots.

For a deeper analysis of the data, we designed a correlation network based on Spearman correlations based on all agronomic and metabolite data using Cytoscape [20] (Figure 5). This analysis showed strong positive correlation between root yield and total carotenoid content irrespective of whether it was determined by LC–MS or colorimetric (iCheck) methods, and a positive correlation was observed between root yield and β-carotene composition. Weak positive correlations were

observed between storage root mass (SRM), storage root count (SRC), harvest index (HI), and carotenoid content. A strong negative correlation was observed between DMC and carotenoid content in both planting seasons. As could be expected, a strong positive correlation was observed between dry matter content and percent starch in planting season 1 (S2); however, there was a much weaker positive correlation observed with planting season 1 (S1).

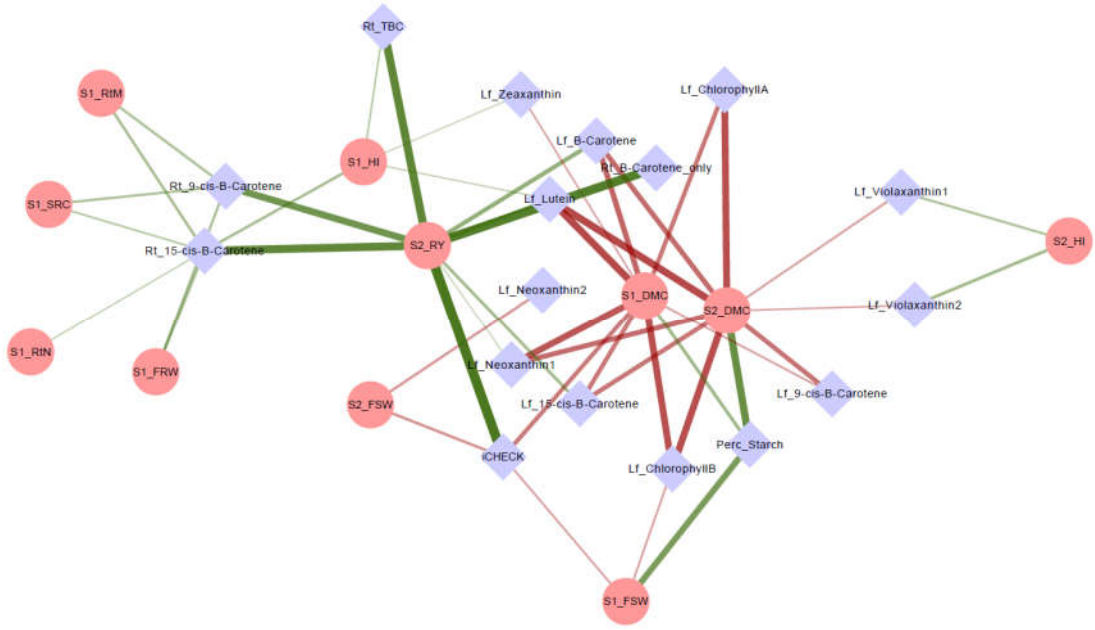

Figure 5. Correlations between agronomic traits and carotenoids. Red circular nodes are agronomic traits, while purple diamond nodes are metabolites. Green lines represent positive correlations, while red lines represent negative correlations. The thickness of the lines reflects the strength of the correlation. Spearman correlation cutoff are values greater than 0.5 or less than −0.5 (Rt = roots, Lf = leaf, S1 = season 1, S2 = season 2).

3.4. Gene Expression

Gene expression levels were determined in the storage roots of six cultivars: Kaleso, IBA011231, IBA083724, IBA011663, IBA141092, and TMS60444 (Figure 6 and Table S3). These genotypes have low to high carotenoid levels based on the LC–MS data. Because this study was partly aimed at selecting suitable genotypes for biotechnological biofortification, we included the cultivar, TMS60444, which is not a Nigerian landrace. While this genotype is susceptible to cassava mosaic virus infection and therefore not ideal for cultivation, it is easy to handle in tissue culture and relatively easy to transform, and can thus be used for proof-of-concept for various genetic modifications.

The following five genes encoding enzymes in the carotenoid biosynthesis and cleavage pathways were analyzed by qPCR: phytoene synthases 1 and 2 (*PSY1* and *PSY2*), which catalyzes the condensation of two molecules of geranylgeranyl pyrophosphate to form phytoene; β-carotenoid hydroxylase (*CHYβ*), which catalyzes the hydroxylation of β-carotene to β-cryptoxanthin and zeaxanthin; lycopene-epsilon cyclase (*LCYε*), which catalyzes the cyclization of lycopene to *α*-carotene; and lastly, 9-cis-epoxycarotenoid dioxygenase (*NCED1*), which catalyzes the formation of abscisic acid.

The genotype IBA011663 (D), had the highest expression of *PSY2*, *CHYβ* and *LCYε*, while IBA141092 (E) showed the highest expression of *PSY1* and relatively high expression of *PSY2* compared to other cultivars. Kaleso (A), a white root cultivar, had the lowest expression for all five genes and genotype IBA011231 (B) had the highest expression level of *NCED1*. The differences in carotenoid gene expression levels and carotenoid composition between the genotypes show the complexity in the regulation and accumulation of carotenoids.

We compared gene expression, carotenoid composition, and starch in five selected genotypes and observed a negative correlation between *PSY2*, *LCYε*, and *NCED1*, and starch (Figure 6).

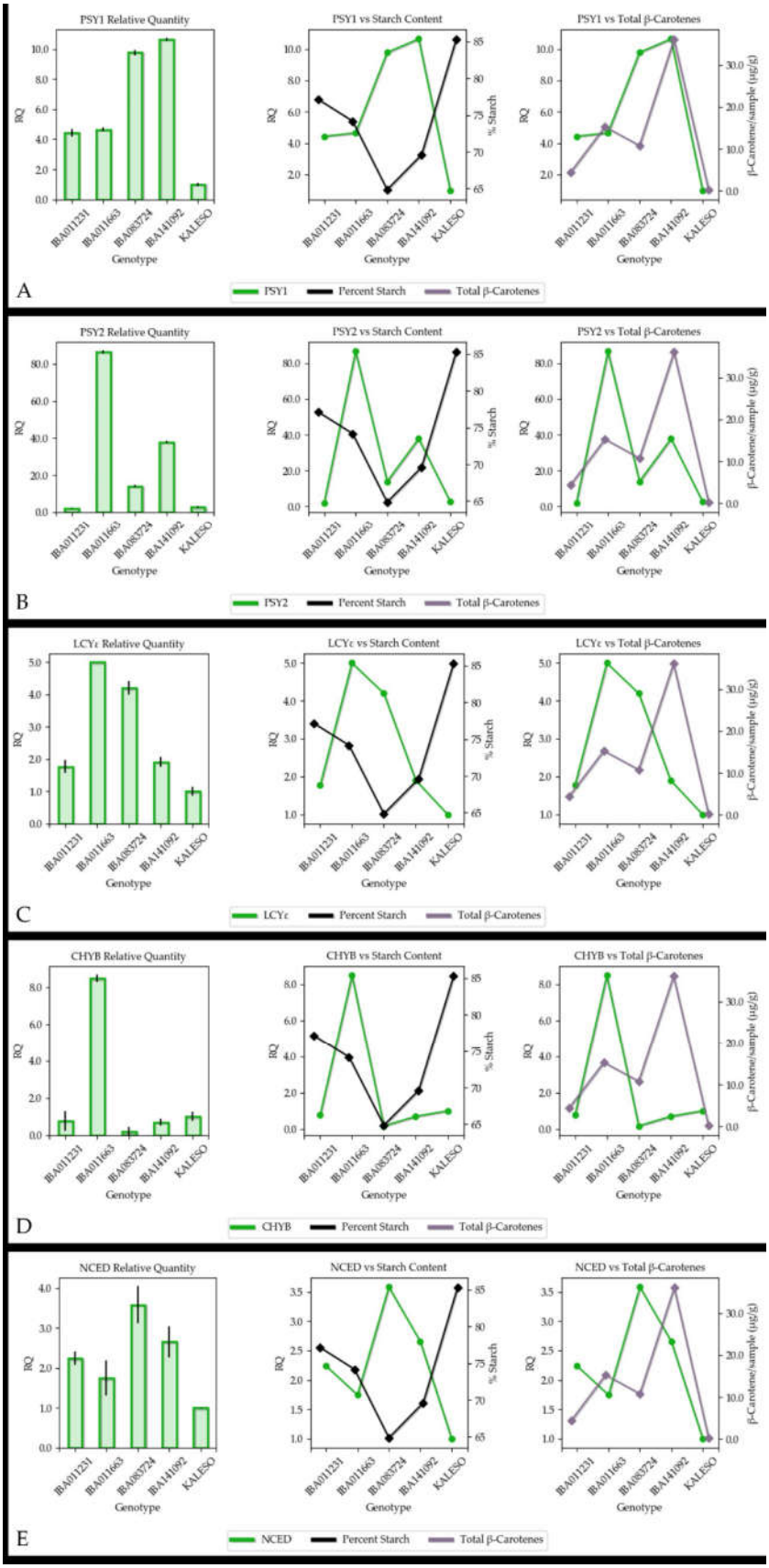

Figure 6. Relative gene expression (RQ) and comparison between selected metabolites across five cassava genotypes.

4. Discussion

4.1. Agronomic Traits and Metabolite Profiling

In this study, we identify some cultivars with high β-carotene content and high starch and dry matter content (Figures 2 and 3). This proves that some yellow root cultivars can be an adequate source of both starch and provitamin A carotenoids, and partly contradicts the generally reported negative correlation between DMC and total carotenoid content in cassava storage roots [13]. According to Ceballos et al. [9], dry matter content increased along with carotenoid content following years of recurrent selection, suggesting that cassava germplasms are able to combine high total carotenoid (TC) content with adequate DMC as observed in a genotype with 25.8 mg g^{-1} TC and an average dry matter content of 37.1%.

We observed a positive correlation between root yield and carotenoid content, coupled to a low level of root rot in cultivars with high carotenoid content (Figure 5). This may be as a result of the antioxidant properties of carotenoids, which are thought to delay the onset postharvest physiological deterioration (PPD) in cassava. Sánchez et al. [22] previously reported an inverse association between total carotenoid content in cassava roots and PPD, suggesting that the onset of PPD is reduced or delayed in cassava roots with high carotenoid content. We believe the higher root yield observed at time of harvest in the cassava cultivars with high carotenoid content can also be attributed to the antioxidant properties of carotenoids.

Only three of the eleven identified carotenoids were detected in storage roots: all-trans β-carotene and its cis isomers, 9-cis-, and 15-cis-β-carotene. A similar finding was published in a study by Ceballos et al. [9] in which most carotenoids detected by HPLC–DAD were in the form of all-trans β-carotene, accounting for 61.2% of total β-carotene (TBC). Other isomers detected in the study were 9-cis-, 13-cis-, and 15-cis-β-carotene, which accounted for 82.2% of total carotenoid. Contrary to our findings, the study of Ceballos et al., which also included a pink variety, identified xanthophylls such as violaxanthin, antheraxanthin, lutein, lycopene, and β-cryptoxanthin in several lines of yellow cassava root.

Chlorophylls are leaf photosynthetic pigments and harvesters of light in plants. In the present study, the chlorophyll A/B ratio ranged from 6.7 to 8. Even if this ratio is species-specific, this value is high compared to a previous report from two cassava cultivars [23]. The proportions of chlorophyll A and B varies according to leaf age and physiological stress [24]. For instance, a decrease in chlorophyll A/B ratio in rice seedlings has been linked to leaf senescence.

4.2. Association between Carotenoid Content and Gene Expression

Carotenoid levels and composition are regulated in many different ways [25]. In our study, *PSY2*, *LCYε*, and *CHYβ* expression were generally positively associated to β-carotene content (Figure 6). This is in line with the findings of Carvalho et al., which showed a correlation between *LCYε* expression and total β-carotene content, as well as correlation between PDS and total xanthophyll content [15]. A high expression of PSY is thought to drive the flux through the carotenoid synthesis pathway which results in the formation of lycopene [4]. LCYβ and *LCYε* both compete for lycopene [6]. Regulation of carotenoid accumulation is seen at the molecular level in the expression of the *LCYε* and *CHYβ* which serve to drive carotenoid synthesis further down the pathway. This leads to the formation of either lutein or zeaxanthin. Subsequent epoxidation and de-epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) forms the xanthophyll cycle. This may be the explanation for the high expression of *CHYβ* in the cultivar IBA011663. It could therefore be inferred for IBA011663 that both α- and β-branches of the carotenoid synthesis pathway were favored equally, and that higher expression of one gene produces a bias for its associated branch.

We see a positive correlation between *CHYβ* expression and total carotenoid content in cassava roots. Even if the number of lines tested for expression in this study is limited, this is in contrast to many transgenic studies. It has been shown a number of times, also in other species, that high *CHYβ* expression is linked to low β-carotene content. For example, RNAi-silenced *CHYβ* expression in white

sweet potato increased the overall carotenoid contentt and also increased salt stress tolerance. In fact, the suppression of *CHYβ* expression in white sweet potato led to a doubling of β-carotene in storage roots[25]. Silenced *CHYβ*1 and *CHYβ*2 in potato also achieved a 38-fold increase in β-carotene [26]. However, during testing of South American landraces, a white cultivar with low β-carotene content was associated with high *CHYβ* expression [15]. These findings suggests that the level of expression of *CHYβ* influences the β-carotene content. In a similar manner, *LCYε* expression levels were also shown to be negatively correlated with β-carotene content. For example, in potato, an RNAi construct downregulating *LCYε* led to an increase in β-carotene levels [26].

Various other factors could affect the synthesis and accumulation of carotenoids in cassava. Plastids are the sites for carotenoid biosynthesis in plants, and the type and size of plastids influence carotenoid accumulation and stability [4]. While chloroplasts are the site of carotenoid synthesis, chromoplasts are the carotenoid-accumulating plastids and the structure of the chromoplasts determines the amount of carotenoids accumulated. Crystalline chromoplasts are known to overaccumulate lycopene and β-carotene [5]. Chromoplasts are thought to exert a great influence on carotenoid accumulation. For instance, in cauliflower, the transcript levels of carotenoid synthesis pathway genes are similar in both the white and orange curd of the *Or* mutant and no significant differences in the expression of carotenoid pathway genes were seen in orange fleshed and nonorange fleshed melon fruits [5]. In addition to this ongoing study, further investigation is needed to understand the underlying mechanism of chromoplast biogenesis in cassava.

5. Conclusions

Biofortification of staple crops is one possible way to alleviate vitamin A deficiency in sub-Saharan Africa. Several studies observed a negative correlation between carotenoid content and dry matter content in African cassava germplasm. However, this present study identified cassava genotypes with high carotenoid content and adequate amount of dry matter and starch content. Expression of carotenoid genes indicated a positive correlation between *PSY2*, *LCYε*, and *CHYβ* expression and total carotenoid content of cassava landraces. A major observation was that gene expression and regulation of carotenoid synthesis may differ between cassava genotypes and there is a high possibility that more than one regulatory mechanism is involved in accumulation and maintenance of carotenoid accumulation. This study identified a suitable cassava genotype, IBA011663, for *in vitro* transformation. Since this cultivar has an adequate amount of dry matter and starch content, we would like to see if it is able to retain these properties if the carotenoid content is increased by genetic engineering.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/10/3/424/s1, Table S1: qPCR primer sequences, Table S2: Agronomic data for average dry matter (DM) and average total carotenoid (TC) composition in storage roots at 13 months, Table S3: Relative expression determined by qPCR of carotenoid genes in six cassava genotypes, Figure S1: PCA loadings of leaf (A) and root (B) metabolites.

Author Contributions: P.O., L.S., and E.A. planned the experiments and wrote the first draft of the manuscript; HPLC and starch analyses were done by L.S.; UHPLC–MS analysis was done by S.B.; P.O. conducted field sampling and performed qPCR; I.R. provided the agronomic field data; A.L. did the statistical analysis and correlation plots; P.O., L.S., and E.A. did the main interpretation of the results. All authors have read and agreed to the published version of the manuscript.

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