Chapter 12

Carotenoids in Cassava Roots

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Additional information is available at the end of the chapter

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Abstract

Vitamin A deficiency (VAD) is a preventable tragedy that affects millions of people, particularly in sub-Saharan Africa. A large proportion of these people rely on diets based on cassava as a source of calories. During the last two decades, significant efforts have been made to identify sources of germplasm with high pro-vitamin A carotenoids (pVAC) and then use them to develop cultivars with a nutritional goal of 15 μg g^{-1} of β-carotene (fresh weight basis) and good agronomic performance. The protocols for sampling roots and quantifying carotenoids have been improved. Recently, NIR predictions began to be used. Retention of carotenoids after different root processing methods has been measured. Bioavailability studies suggest high conversion rates. Genetic modification has also been achieved with mixed results. Carotenogenesis genes have been characterized and their activity in roots measured.

Keywords: carotenogenesis, conventional breeding, dry matter content, physiological deterioration

1. Introduction: cassava as an important food-security and industrial crop

Cassava (*Manihot esculenta* Crantz) has a Neotropical origin and significant economic relevance. It is an important crop in tropical and subtropical regions of the world, growing from sea level up to 1800 m. Its most common product is the starchy root, but cassava foliage has an excellent nutritional quality for animal and human consumption and offers great potential [1, 2]. Stems are used for commercial multiplication. Therefore, every part of the plant can

be used and exploited. Cassava is the fourth most important basic food after rice, wheat, and maize worldwide, but is the second most important food staple (in terms of calories consumed) in sub-Saharan Africa [3–6]. The crop is called Africa's food insurance because it offers reliable yields even in the face of drought, low soil fertility, low intensity management, and because of its resilience to face the effects of climate change [7–9].

Between 2010 and 2014, an average of 22 million ha was annually grown with cassava worldwide (70% in Africa, 18% in Asia, and about 12% in the Americas). The area planted to cassava has grown steadily, and it was 2011 when, for the first time, more than 20 million ha were planted with the crop (FAOSTAT). Markets in cassava are diverse. The crop was initially domesticated for the direct consumption of the roots, which contain plenty of carbohydrates. However, roots contain little protein and few micronutrients [10], when compared to sweet potatoes, potatoes, beans, maize, or wheat. Globally, in the period 1970–2003, the main uses of cassava roots were for food (54%), followed by feed (30%), and other uses including starch production (4%) [11]. Global use of cassava for feed was affected by the reduction of imports from the European Union in the 1980s. Production of starch, on the other hand, increased considerably in the same period (by 17.5% annually [11]). Today, cassava is the second most important source of starch worldwide [12]. In the 2000s, a considerable amount of cassava roots started to be used for the production of fuel ethanol as well [13, 14].

A comprehensive screening of root quality traits from more than 4000 cassava clones has been published [15]. On average, roots from these genotypes had 33.6% of dry matter content (DMC) of which 84.5% is starch (the main and most valuable product in cassava roots). Cassava starch quality is excellent and has, on average, 20.7% amylose (the remaining 79.3% is amylopectin). Cassava roots spoil quickly (2–3 days after harvest) because of a process called postharvest physiological deterioration (PPD). Therefore, roots need to be processed or consumed soon after harvest.

Vitamin A is an essential micronutrient for the normal functioning of the visual and immune systems, growth and development, maintenance of epithelial cellular integrity, and for reproduction [16]. Vitamin A deficiency (VAD) is the leading cause of preventable blindness in children and increases the risk of disease and death from severe infections. In pregnant women, VAD causes night blindness and may increase the risk of maternal mortality. VAD is a public health problem in more than half of all countries, especially in Africa and South-East Asia, hitting hardest young children (visual impairment and blindness, and significantly increases the risk of severe illness, and even death) and pregnant women (especially during the last trimester when demand by both the unborn child and the mother is highest) in low-income countries. An estimated 250 million preschool children show VAD, and it is likely that, in areas where VAD is prevalent, a substantial proportion of pregnant women is also affected. An estimated 250,000 to 500,000 vitamin A-deficient children become blind every year, half of them dying within 12 months of losing their sight [17].

Vitamin A exists in natural products in many different forms: as preformed retinoids (stored in animal tissues) and as provitamin A carotenoids (pVAC), which are synthesized as pigments by many plants and are found in different plant tissues [16]. The carotenoids are present in

both plant and animal food products (in animal products their occurrence results from dietary exposure). Retinoids, on the other hand, are only found in animal products. A comprehensive review on carotenoids has been recently published [18].

Different strategies (dietary diversification, food fortification, and/or supplementation) and considerable efforts have been made to reduce VAD worldwide [19]. These strategies are relatively cost-effective, but have failed to completely eradicate the problem for a diversity of reasons [20]. Moreover, prevalence of VAD has remained unchanged in sub-Saharan Africa and south Asia during the 1991–2013 periods [21]. Recently, different programs such as HarvestPlus (www.harvestplus.org), involving a global alliance of research institutions, initiated the implementation of a fourth strategy (biofortification) to develop micronutrient-dense staple crops [19, 22, 23]. A diet rich in pVAC, in addition to reducing the problems related to VAD would also result in other health benefits, including a reduction of cancer incidence [24, 25].

The color of root parenchyma (e.g., pulp) in cassava varies from white to yellow. Pinkish pigmentation has also been reported [26]. Pigmentation of the parenchyma is closely linked to carotenoids content. Most cassava varieties worldwide produce roots with white parenchyma. This is particularly appreciated by the starch industry. However, breeding to develop cassava varieties need to target different end users and it is only recently that the efforts to develop high-carotenoids biofortified germplasm were initiated by HarvestPlus. This is an international, interdisciplinary research initiative that seeks to reduce human malnutrition by increasing micronutrients in staple crops, including cassava. The progress already attained by HarvesPlus and the awareness that has elicited in the plant breeding community resulted in the 2016 World Food Prize Award. Biofortified cassava makes sense considering the importance of this crop in sub-Saharan Africa and the reported prevalence of VAD in that region of the world. The target for these varieties will be not only for human consumption but also for animal feed, particularly poultry [27].

2. Nutritional value of cassava roots

The nutritional quality of cassava roots in general is low, and contains mainly carbohydrates. Per 100 gram raw weight, white cassava provides 160 kcal mainly as carbohydrates (38 g) and contains further water (60 g), a little protein (1.4 g), fat (0.3 g) and trace elements of iron (0.3 mg), niacin 0.9 mg), thiamin (0.09 mg), riboflavin (0.05 mg), calcium (16 mg), potassium (271 mg), zinc (0.3 mg), and vitamin C (21 g) [28]. People depending on a diet predominantly based on white cassava roots are at greater risk of having iron, zinc and VAD, as was shown in children in Kenya and Nigeria [29].

Cassava also contains variety-dependent concentrations of cyanogenic glucosides (CG), which are toxic for humans and needs to be eliminated during processing before consumption. Therefore, cassava with high concentration in cyanide are better suitable for more processed products like porridge made out of flour and those low in cyanide are more suitable for boiled consumption [30]. CG levels range from low (≤100 ppm) in roots from sweet/cool

varieties to very high (≥3000 ppm) in bitter cassava cultivars. CG are eliminated through alternative processing techniques [2]. However, health of people may be affected [31], particularly in years when drought has affected other crops, which increases people's dependency in cassava. In addition to higher consumption of cassava roots, drought generally increases CG levels in them [32].

3. Genetic variation of carotenoids content in cassava roots

Carotenoids perform essential physiological functions in plants. They are involved in the photosynthesis process, protecting the plant from photo-oxidative damage, and as precursors of regulatory molecules, such as abscisic acid (ABA) and strigolactones [33, 34]. In cassava, carotenoids can be found in leaves and roots. Concentration is much larger in leaves than in roots [35–37]. The earliest reports on pVAC contents in cassava roots (written in Portuguese) were based on cassava samples from the Amazon region of Brazil and the earliest dates back to 1964 [38–40]. Interest in increasing pVAC content in cassava roots began in the 1990s in India [41, 42] and few years later at International Center for Tropical Agriculture (CIAT) in Colombia [43] and at IIITA in Nigeria [37]. HarvestPlus provided financial resources and encouraged systematic work that resulted in the screening of a large sample of cassava germplasm [26, 36].

Quality of data improved over the years. Initially most of the information focused on assessing differences in the intensity of parenchyma pigmentation [44] and often relied on measuring total carotenoids content (TCC) by spectrophotometer. HPLC analysis restricted the number of samples that could be screened. Moorthy reported in 1990 a range of variation for β-carotene from negligible to 7.9 μg g^{-1} [41]. Unless otherwise specified, all concentration values for carotenoids will be expressed on fresh weight basis. A comprehensive screening of carotenoids content reported in 2005 (1789 genotypes) had an average total carotenoids content (TCC) of 2.46 μg g^{-1}, ranging from 1.02 to 10.40 μg g^{-1} [26]. **Figure 1** illustrates the strong skewness in frequency distributions for TTC by 2005 with a long tail to the right (low frequency of genotypes with high TCC values).

The basic concept in breeding is to cross outstanding genotypes (e.g., clones) to generate a new generation of segregating progenies that will hopefully have a better average performance. At CIAT those individuals, at the right of the plot in **Figure 1**, were crossed among themselves to obtain botanical seed. Each seed represented a new, genetically unique, genotype. The seed was germinated and the roots from the plants generated were evaluated for their carotenoids content. The best materials (highest pVAC) were then selected and crossed to produce a new cycle of selection. The basic scheme was described by Ceballos and coworkers in 2013 [45]. Very early in this process, it became clear that carotenoids content was not only closely associated with pigmentation of the root parenchyma [41, 43] but also with a reduction or delay of PPD [26, 46, 47]. The beneficial effect of increased TCC in lengthening the shelf life of cassava roots was an important finding that would encourage the adoption

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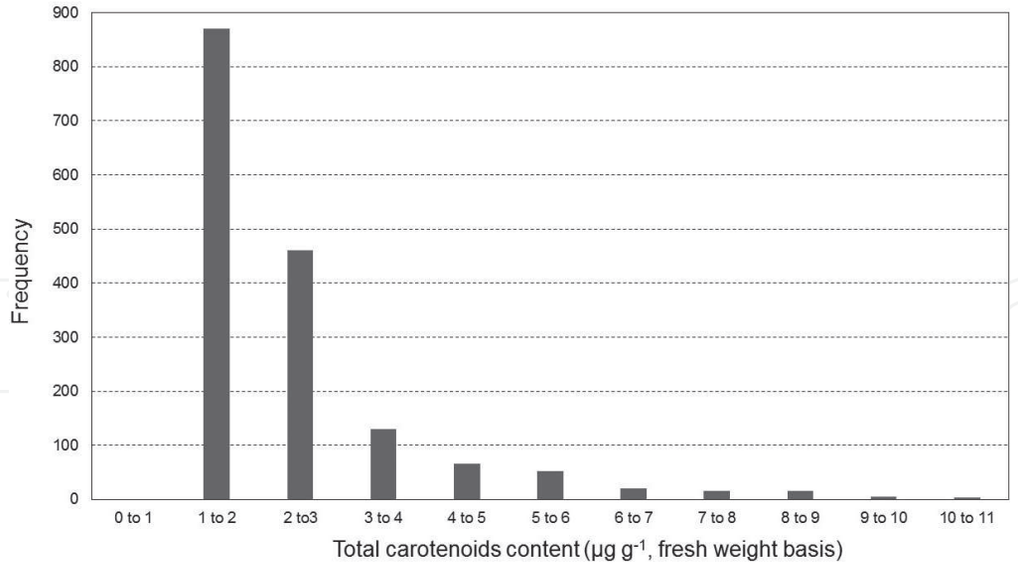

Figure 1. Frequency distribution for total carotenoids content (μg g^{-1}, fresh weight basis) of cassava germplasm by 2005.

of biofortified varieties. It has been suggested that reduced PPD in high pVAC roots may involve β-ionone-like molecules, derived from β-carotene catabolism, which play a role in the response to biotic stress such as fungal infection [48]. However, PPD is a very variable trait, difficult to measure visually, influenced by the environment, and which depends very much on the storage conditions of the harvested root.

The relationships depicted in **Figure 2** are relevant for the impact of the biofortified varieties released through the HarvestPlus initiative. It is not enough to develop cassava cultivars with high pVAC. The roots from these cultivars should meet consumer preferences. Key parameters defining consumer acceptance are dry matter content (DMC) and cyanogenic potential (particularly in regions of the world where roots are boiled). DMC influences texture after boiling and is also a key parameter in the production of gari, for example (a popular way to consume cassava in Africa). The relationship between carotenoids and DMC is basically nonexistent (**Figure 2**). It is possible, therefore, to identify genotypes with high pVAC and acceptable levels of DMC. The first target of the HarvestPlus initiative was to develop biofortified clones for Africa. The key related trait for the most important ways to consume cassava in Africa would be DMC. Cyanogenic potential is not critical for gari production, but it is critical for table consumption after boiling. This is the most common way to consume cassava in many Latin American and Caribbean (LAC) countries. Only recently CIAT started the development of biofortified cassava with low cyanogenic potential targeting LAC. The relationship between carotenoids and cyanogenic potential in **Figure 2** shows a negative trend, although the coefficient of determination is low ($r^2 = 0.15$). For a variety to be considered "sweet" and apt for table consumption, the maximum HCN levels would be about 150 ppm. At this stage of the breeding process, only a few dozen genotypes have been found to have low HCN, high pVAC and acceptable cooking quality. Efforts are currently made in making crosses among these genotypes to increase the number of segregating materials that can fit the consumer preferences in LAC.

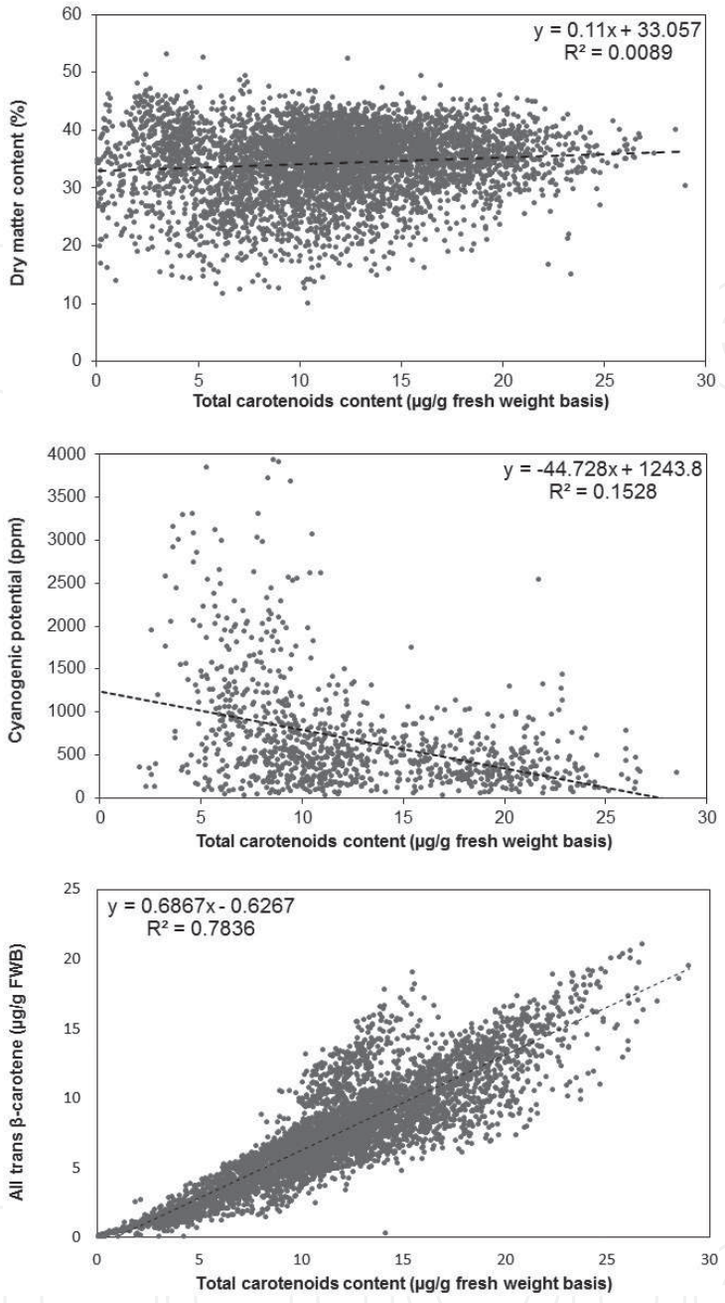

Figure 2. Relationship between total carotenoids content (µg/g, fresh weight basis) with dry matter content (TOP); cyanogenic potential (MIDDLE) and all-trans β-carotene (bottom) contents.

About 600 carotenoids have been isolated and characterized in nature, and approximately 10% of these can be metabolized into vitamin A by mammals. The most important carotenoids with vitaminic activity are β- and α-carotenes and cryptoxanthins. Some carotenoids that cannot be converted into vitamin A (e.g., lutein, zeaxanthin, and lycopene) can be found in the parenchyma of cassava root as well. Not all pVAC carotenoids have the same activity. β-carotene has about twice as much vitamin activity as the remaining pVAC carotenoids [49]. Fortunately, as illustrated in **Figure 2**, most of carotenoids in cassava roots are β-carotene. Assessing the nutritional potential of cassava roots, therefore, requires partitioning TCC

into its different individual carotenoid components. This is usually done through HPLC chromatograms.

Table 1 presents information on key root quality traits such as DMC and HCN and a description of the different carotenoids quantified in roots from a large sample of cassava genotypes. The information presented in **Table 1** is clearly unbalanced with large variation in the number of samples used for quantifying different parameters. For example, DMC is available for 4913 samples, whereas HCN was only measured in 981genotypes. Data for all-trans β-carotene is available from 4952 chromatograms, whereas only 49 samples allowed measuring α-carotene. To a large extent, the limited information on α-carotene is due to the low concentration, often below detection level, observed for this carotenoid. It is clear from data presented in **Table 1** that most carotenoids present in cassava roots are β-carotene (TBC), with a prevalence of its all-trans isomer. Similar conclusions were reported by Maziya-Dixon and Dixon in 2015 [50].

	Count	Average	(Standard deviation)	Range		Skew
Dry matter content (%)	4913	34	(±6)	10	−53	−1
HCN total (ppm)	981	684	(±631)	22	−3927	2
Precursors in α- and β-carotene synthesis μg g^{-1}, fresh weight basis						
Phytoene	3992	4.12	(±3.10)	0.00	−22.3	1.17
Phytofluene	2635	2.12	(±1.87)	0.00	−12.2	1.36
Carotenoids concentrations measured in μg g^{-1}, fresh weight basis						
Total carotenoids (spectroph.)	4922	11.59	(±5.16)	0.07	−29.5	0.14
Total carotenoids (HPLC)	4952	11.51	(±5.08)	0.11	−29.0	0.12
β-carotene and derived carotenoids μg g^{-1}, fresh weight basis						
All-trans β-carotene	4952	7.28	(±3.94)	0.00	−21.0	0.31
15-cis β-carotene	4107	0.15	(±0.15)	0.00	−2.99	2.18
13-cis β-carotene	4920	1.01	(±0.60)	0.00	−2.24	0.74
9-cis β-carotene	4895	0.99	(±0.54)	0.00	−3.95	0.82
β -Criptoxanthin	4074	0.14	(±0.13)	0.00	−3.77	1.20
Anteroxanthins	4038	0.30	(±0.26)	0.00	−1.46	2.03
Violaxanthins	4469	0.38	(±0.25)	0.00	−3.74	1.12
α-carotene and derived carotenoids μg g^{-1}, fresh weight basis						
α-carotene	49	0.08	(±0.01)	0.04	−1.09	−0.22
Lutein	4853	0.38	(±0.41)	0.00	−0.10	2.17

Table 1. Root quality traits and carotenoid components quantified in a large sample of cassava roots.

4. Inheritance of carotenoids content in cassava roots

Early attempts to explain the inheritance of carotenoids content in cassava roots were actually based on the visual assessment of intensity of pigmentation in root parenchyma, which was not always linked to the quantification of carotenoids content [44, 51]. Iglesias and co-workers [43] suggested in 1997 that relatively few major genes were involved, based on a hypothesis of two genes with epistatic effects controlling root color. One of these genes would show complete dominance while the second would have partial dominance. These early studies typically describe three classes for the intensity of root pigmentation (white, cream and yellow parenchyma) and agree that the trait shows dominance and is controlled by few genes, thus suggesting high heritability. Akinwale and co-workers [52] also suggested that segregations in TCC can be explained by the genetic control of two genes showing complete dominance.

During the past few years, several studies have been published on the heritability of carotenoids content in cassava roots, but based on their systematic quantification, rather than indirectly assessing the intensity of pigmentation. Parent-offspring regression analysis confirmed high heritability (>0.60) for the trait [53–55]. High heritability for carotenoids content in cassava is further confirmed in stability studies assessing the relative importance of genotype, environment, and genotype-by-environment interaction [56–58] and estimations of general and specific combining ability effects [59]. An analysis of the segregation for TCC in large full-sib and self-pollinated families was conducted aiming at identifying patterns that could be explained by Mendelian genetics [53, 54]. **Table 2** reproduces the segregating values in 14 self-pollinated (S_1) families. It is clear that there is an association between the TCC values of the progenitors and the average of their respective S_1 progenies (further validating the high heritability of the trait). The range of variation in the resulting S_1 progenies is wide, and in most cases, there are individuals with TCC values above those of the respective progenitors (except for one family that only had two individuals). Based on the results of these segregations, it was postulated again that there are at least two genes explaining the high, intermediate, and low TCC values [53, 54].

However, one of these genes (*I*-) would drastically reduce the accumulation of carotenoids and shows complete dominance (one copy of the gene is enough to reduce TCC levels). Breeders may be interested in the homozygous recessive genotype for this gene (*ii*). The other gene (*C*-), would contribute to carotenoids accumulation and shows partial dominance.

The large variation for TCC values in the S_1 family AM702 is worth highlighting (**Table 2**). This family had 29 individual genotypes and the range of variation for TCC was from 0.63 to 19.1 μg/g. The performance in family AM697 is also interesting because none of its 38 members had TCC values above 1.0 μg/g. The progenitors of these two families are related to each other since they were siblings from the same full-sib family (GM708). It has been proposed that the progenitor of AM697 was homozygous for the allele reducing TCC accumulation (*II*) and/or was lacking the allele that promoted the accumulation of carotenoids (*cc*) [53, 54].

Family	TCC in progenitor	n	Minimum	Maximum	Average	Standard deviation	Skewness
S_1 family from genotypes derived from full-sib family CM9816							
AM690	9.99 (H)	90	2.89	13.10	6.51	1.82	−0.599
AM689	6.67 (I)	71	0.46	11.37	4.73	2.69	−0.045
AM692	5.04 (I)	48	4.85	8.95	6.38	0.77	0.026
AM691	1.87 (L)	73	0.40	5.89	2.34	1.18	0.892
S_1 family from genotypes derived from full-sib family GM708							
AM702	11.16 (H)	29	0.63	19.1	6.46	5.92	0.734
AM700	7.32 (I)	2	2.01	2.79	2.40	0.55	n.d.
AM698	0.54 (L)	34	0.30	6.56	1.10	1.25	3.298
AM697	0.27(L)	38	0.26	0.89	0.54	0.15	0.34
S_1 family from genotypes derived from full-sib family GM893							
AM710	8.77 (H)	29	4.85	8.95	6.38	0.77	−0.299
AM718	8.14 (H)	38	3.19	9.24	5.90	1.28	−0.025
AM712	6.06 (I)	57	0.44	8.41	3.31	2.06	0.478
AM720	2.04 (L)	40	0.14	9.72	2.28	2.22	0.432
S_1 families derived from commercial clones MTAI 8 and CM 4919-1							
AM320	2.14 (L)	177	0.37	8.10	2.22	1.37	1.084
AM324	3.83 (I)	11	0.75	8.58	3.83	2.35	0.313

Table 2. Descriptive statistics in roots from S_1 families derived from progenitors selected because of their high (H), intermediate (I) or low (L) levels of TCC (μg/g fresh weight basis).

It may be convenient to relate the three intensities of pigmentation used in earlier studies to the TCC values of the progenitors in **Table 2**. A root with white parenchyma has TCC values up to 1.5–2.0 μg/g, those with a cream pulp may show TCC values ranging from 1.5 to 3.0 μg/g, whereas a TCC value above 3.0–3.5 μg/g is observed only in yellow roots. It is useful to visualize two levels of variation in carotenoids content in cassava roots. The first level of variation is **qualitative** and relates to the three phenotypes regarding intensity of pigmentation (e.g. white, cream, or yellow roots with the TCC values mentioned above). It is expected that this qualitative variation relates to some of the genes in the carotenoids biosynthesis described below. There is, however, a second level of variation that is **quantitative** in nature. It involves genotypes only with yellow roots and covers a very wide range of variation (from 3.0 to 30.0 μg/g). Understanding the genetic factors controlling the large quantitative variation for TCC among yellow-rooted cassava is critical for breeding cassava with higher levels of pVAC. The distinction between white, cream and yellow roots, on the other hand, is easy to make visually and has already been linked to some of the genes in the carotenogesis pathway.

The pathway in carotenoids biosynthesis has been known for more than three decades. However, it was only after the 1990s that the genes involved in it could be cloned [60]. The diagram in **Figure 3** illustrates the key steps in the process and is helpful for understanding the inheritance of carotenoids synthesis and their accumulation, as crystals, in root chromoplasts. Few conclusions can be drawn from **Figure 3**: (a) The carotenogenesis pathway is relatively simple with few key steps (and therefore few genes); (b) breeding should downregulate the activity of lycopene ε-cyclase (LCYE) to favor the synthesis of β-carotene on the right side of **Figure 3**; and (c) breeding should try to reduce catabolic conversion of β-carotene into zeaxanthin, which eventually leads to the production of abscisic acid (ABA).

Phytoene synthase (PSY) is the enzyme responsible for the synthesis of phytoene, which is the first reaction specifically related to the carotenoid synthesis pathway as illustrated in **Figure 3** and demonstrated in cassava [34], also see later in this chapter. There are three copies of PSY in cassava but transcripts for one of them (PSY3) were negligible [61]. It has been proposed that PSY1 is mostly involved in responses to stress (through ABA), whereas PSY2 would be involved in carotenoids synthesis and accumulation [61]. Genetic transformation works to enhance carotenoids content in cassava roots through the simultaneous over-expression of PSY and CRTI (a bacterial version of phytoene desaturase—PDS) have been successful. Transformed cassava produced yellow roots with considerably higher TCC values compared

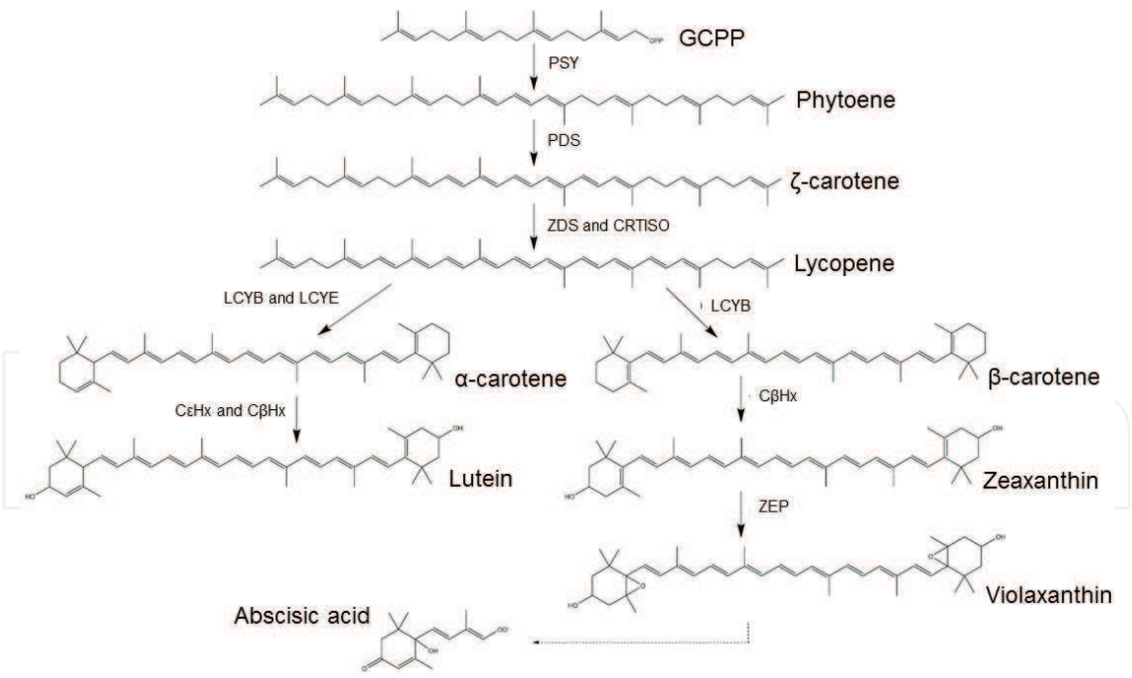

Figure 3. Diagram of carotenoids biosynthesis in plants (adapted from Stange and co-workers [33]). PSY: phytoene synthase; PDS: phytoene desaturase; ZDS: z-carotene desaturase; CRTISO: carotene isomerase; LCYE*:* Lycopene ε-cyclase; LCYB: Lycopene β cyclase; CεHx: ε-carotene hydroxylase; CβHx: β-carotene hydroxylase; ZEP: zeaxanthin epoxidase.

with the untransformed version of the same genotype that produces roots with white parenchyma [34, 62, 63]. It is clear, therefore, that PSY explains the variation between white and yellow roots (e.g., TCC values below 2.0 μg/g versus those > 3.0 μg/g). It is reasonable to hypothesize that the partially dominant factor (*C*-) in Morillo-Coronado and co-workers [54] is related to PSY.

There is no need to deregulate the activity of LCYE in cassava because the pathway naturally favors the accumulation of β-carotene as demonstrated by the values presented in **Table 1**. In fact, only trace values of α-carotene and lutein are found in cassava roots. Downregulation of β-carotene hydroxylases (*CβHx*), the third conclusion above, on the other hand, may be desirable. Reduced activity for this enzyme has been shown to enhance β-carotene concentrations in potato and sweet potato [64, 65]. No information has been published regarding variation for *CβHx* in cassava. However, the putative recessive trait in phenotypic studies (*ii*) may be related to a reduced activity of this enzyme [54].

Molecular markers ranging from microsatellite (SSR) to single nucleotide polymorphisms (SNP), identifying regions in the genome responsible for carotenoids content have been reported as well [34, 66–70]. It would be expected that some of the QTLs identified in these studies are related to the PSY gene. In fact, that was the case reported by Esuma and co-workers in 2016 [67]. Other markers may be linked to other genes in the carotenogenesis pathway (perhaps the putative *I* factor mentioned in Morillo-Coronado et al. article [54]. which may be related to *CβHx*). These genes are likely linked to the qualitative variation mentioned above (e.g., distinguishing white, cream, and yellow roots). From the nutritional point of view, however, much more relevant is the quantitative variation observed among genotypes producing yellow roots (ranging from 3.0 to 30.0 μg/g). Interesting studies in carrot compared roots from three cultivars: one producing white roots with negligible amounts of carotenoids crystals (CC) and two cultivars producing yellow roots with vast differences in CC [71]. In this study, the authors concluded that the difference in CC among the two cultivars producing yellow roots was not due to increased numbers of carotene-containing chromoplasts but rather greater accumulation of carotene per chromoplast. In other words, the carotenogenesis pathways were similar in both cultivars producing yellow roots, but the chromoplasts of the cultivar bred for higher levels of CC show a higher demand or capacity to store them. Perhaps future molecular work in cassava should focus in identifying QTLs related to the *sink strength* of chromoplast in their demand for carotenoids and/or capacity to store them as crystals. These studies should only focus on cassava genotypes producing yellow roots ranging, for example, from 10 to 30 μg/g of TCC.

The relationship between carotenoids and ABA (**Figure 3**) is relevant. ABA has been shown to have important effects as a plant growth regulator [61, 72]. As illustrated in **Figure 2**, there is no relationship between TCC and DMC in the germplasm screened at CIAT. However, many studies in Africa report a clearly negative correlation between the two traits [61, 72]. Perhaps African breeding populations have restricted genetic variability (particularly for *CβHx*) and some of the β-carotene initially produced is further converted into ABA. This hypothesis

would be supported by a genetic transformation work in which, parallel to an increase in TCC, there is a reduction in DMC and increase in ABA. The performance of six transformed genotypes with high pVAC has been reported [63]. TCC in the wild type was 0.38 μg/g, whereas the best transgenic line showed 5.73 μg/g. Similarly, TBC values increased from 0.12 μg/g in the wild type to 4.67 μg/g in the best transgenic line. One distinctive feature of the transgenic genotypes is a drastic reduction of DMC. The wild-type roots had 32.6% DMC, whereas the average DMC of the six transgenic lines was 20.2% (ranging from 15.8 to 23.8%). There seems to be a generalized fact that increases of pVAC through genetic transformation result in a (pleiotropic) decrease in DMC. A reasonable explanation for the simultaneous increase in pVAC and reduction of DMC in the transgenic lines would be that the pathway did not stop at the step accumulating β-carotene but continued resulting in a higher production of ABA.

5. Evolution and improvements of sampling protocols for measuring carotenoids in cassava roots

Only a small tissue section (5 g) is needed for extracting and quantifying carotenoids in cassava roots. At the inception of the HarvestPlus initiative, there was no information regarding the uniformity of pVAC concentration within the root, among roots of the same plant, among roots from different plants of the same genotype, and on the relative importance of environment, age, and genotype-by-environment interaction. For early work, roots were cut longitudinally in four sections: two diagonally opposed sections were used for DMC determination and the remaining two quarters were chopped into small pieces which were thoroughly mixed, and from this bulked tissue, a random sample was used for pVAC quantification.

The first systematic study analyzing sampling variation of pVAC concentration in a cassava clone that produces yellow roots (average TCC 3.90 μg/g) was published in 2008 [73]. Samples along the longitudinal (proximal, central, and distal section) and across the transversal (periphery, mid-parenchyma, and core) axes of the roots were analyzed. Carotenoids and dry matter content were quantified individually in 243 root samples. Average TCC values, on a fresh weight basis, were higher in the proximal sections (4.10 μg/g) and gradually lower in the central (3.86 μg/g) and distal portions (3.73 μg/g). An opposite trend was observed when carotenoids were quantified in dry weight basis. Carotenoids concentrations were higher in the core (4.13 μg/g) and lower toward the mid-parenchyma (4.04 μg/g) and the periphery of the root (3.52 μg/g), both fresh and dry weight basis. Plant-to-plant variation was only significant for dry matter content.

Breeding progress was successful increasing pVAC levels. Along this process, however, laboratory personnel began to observe an increase in the variability of intensity of pigmentation within the root and among roots from the same genotype, even when coming from the same plant. **Figure 4** illustrates some cases with clear differences in intensity of pigmentation particularly across the root. It should be pointed that these differences are not always present and the bottom left photograph shows a case with a more uniform pigmentation. A new and more complete study on sampling variation was published in 2011 [74]. In this new study of many genotypes, most of them with considerably higher levels of pVAC compared with the earlier

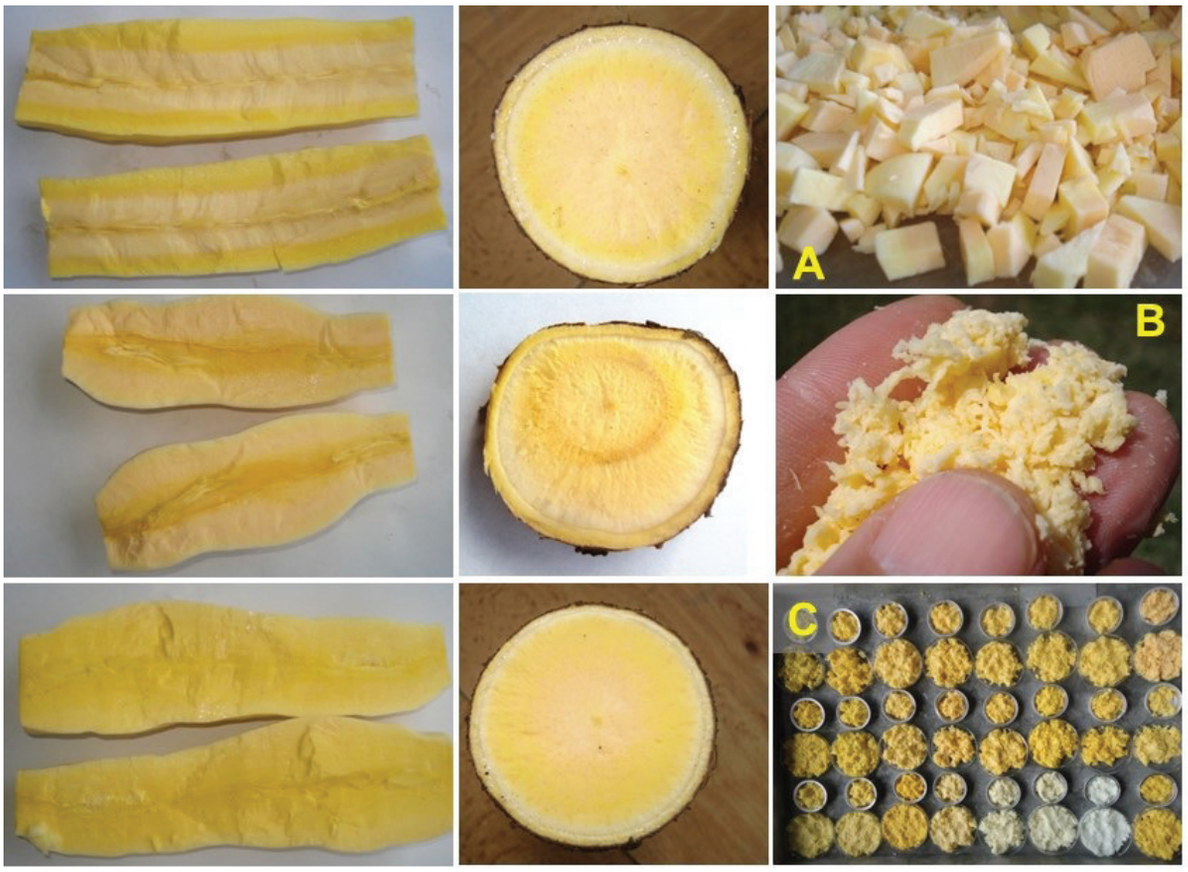

Figure 4. Illustration of longitudinal (left) and transversal (center) variation in the intensity of parenchyma pigmentation. On right: (**A**): chopped roots; (**B**): homogenized paste after processing roots with a food processor; (**C**): illustration of variation in root color in a full-sib family.

work [73] were analyzed. Variation in aliquot quantifications from the same root was negligible indicating a reliable experimental procedure. A large source of variation for carotenoids was due to differences among the 26 genotypes analyzed (ranging from 2.87 to 12.95 μg/g). In contrast to earlier results, root-to-root variation from the same plant was surprisingly high in some cases and accounted for an average of 25% of the total variation. Plant-to-plant variation was not as high and accounted for 20% of the total variance. Carotenoid content was shown to vary depending on the age of the plant as well, particularly comparing samples harvested at 8 and 10 months after planting. Single-plant evaluations for carotenoid content in cassava, which is a requirement for rapid cycling recurrent selection (described later in this chapter) was acceptable, considering that it reduces in half the time required for evaluation and selection. However, it was suggested that two to three roots per plant are combined together in a sample to better represent each genotype at a standard plant age (10–12 months after planting).

CIAT laboratory began using an industrial-grade food processor that quickly grinds the root samples in to a uniform paste (**Figure 4B**). This approach allowed overcoming the problem of variation in pVAC concentration along and across the root (which resulted in variation in the coarsely chopped pieces, **Figure 4A**), as well as the operational problems of having to use

two to three roots per genotype. Early studies made apparent that the variation in DMC along the root influenced the concentrations of pVAC [73]. Variation of DMC across the root, on the other hand, seemed to be less relevant. The influence of variation in DMC in different sections of the root on the quantification of carotenoids was also shown in Ref. [75]. It is important to recognize, therefore, that there is not always a linear relationship between pVAC concentrations reported on a fresh and a dry weight basis. Since DMC is generally around 30–35%, the rule of the thumb dictates that the relationship between TTC and TBC reported on a dry weight basis should be around threefold higher than when reported on a fresh weight basis. However, DMC can vary considerably (from 10 to 50% as shown in **Table 1**) in experimental material. **Figure 5** has been presented to illustrate how a pVAC concentration of 15 μg/g (on a fresh weight basis) will vary when expressed on a dry weight basis, depending on the DMC values of the root. It is important for researchers reporting data on pVAC concentrations to also provide the respective levels of DMC.

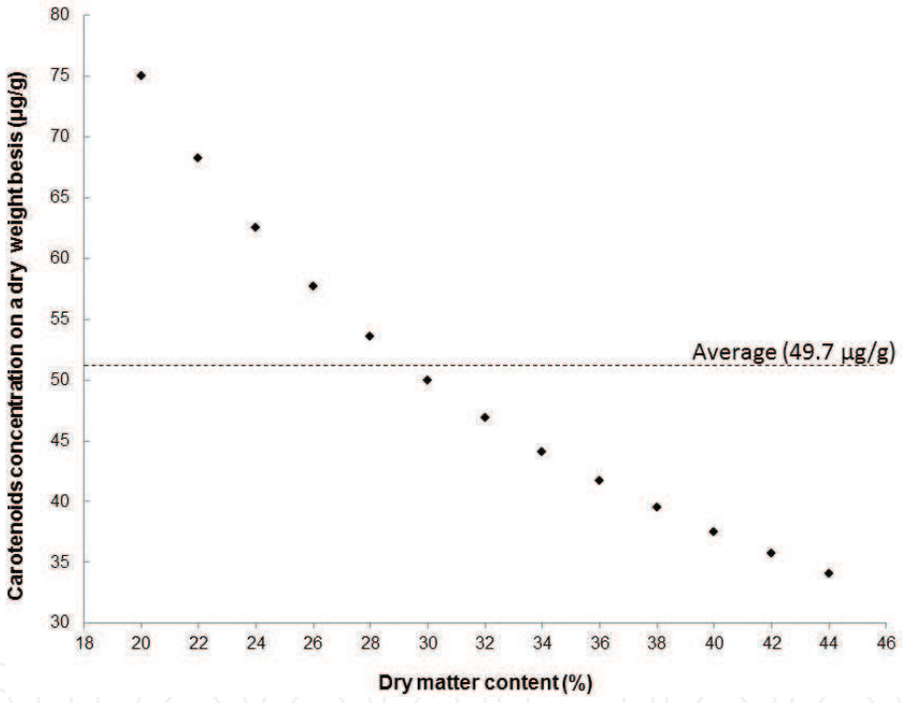

Figure 5. Illustration of how DMC in the root will affect the concentration of carotenoids expressed on a dry weight basis in a sample that showed 15 μg/g of pVAC on a fresh weigh basis. When DMC is low (22%) pVAC on a dry weight basis tends to be very high (75 μg/g), whereas roots with higher DMC (a desirable trait) show lower pVAC values, if expressed on a dry weight basis (e.g., <45 μg/g).

6. Evolution and improvements of quantifying protocols for carotenoids in cassava roots

Early work quantifying carotenoids content relied on standard spectrophotometry and HPLC quantifications. Adjustments in the extraction protocol these techniques require, however, had to be made [76, 77].

One of the adjustments was in the separation of the solid and liquid phases that was carried out by centrifugation and not by filtration [26]. Carotenoids are sensitive to ultraviolet (UV) light, pro-oxidants or associated compounds, and high temperature. Thus, steps need to be taken to avoid any adverse changes in this pigment due to such effects protecting them from UV light and avoiding excessively high temperatures. Therefore, special care to avoid direct exposure of ground tissue samples to sunlight is critical. Likewise, the lights in the laboratory need to be protected with UV filters. The typical extraction protocol requires 5 g of root tissue (either fresh or boiled) which is added to a vial with 10 mL acetone. After 10 minutes, 10 ml of petroleum ether are added and mixed using an ultra-turrax for 1 min. Samples are then centrifuged at 3000 RPM, for 10 min, at 10 °C. The organic phase is collected and extraction repeated on the residue with 5 ml of acetone and 5 ml of petroleum ether, followed by centrifugation. Extractions are optimized until it residues turn colorless. Based on preliminary analysis, it was decided that three iterative extractions would be used for fresh root samples and four for boiled samples. The organic phases are combined with 10 mL of 0.1 M NaCl solution and centrifuged (3000 RPM, for 7 min, at 10 °C). This washing process is repeated two additional times. The aqueous phase is extracted with a pipette. Petroleum ether is added to the extracts to adjust volume to 15 ml.

With the extracts obtained, TCC can be determined by visible absorption spectrophotometry, at an absorbance at 450 nm and using the absorption coefficient of β-carotene in petroleum ether (2592) [76, 77]. Partitioning of TCC into concentrations of individual carotenoids is done by HPLC. From the organic phase used for spectrophotometric quantification of TCC, aliquots (15 mL) are taken and partially dried by nitrogen evaporator. Immediately before injection, the dry extract is dissolved in 1 mL of (1:1) methanol and methy tert-butyl ether HPLC-grade and filtered through a 0.22 μm PTFE filter. Separation and quantification of carotenoids are achieved at CIAT using an YMC Carotenoid S-5 C30 reversed-phase column (4.6 mm × 150 mm: particle size, 5 μm), with a YMC carotenoid S-5 guard column (4.0 × 23 mm) in a HPLC, using DAD detector with wavelength set at 450 nm. Peaks are identified by comparing retention time and spectral characteristics against a pure standard and available literature [78].

Breeding projects to increase carotenoids content require screening hundreds of samples in a short period of time. Quantification of carotenoids by spectrophotometry or HPLC limits the number of samples that can be analyzed. Therefore, a large data set linking TCC and TBC data with Near-infrared spectroscopy (NIRS) spectra was gradually developed.

NIRS is based on the absorption of electromagnetic radiation at wavelengths in the range of 780–2500 nm. The interaction between the electromagnetic radiations and the vibrational properties of the chemical bonds results in absorption of a part of the radiation energy. The electromagnetic spectrum is divided into several regions, each of which induces specific molecular or atomic transition and is therefore suited to a specific type of spectroscopy. NIR spectroscopy belongs to the class of methods called vibrational spectroscopy techniques. This class of techniques aims to analyze a product in order to obtain qualitative and/or quantitative information. The principle of absorption can be interpreted as a resonance phenomenon: when the vibrational frequency of a specific chemical bond is equal to the frequency of the infrared radiation, a part of the energy is absorbed by the chemical bond. NIR spectra of foods comprise broad

bands arising from overlapping absorptions corresponding mainly to overtones and combinations of vibrational modes involving C-H, O-H, and N-H chemical bonds [79]. Additional information on the theory regarding vibrational NIR spectroscopy is found in more detail in several Refs [80].

The key point is that a NIR spectrum is the resultant of all the elementary absorptions due to the chemical constituents of the product analyzed; the spectrum is as a fingerprint of the sample. This fingerprint contains qualitative and quantitative information about the physical and chemical composition of the sample. Due to this complexity, spectra are treated mathematically in order to extract the relevant information within the spectra linked to the property of interest (carotenoids or other). This step, called calibration, aims to develop mathematical models, which link the reference values to a linear combination of the values of absorbance. The calibration step is based on chemometric methods that applies multivariate analyses such as multiple linear regression (MLR), partial least squares (PLS), or principle components analysis (PCA) to the spectra in order to quantify the property analyzed. Thus, NIRS is a secondary, indirect method, and the calibration step requires a primary method that provides the value of the property for each samples. Once the calibration is developed and validated, it can be applied to new samples and used to directly quantify (actually predict) the property from the spectrum.

In the routine analyses, NIRS method is simple, nondestructive, and rapid, with minimum sample preparation and environmentally friendly. The NIR technique is widely used in the agriculture sector. However, a few studies have been conducted on nutritional properties of fresh tubers or roots using NIR spectroscopy. McGoverin and collaborators inventoried studies on carotenoids in various crops and one related to carotenoids in potato [81]. The efficiency of NIR spectroscopy for predicting TCC, TBC, and DMC in fresh cassava roots has been demonstrated [82]. The models were based on partial least squares (PLS) regression. PLS regression ranged within the linear methods, which assume that the relationship between the independent and dependent variables are linear in nature. However, predictions of a new harvest based on the PLS models were actually "extrapolations" because, year after year, cassava genotypes with higher carotenoids content were obtained by the breeding project. This resulted in a nonlinear response and a tendency to underestimate the highest contents. The use of LOCAL regression algorithm based on large database has circumvented this restriction [83].

The cassava database (6026 samples) has been built over 6 years (2009–2014). For each genotype, two to three commercial-size roots were taken to the lab where they were washed, peeled, and homogenized with a food processor into a homogenous paste. Further analyses were made using aliquots from this homogeneous paste. For NIR analysis, approximately 8 g of ground root tissue was placed in NIR spectroscopy capsules for analysis using a FOSS 6500 monochromator with autocup sampling module. All spectra were recorded from 400 to 2498 nm at 2 nm intervals and saved as the average of 32 scans. Each sample was duplicated. Therefore, spectra from two root subsamples were obtained per genotype. Each of these two samples was measured once. Further analyses were made on the average of the two spectra available per genotype. Spectra were corrected for light scattering using the standard normal variate and de-trend (SNVD) correction. Then, the second derivative of the Log(1/R) spectrum, calculated

on five data points and smoothed using Savitzky-Golay polynomial smoothing on five data points, was used in combination with LOCAL regressions to develop prediction models [84].

The laboratory analyses led to 4277 TCC values that ranged from 0.11 to 29.0 μg g^{-1} with an average value of 11.6 μg g^{-1} and 4288 TBC values that ranged from negligible to 20.1 μg g^{-1} with an average value of 6.9 μg g^{-1}. The standard deviation (SD) was 5.1 μg g^{-1} for TCC and 3.6 μg g^{-1} for TBC. All values were measured and expressed on a fresh weight basis. The DMC values (n = 5578) ranged between 12.3 and 52.4% with a SD of 5.9%. Between 2009 and 2014, increases in TCC and TBC were 86 and 122%, respectively. The standard error of prediction were 1.38 μg g^{-1} for TCC and 1.02 μg g^{-1} for TBC and 1.09% for DMC using LOCAL regression. The scatter plots of NIRS values versus HPLC values for TBC and TCC illustrate the high performances of the models (**Figure 6**). The multiple determination coefficients were higher than 0.9 for both constituents.

After 5 years of harvest and database building, NIR spectroscopy coupled with LOCAL regression led to accurate and robust calibrations for breeding programs aiming at increasing carotenoids content in fresh cassava roots [85]. These results offer immense prospects for many cassava-breeding projects in the world; NIRS overtakes the bottleneck of conventional carotenoids quantification methods. Classically, in a well-equipped laboratory with experienced personnel, 20–30 samples per day can be analyzed; the implementation of NIRS in the analytical chain boosts this number by five to ten. Moreover, the possibility to share data and calibrations between spectrometers make it possible to develop a network for high-throughput phenotyping of fresh cassava roots.

An alternative quantification protocol has been implemented at the International Institute of Tropical Agriculture (IITA) in Nigeria and other cassava research programs in Africa. This method is based on the iCheck™ Carotene technology [86].

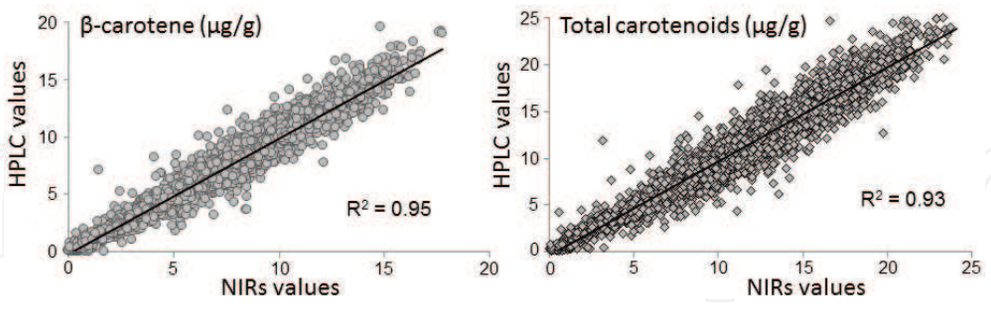

Figure 6. Scatter plots of TBC (left plot) and TCC (right plot) NIR spectroscopy values versus HPLC values.

7. Evolution and improvements of breeding methods to increase carotenoids in cassava roots

Cassava breeding relies on a method known as phenotypic recurrent selection. Although it is a simple approach, each cycle of selection requires about 8 years for completion. Elite clones

are crossed to produce full- or half-sib families. In the former, the identity of both (male and female) progenitors is known. In the latter, only the female progenitor is known [78]. Large number of botanical seed from the crosses of elite germplasm is generated each year. The seeds are then germinated to produce seedling plants. At this stage, called F1, only one plant per genotype is available. The seedling plants are grown for 11–12 months (the standard age for harvesting commercial cassava) when they are selected based on different traits such as vigor, plant architecture, resistance/tolerance to pest and diseases, and/or starch and root quality traits. Stems of selected plants are harvested and used as a source of planting material for the next stage of selection (single-row trials—SRT). Typically, eight stem cuttings are used to represent each genotype in SRT, which is the first stage where cloned plants are evaluated. The rate of vegetative multiplication (number of cuttings that can be obtained from a given plant) is relatively low in cassava (1:8 to 1:10) and, therefore, it takes several years to have enough planting material from a given genotype to be evaluated in multi-location trials.

Following SRT are the preliminary yield trials (PYT), advanced yield trials (AYT), and uniform yield trials (UYT), which are usually conducted for two consecutive years. Each of these types of trials increases the size of the plots, the number of replications, and/or the number of locations used in the evaluation process. Usually, 4000–5000 seeds are germinated, and about 4000 seedlings transplanted for the F1 stage. The number of genotypes in SRT, PYT, ADYT, and UYT is gradually reduced from about 2500, 200, 60, and 20, respectively. The entire process takes about 8 years for completion. This lengthy process is necessary because yield data is prone to large experimental errors and affected by genotype-by-environment interaction.

Breeding for increased levels of pVAC, however, is much easier because of the high heritability of the trait. Breeders do not need data from replicated trials using large plots in many locations to identify a genotype with higher levels of pVAC. In fact, a single plant is enough as results from studies on sampling variation demonstrated. Therefore, a special breeding approach—rapid cycling recurrent selection RCRS—was implemented [45]. In this scheme, seedling plants were evaluated for carotenoids content and the best genotypes preselected based on single plant evaluations. Preselection was based on a visual assessment for the intensity of pigmentation that discarded genotypes producing roots with white, cream, or pale yellow roots. Yellow roots from genotypes preselected in the field were then sent to the laboratory for carotenoids quantification. Selection was based primarily on TCC/TBC levels but other traits such as DMC and root yield potential were also taken into account. Selected genotypes were immediately incorporated into the crossing blocks to be used as progenitors.

Each recurrent selection cycle, therefore, lasted 2–3 years (depending on how quickly the selected materials flowered). Selected genotypes were also incorporated into the normal selection process described above (SRT, PYT, AYT, and UYT) to identify genotypes that not only had excellent levels of pVAC but also acceptable to outstanding agronomic performance. Number of genotypes involved was different compared with ordinary cassava breeding. The F1 seedling stage was considerably larger with 8000 to 10,000 seeds germinated and about 5000 to 8000 plants grown through the season. About 1000 to 1500 plants were selected in the field and about 500–800 of them eventually screened for pVAC levels in the laboratory. This breeding

approach was very successful and resulted in three to fourfold increases in TCC and TBC in the maximum values observed at the F1 trials in a decade [45]. This kind of genetic progress was in fact unprecedented in cassava and is largely due to the high heritability of the trait.

RCRS, however, faced some limiting bottleneck in its initial scheme. Selection for TCC/TBC of seedling plants required extraction and quantification of pVAC that was time-consuming. Only six to eight samples per day could be analyzed through HPLC. This resulted in many logistic problems that were gradually identified. The harvesting season lasted up to 4 months rather and 2–3 weeks. This was necessary to screen at least 500–800 samples in the laboratory. Extending the harvesting season for such a long period implied that some genotypes were harvested during the dry season and others after the arrival of the rains. This, in turn had some impact on DMC of the roots because this parameter is highest at the end of the dry season but is considerably reduced after the rains began. The availability of irrigation at CIAT reduced the difference in dry and wet season, but only partially. As the problem of a lengthy harvesting season became evident, CIAT began the development of the protocol for predicting pVAC and DMC based on NIRS, as described above [82, 84]. The possibility of selecting for high pVAC based on reliable NIRS predictions was a major breakthrough. It allowed increasing the number of samples analyzed (from 500–800 to 2000–2500), while reducing the harvesting season (from 4 months down to 3–4 weeks).

An improved RCRS scheme has been recently described [85]. In this scheme, seedling plants (F1 stage) are grown only for 6 months. Since plants are young, only three vegetative cuttings can be taken from selected genotypes. These cuttings are planted to grow a new stage (F1C1) that was not used previously. In the F1C1, each genotype has been cloned and is represented by three plants. Selection is conducted in two steps. The visual assessment done in the field in the old system is still done at the F1 stage, with the difference that it is done when harvested plants are only 6-months old. Only genotypes producing yellow roots are selected, but other traits such as resistance to thrips, adequate vigor, and acceptable yield potential are also taken into consideration. A key feature is that seedlings are transplanted off-season, and the harvest of the plants takes place during the normal harvesting/planting season. Therefore, planting of the F1C1 is done in the usual season. The F1C1 is then grown for a full season and plants harvested at 10–12 months of age. However, in the new system, three plants per genotype are available.

One of these plants is harvested at the end of the dry season for NIRS quantification of pVAC and DMC. Stems from selected genotypes can be used for planting a new crossing block. The remaining two plants of selected genotypes are left in the field and are used as a source of planting material for further phenotypic selection for good agronomic performance in two separate SRT planted in two locations. In the old system, the seedling plant was used for two purposes: as a source of roots for pVAC quantification and the stems were used as a source of planting material. In the new system, these functions are performed by different plants. Quantification of pVAC is done only during the dry season, thus avoiding the variation due to changes in DMC that is somewhat related to the timing of harvest. Harvesting of the stems to be used as a source of planting material takes place only when the rains have arrived in the target environment. The planting material does not need to be stored for (sometimes) a long period of time, waiting for the rains to arrive.

8. The transgenic approach

As already demonstrated in this chapter, conventional breeding has been very effective in increasing carotenoids content in the storage roots of cassava. However, breeding cassava is time-consuming and cumbersome due to its heterozygous nature. Adoption of new, improved varieties where cassava plays a key food security role is often low. Farmers tend to be reluctant to shift away from the varieties they have grown for decades [6, 87]. The alternative of turning farmers' preferred varieties into vehicles for delivering pVAC through genetic transformation is very appealing. The technology could deliver exactly the same variety that farmers have grown for years but with increased nutritional value. This is a product that conventional breeding could not offer. Therefore, biotechnology tools have also been included among the strategies that have been considered to deliver biofortified cassava to farmers.

The accumulation of carotenoids in the root involves several genes that, as described above, may have anabolic or catabolic function. This further complicates the conventional breeding strategy, since putting all the desirable alleles of the relevant genes into a single variety are complex and takes time. This would be particularly true if the objective is to silence a gene whose function is to catabolize carotenoids, for example, into ABA (**Figure 3**). Not only enzymes that are directly involved in the making or degrading of carotenoids should be the target of breeding. It has been recently shown that the ORANGE (OR) protein is a posttranscriptional regulator of phytoene synthases (PSYs) in plants [88], which adds another level of intervention (conventional or transgenic) for enhancing carotenoids in cassava roots.

Genetic transformation is not only a viable approach to produce cassava clones with increased pVAC in the roots, but it is also a powerful tool for understanding the individual impact of different genes and alleles in carotenogenesis. Examples of the importance of polymorphisms of single nucleotides—SNPs—from relevant genes such as phytoene synthase have been published [34, 63, 89]. The different studies have demonstrated that the substrates necessary for the activity of relevant enzymes were present in roots of most genotypes. Without this precedent, it would be more difficult to design a genetic modification strategy to increase pVAC contents by inserting new gene combinations in commercial varieties, well established in the markets and accepted by consumers.

As stated above, genetic transformation would add nutritional value to farmers' preferred varieties. In addition, this approach would reduce the time required for developing new varieties since it implies handling few genes of the carotenoid synthetic pathway alone, not an entire genome. Finally, it could ensure that the transgenic variety produces a minimum pVAC as has been the case for genetically modified potato or the Golden Rice [90, 91].

There are already at least five examples of crops in which pVAC contents have been substantially increased using transgenes from the pathway of carotene synthesis: rice, maize, potato, tomato, and canola. They have been guided by promoters that express these genes in specific organs, or constitutively. Genetic transformation of rice, with genes from the carotene pathway [92, 93] has shown that it was possible to increase the TCC in the grain up to 27 times (maximum 37 μg/g, DW), of which more than 80% (>30 μg/g, DW) corresponded

to β-carotene. In canola, the β-carotene increase was 50-fold [94]. It has been shown that β-carotene in the potato tuber can be increased >3600 times, reaching 47 μg/g DW, with which 250 g of potato satisfy half the RDA [90].

Both the rice grain and the potato tuber have complemented the carotene synthesis route, which in the case of rice was not very active, with encouraging results suggesting that a similar strategy could be attempted for cassava. In the case of maize, genes of the carotenoids synthesis pathway have been introduced in different combinations producing increases β-carotene and other carotenoids, including complex mixtures of hydroxycarotenoids and ketocarotenoids [95, 96].

The synthesis and accumulation of carotenes in plant storage roots such as cassava and sweet potato are just beginning to be understood at the molecular level. It is not yet very clear how the promoters of carotene synthesis genes are regulated in roots. On the other hand, the perception of foods derived from transgenic crops is not yet totally favorable, with exceptions, although they are safe for human consumption [97]. This forces us to think of strategies to reduce opposition to acceptance, such as replacing bacterial genes, which are used today to genetically modify crops, by plant genes. Genome edition using CRISPR/Cas9 or similar molecular scissors is an alternative for modifying alleles in cassava [62]. However, there are at least three factors that must be taken into account when using genetic modification to increase carotene content in cassava roots: the genes themselves (their coding sequences and their origins), controlling sequences for transcriptional control, and interacting proteins such as PSY and OR for posttranslational control of carotenoid production in plants.

As an example, in the case of cassava, having genetically transformed a wild-type genotype that produces white roots with combinations of the bacterial versions of key carotenoid biosynthetic genes (crtB, crtY, and crtI) significantly increased pVAC levels. However, transgenesis could not exceed the maximum pVAC levels attained by conventional breeding (about 90 μg/g DW in the 2016 harvest, unpublished data). These experiments, however, demonstrated that there were bottlenecks for the synthesis of carotenoids in roots with white parenchyma, such as the absence of a PSY capable of effectively synthesizing phytoene, to keep the route operating so that enough carotenoids were produced and accumulated in the root until the harvest time (usually 11–12 MAP). This bottleneck was solved with the introduction of the CRTB (the bacterial version of PSY in plants) enzyme alone. This modification was responsible for increasing TCC from 0.4 to 22 μg/g (DW) and turning roots from white to yellow [34]. In addition, in the same work, it was confirmed that the PSY enzyme was a limiting step of the pathway, demonstrating that a single SNP in the coding region of the PSY gene could partially explain the difference between white and yellow roots. Thus, deficiency in the carotenoid synthesis pathway in the white cassava root was complemented by providing enzymes more effective than the endogenous ones, possibly not regulated by the plant [34, 62], and by showing allele diversity correlated with better efficiency among PSY endogenous enzymes.

Compared with the increases in TCC obtained in potato by transgenesis (maximum 114.4 μg/g DW; [90]), the 13–31 μg/g (DW) obtained in cassava [34, 63, 98, 99], can be considered only moderate. However, these comparisons are made on a dry weight basis that would favor potato (**Figure 5**) because of its considerable lower DMC and starch contents compared with

cassava. The same three genes (crtB, crtY, and crtI) were used in cassava and potato, under the control of the same patatin promoters (in fact it was the same construct). However, the TCC baseline of the nontransgenic control in potato was 5.8 μg/g DW, while for cassava, it was only 1.1 μg/g DW, which improved the chances of increasing TCC in the former. If we accept that the genetic modification of cassava still has a potential to raise the carotenoid content in the root, at levels similar to or higher than those reached in potato, the results with the tuber would indicate the way forward with cassava: raise TCC would be more effective by transgenesis if yellow-rooted (nonwhite) plants were modified.

9. Retention and bioavailability studies

The impact of biofortified cassava roots in the reduction of VAD depends on two factors: (a) how much of the carotenoids present in the raw root at harvest time reach the individual consuming the cassava product; and (b) how much retinol can the individual make with the consumed cassava product. This section describes information regarding these two key steps.

As stated in the introduction, cassava roots have a short shelf life due to PPD. Roots therefore need to be consumed or processed 1–3 days after harvest. A diversity of processing methods has been developed by different cultures resulting in many ethnic products. The easiest and most direct way to consume cassava roots is boiling or steaming them. However, this approach does not allow storing the roots and other processing methods that allow storage for long periods have been developed. Roots can be dried and ground into flour. Drying can be done in an oven, in open air under shaded conditions or by sun drying. Two popular processing methods in West Africa are fufu and gari [100].

A comprehensive review of true retention of carotenoids in cassava roots after alternative processing methods and different storage period has been published [101]. Different authors have highlighted that retention of carotenoids is not only affected by the processing method and length of storage period but also by the cassava genotype [102–104]. Boiling the root is a very simple and popular processing method in many regions of the world, which results in relatively high retentions. There is large variation reported depending on genotypes. Average retention after boiling ranged from 68 [105], around 70 [104], and 74% [100]. DMC in the roots influences true retention of carotenoids after boiling [75, 106]. When the effect of DMC prior to boiling is taken into consideration, retention of carotenoids after this processing method was relatively high (87%) and uniform (retention ranged from 76 to 97%) [75].

Drying the roots soon after harvest is an important method to prevent PPD. Significant differences were observed regarding the drying method employed. The highest β-carotene retention was obtained by oven drying (72%), followed by shade drying (59%), and sun drying (38%) [102]. Similar conclusions were obtained by other authors but with some variation in the average retentions that may be due to the genotype effect [43, 101, 104, 107].

Retention of carotenoids after gari preparation varies widely. Final carotenoids content in gari is a function of the intensity and duration of roasting as well as the duration of the

fermentation prior to roasting [101]. Retention reported by Failla and collaborators in 2012 was about 66, 62, and 29%, respectively, for boiling, fufu, and gari [63]. On the other hand, a different study reported average retentions of 74% after boiling, 41 and 22% in raw and cooked fufu, and about 45% in gari [100].

Carotenoids are also lost during storage of the processed product. Chávez and co-workers measured the β-carotene retention during a 4-week storage period with flour and chips that had been either oven-dried or sun-dried [102]. Oven-dried cassava initially retained 72% of β-carotene, which decreased to 40 and 32% after 2 and 4 weeks of storage at room temperature, respectively. Sun-dried cassava had a lower initial β-carotene retention (38%), which was further decreased to 24 and 18% after 2 and 4 weeks of storage, respectively. Retention values for cassava chips were very similar to those of cassava flour. Lower retention levels after storage (at room temperature and in the absence of light) have been reported [108]. Carotenoid content of gari products decreased markedly with time and temperature [109].

The absorption, bioavailability, and conversion of β-carotene, the most prominent carotenoid in yellow cassava, into retinol in the human body depend on several factors. These factors are food- and host-related and depended on, for example, host genotype, availability of fat in the diet, and the food matrix [110]. Beta-carotene in cassava is stored in parenchyma cells, which are more easily destructed in the gastrointestinal tract than, for example, chloroplasts membranes, the primary location of β-carotene in green leafy vegetables. Little is known on the histology of carotenoids stored in cassava roots because of the density of amyloplast. An animal study with gerbils showed a bioconversion factor of 3.7 μg β-carotene to 1 μg of retinol [111]. In a study with 10 healthy American adults, this conversion factor was estimated to be 4.2 μg, but the individual variation between the humans was high (range 0.3–10.6) [112]. A randomized-controlled efficacy study with Kenyan primary school children showed a significant increase of both retinol and β-carotene in the blood after 4 months of feeding with boiled yellow cassava as compared to boiled white cassava [113]. In conclusion, we can say that the β-carotene from cassava is absorbable, bioavailable and converted into retinol in the human body. More research is currently being conducted to provide evidence on the effect of processing in different recipes, as well as for different age groups countries.

10. Conclusions and perspectives

Most of the information described in this chapter relates to the work coordinated and financed by the HarvestPlus initiative. It all began about two decades ago with the simple, yet relevant, idea that the nutritional value of crops could be improved. Since then, the significant progress achieved in different crops was highlighted with the 2016 Food Prize Award to a group of researchers lead by Dr. Howard Bouis.

The significant gains in carotenoids content in cassava roots could only be obtained with the concerted effort of many researchers working in a wide range of disciplines. The first group of cassava varieties with increased levels of carotenoids has been already released in Brazil and in Africa. A second generation of new varieties with higher levels of pVAC and

better agronomic performance will soon follow. In addition to the strategic relevance of the germplasm generated, valuable information has been generated ranging from the relationship between carotenoids and DMC in the roots to retention and bioavailability information. Enhanced levels of carotenoids resulted in an unexpected reduction of PPD. The new protocol to screen for carotenoids content based on NIR was developed and prompted further changes in the breeding approach. The different constructs for genetic transformation not only resulted in varying degrees of success but also exposed unexpected responses from the plant, such as the reduction in DMC.

The release of varieties, rich in pVAC, requires strategies for the efficient production of planting material to be distributed to farmers as well as participatory approaches to promote their adoption. Deploying these varieties will provide excellent opportunities for nutritional studies and development of new food products and alternative processing methods. The concerted effort of many researchers and institutions and the valuable financial support of key donors and investors have been motivated by the magnitude of VAD. Hopefully, the scientific community will soon be able to document the impact of these efforts in the livelihood of millions of people affected by VAD.

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