Research Article

Studies on Variation of Carotenoid-Proteins Content in Cassava (*Manihot esculenta* Crantz) Storage Root Reveal Implications for Breeding and the Use of Induced Mutations

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Abstract

Carotenoid-Protein content in cassava storage root (CSR) is low but variable, and characterization of this variability is lacking. Accumulation of carotenoids occurs in chromoplast and depends on a broad class of proteins named carotenoid associated proteins (CAP), lipids and the biosynthesis of carotenoids. Twenty-nine landraces and progeny of 200 individuals were accessed for CAP and carotenoid content varied in two ways. First, related to landrace diversity, total buffer extractable proteins (TBEP), buffer insoluble proteins (BIP) and total carotenoid and β-carotene content were assessed. Significant differences were observed in the tested genotypes. Secondly, analyses related to storage root tissue age were assessed by TBEP. This showed protein content decreased and total carotenoid content increased as secondary growth proceeds. Further carotenoid-proteins complex (CPC) identified in carotenoid contrasting landraces showed different proteins profile in SDS-PAGE with proteins size of 18 and 33 kDa in low carotenoid (IAC12.829) and 18-20-30-33 kDa in a high total carotenoid landrace (Cas74.1). Progeny analysis for TBEP and total carotenoid content confirmed the interdependence of carotenoid-proteins association by correlation analysis, estimated heritability of individual traits and grouping clones for carotenoid-proteins content. Results allow us to conclude that: natural carotenoid-protein content varies due to differential genetic background and storage root tissue age; carotenoid-protein complex showed variation in protein and carotenoid types; estimated heritability of proteins and carotenoids traits showed different values. The establishment of a genetic component allows future strategies including traditional breeding and the use of induced mutations to create novel variation for the nutritional improvement of cassava tubers.

Introduction

In tropical regions of the globe cassava storage root (CSR) represents 20% of the world crop production (FAO 2010). Nutritionally, dietary intake provided by CSR is carbohydrate (85% starch), pro-Vitamin A carotenoids [1-3] and low protein content [3, 4]. Progress in conventional breeding programs to boost carotenoid-protein content in CSR depends largely on natural occurrence of variation in germplasm collection [3, 5]. Detailed characterization of carotenoid-protein content variation is missing mainly due to the lack of critical information especially on the sink capacity of CSR. Chromoplast is a relevant target sink because it is stable and requires a significant amount of functional proteins to fulfil its cellular function during carotenoid accumulation [6-10]. Natural massive accumulation of intermediary carotenoids in the membranous system of chromoplasts of storage organs depends on a broad set of proteins named carotenoid-associated proteins (CAP) and lipids [11], and enzymes for the biosynthesis of carotenoids [12]. We hypothesize that CAP and carotenoids content variation observed in the diversity of pigmented CSR landraces may lead to the formation of a relevant carotenoid-protein sink.

The present study focused on searching for carotenoid-protein content variation in pigmented CSR in landrace diversity, and estimation of genetic parameters from crosses of selected progenitors in a 200 offspring population.

Materials and methods

Plant material and tissue preparation

Twenty-nine accessions of pigmented cassava landraces from a GENEBANK collection and 200 individuals from cross of selected parentals cultivated in field plots at Embrapa Genetic Resources and Biotechnology or Embrapa Cerrados in Brasília were used in the present study. Material from storage roots was extracted in three ways and used for analysis as follows: (1) the bulk of the

root tissue from the central cylinder was freeze-dried and extracts were used for quantification of proteins, total carotenoid, β-carotene and observation of the protein profile in SDS-PAGE; (2) root tissue layers dissected as illustrated in de Souza et al. [13], were freeze-dried and extracts were used for quantification of proteins and total carotenoids; (3) fresh root tissues were immediately processed and used for preparing chromoplast enriched suspension.

Proteins and carotenoid extraction

Extracts from tissue powder (100-500 mg powder) of storage roots of 29 cassava landraces were buffer fractionated as follows: for Total Buffer Extractable Proteins (TBEP) the tissue powder was added to 2 mL of EB (Tris 80 mM pH 6.8, NaCl 0.2 M, Pefabloc SC 0.1% and DTT 25 mM) and after addition of 4 volumes of acetone the material was vortexed, sonicated, incubated at -20°C, and centrifuged (30000 rpm/4°C/20 min). Total protein was solubilized from the pellet with 2 mL of Protein Suspension Buffer – PSB (Tris 10 mM, NaCl 0,2 M, EDTA 50 mM, Chaps 5 mM, DTT 10 mM, Pefabloc 50 mM) for protein quantification. A new set of tissue powder was treated with 2 mL of EB and the material (without acetone treatment) was vortexed, left at room temperature for less than 1 hour, and subsequently centrifuged (30000 rpm/4°C/20 min). The procedure was repeated twice and the pooled buffer soluble proteins (BSP) were used for protein quantification. The remaining pellet was mixed with 2 mL PSB, vortexed, heated at 60°C, cooled at room temperature, and incubated at -20° for 1 hour, before centrifugation (30000 rpm/4°C/20 min). The Buffer Insoluble Proteins (BIP) was used for protein quantification using Bradford assay according to the manufacturer's instructions (BioRad micro assay).

Tissue powder (100-500 mg powder) from storage roots of 200 individuals from an F1 population was rehydrated with 2 mL extraction buffer (EB) (Tris 80 mM pH 6.8, NaCl 0.2M, Pefabloc SC 0.1% and DTT 25 mM). The solution was vigorously vortexed and sonicated for extraction of total protein and carotenoids using phase separation with the addition of 4 volumes acetone, incubation at -20°C for 1 hour, and centrifugation (30000 rpm/4°C/20 min). The supernatant was collected and pooled and the pellet was washed twice with pure acetone and centrifuged (30000 rpm/4°C/20 min). Acetone was evaporated under N_2 flush, and the formed yellow colour phase was solubilized in anhydrous ether for total carotenoid quantification. The remaining pellet was vortexed in 3mL PSB, sonicated and heated at 60° C for one hour. After centrifugation (30 000 rpm/4°C/20 min), total buffer extractable proteins (TBEP) were quantified as described above.

Total carotenoid extraction and quantification

About 5-20 g each of powder from CSRs of 29 landraces was transferred to a 50 mL 'Falcon' tube, hydrated with an equal volume of ddH_2O for 10 min and mixed with a scoop of Hyflo Super Cel (SIGMA brand) followed by vigorous vortexing with acetone and subsequent centrifugation (5000 rpm/RT/15 min). A pooled supernatant, after three washes with acetone, was sequentially transferred with 1/5 of the acetone volume into 50 ml petroleum ether with centrifugation (2000 rpm/RT/15 min). After 5x washes with ddH_2O, the collected solvent phase passed through anhydrous sodium sulfate before using it to generate the HPLC spectrum and to quantify specific carotenoids using a standard curve for β-carotene as reported in Kimura et al .[14]. In an alternative extraction procedure samples were separated for quantification by spectrophotometry. In brief, samples (100 to 2000 mg powder) were hydrated with ddH_2O, and after adding petroleum ether mixed with politron pulses, sonicated and centrifuged (4000 rpm/4ºC/20 min). The solvent phase was collected, filtrated through anhydrous sodium sulfate, and the volume was adjusted with petroleum ether for OD spectrum readings at 300 to 600 nm and λ_{max}.

Spectrophotometric quantification of total carotenoid

Total carotenoid was estimated using the formula µg/g = OD * 104*V / Acm%11 * DWt, where OD = Absorbance in the higher peak (obtained by light absorption spectrum in petroleum ether), Acm%11= 2592 - carotenoid extinction coefficient in petroleum ether, V = volume of extraction (mL) and DWt = dry weight of powder (g). Optical densities readings were taken in a spectrophotometer SPECTRA_Max (Model 384 PLUS).

Specific carotenoid identification and quantification by HPLC

Carotenoid standard preparation, identification, and sample quantification were carried out as described by Kimura et al. [14]. Specific carotenoid quantification in the storage root calculation considered integration of the peak area in the HPLC chromatogram. A correction factor for a specific carotenoid extinction coefficient [15] was calculated in relation to the extinction coefficient of β-carotene using the standard curve for β-carotene. For those carotenoids with an unknown extinction coefficient, the correction factor was 1. A standard curve prepared with β-carotene extracted from carrot [14] was used for quantification of the corrected areas and the results were expressed as µg/g DW.

Chromoplast enriched suspension preparation

Fresh storage roots from cv. IAC 12-829 and landraces CAS031 and CAS74.1 were sliced and treated with homogenate buffer (Tris-HCl 100 mM (pH 8.2), EDTA 8 mM, KCl 10 mM, $MgCl_2$ 2 mM, sucrose 400 mM, and PMSF 1 mM) for 2 hours in a cold room, and ground with a household blender to obtain a paste

that was filter through three layers of cheesecloth. The filtrate was centrifuged (400 rpm/4°C/20 min), and the supernatant from this was collected and re-centrifuged (20000 rpm/4°C/40 min). The resulting pellet, enriched with chromoplasts, was suspended in 100 mL of homogenate buffer (HB), centrifuged (20000 rpm/4°C/40 min) and washed once with HB. The pellet containing the Chromoplast Enriched Suspension (CES) was collected in 100 ml of suspension buffer (Tris-HCl 100 mM (pH6.8), 250 mM NaCl) and used for carotenoid-protein complex (CPC) separation.

Size exclusion chromatography of chromoplast enriched suspension

A 5 ml aliquot of CES was loaded on to a 1 m x 1.5 cm internal diameter column containing Sepharose CL 6B-200 in suspension buffer (SB). Fractions of 1 ml were collected at a flow rate of 1.5 ml/min and an aliquot of 200 µL used to OD readings at 280 nm and 461 nm using a spectrophotometer (SPECTRA-Max; Model 384 PLUS). Fractions profile revealed three peaks; two of them were pooled for further analysis.

SDS-PAGE analysis

Total buffer extractable proteins (TBEP), chromoplast enriched suspension proteins (CES) and pooled protein fractions from carotenoid-protein complex and non-carotenoid-protein complex were separated by one dimensional SDS-PAGE as previously described (de Souza et al. 2008).

Carotenoid identification in CPC by HPLC

Pooled fractions from size exclusion chromotography (SEC) peak 1 were added with equivalent acetone volume and sonicated for one hour. After centrifugation (20000 rpm/4°C/40 min), the supernatant was collected and the acetone partially evaporated and further dried by filtering through anhydrous sodium sulfate. After passing through a 22 µm Millipore syringe filter, the preparation was thoroughly dried in N_2 and suspended with Methanol:MTBE:ddH_2O (81:15:4). Carotenoid composition was identified by HPLC using a WATERS C18 column.

Data analysis

Statistical analyses were performed using R Statistics (http://www.r-project.org/), SISA (http://www.quantitativeskills.com/sisa/) and XLSTAT package (http://www.xlstat.com/en/my-xlstat.html). Parameters such as heritability (calculated as h^2=Var (A)/Var (P)), data distribution pattern (normality test), and individuals' frequencies (frequency distribution) were calculated. Principal component analysis (PCA) was done using silhouette width technique to establish cluster divergences (genetic structure of the data) and variation within clusters (random genetic variability). Numbers of K-clusters were recognized under width silhouette (sj) test that varies from 0 to 1, where values

close to 0 refer to not well established clusters and values closest to 1 to well established clusters.

Results

Carotenoid–protein content variation

Protein content variation was assessed based on buffer solubility in stepwise fractionation as aqueous buffer soluble proteins (BSP), buffer insoluble proteins (BIP) and total buffer extractable proteins (TBEP) combining both fractions in a direct single extraction step. Total buffer extracted proteins (TBEP) across landraces (Fig.1(a)) varied from 0.27 (cv. IAC 12.829) to 8.0 mg/g DW (landrace CAS006), while buffer soluble proteins (BSP) and buffer insoluble proteins (BIP) varied from 0.8 to 4.7 mg/g DW and 0.2 to 3.3 mg/g DW respectively (Fig.1(b)). This variation corresponded to 69-73% presence of proteins in BSP and 36-41% in BIP (Fig. 1B). Values for TBEP and BIP correspond to 8 and 22 times more protein in the high compared to low carotenoid landraces. Total carotenoid and β-carotene content variation ranged from 0.26 to 61.66 µg/g DW and 0.07 to 49.91 µg/g DW respectively (Table 1).
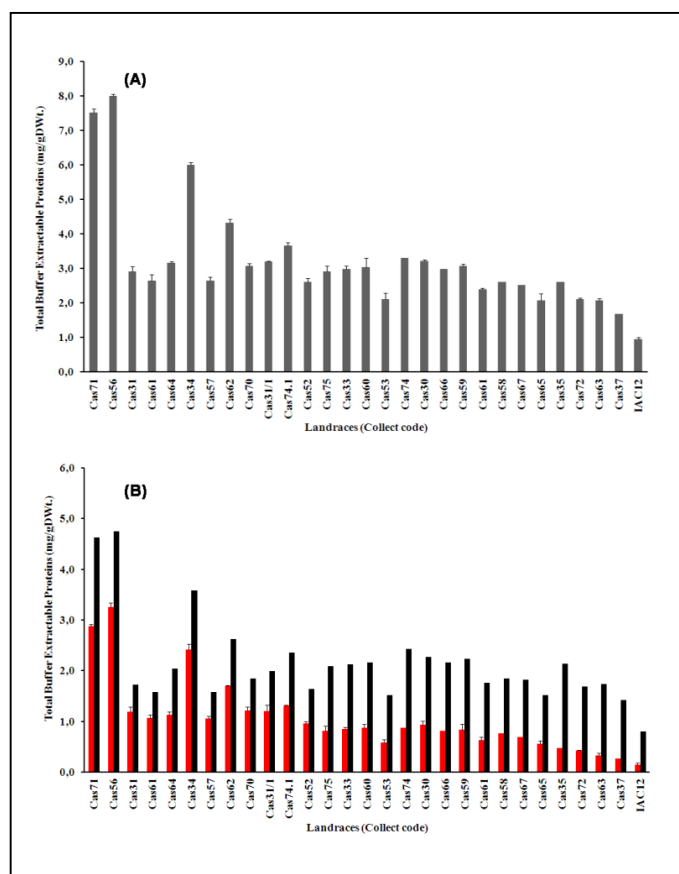

Fig. 1. Protein content variation in bulk tissue of cassava storage root for 29 landraces. (A) Total buffer extractable proteins (TBEP). (B) Buffer soluble proteins (BSP) in black bars, and insoluble proteins (BIP) in red bars. Proteins fractionation and quantification using Bradford assay procedure is described in Materials and methods.

Protein distribution in root tissue layers

The effect of tissue age on protein and total carotenoid content was performed based on dissecting tissue layers as described by de Souza et al. [13]. The distribution of TBEP and carotenoids contents followed a particular pattern with variable magnitude, depending on tissue type (layers 1 and 2), and tissue age (layers 3, 4 and 5) across landraces (Fig. 2). Overall, while values for TBEP where always higher in tissue system I (layer 1, corresponding to phelogen/pheloderm) and tissue system II (layer 2, corresponding to cambium/phloem), in the case of tissue system III (corresponding to secondary parenchyma), these values showed decreasing content from layers 3 to 5 across all landraces studied. Total buffer extractable protein content decreased as the tissue got older, layers 3-4-5, in tissue system III across all the landraces studied (Fig. 2(a)). For the case of total carotenoid content, an opposite pattern of variation was observed (Fig. 2(b)). Taken together, these results indicated a strong influence of tissue type (tissue system I and II) and age (layers in tissue system III) on the accumulation of TBEP and total carotenoids as secondary growth proceeds.

TABLE 1. VARIATION IN TOTAL CAROTENOID (SPECTROPHOTOMETRIC ASSAY) AND TOTAL β-CAROTENE (HPLC PROCEDURE) CONTENT OBSERVED IN STORAGE ROOT OF 29 CASSAVA LANDRACES. NR REFERS TO PEAK WITHOUT RESOLUTION FOR ESTIMATION THE AMOUNT OF B-CAROTENE IN THE HPLC PROFILE.

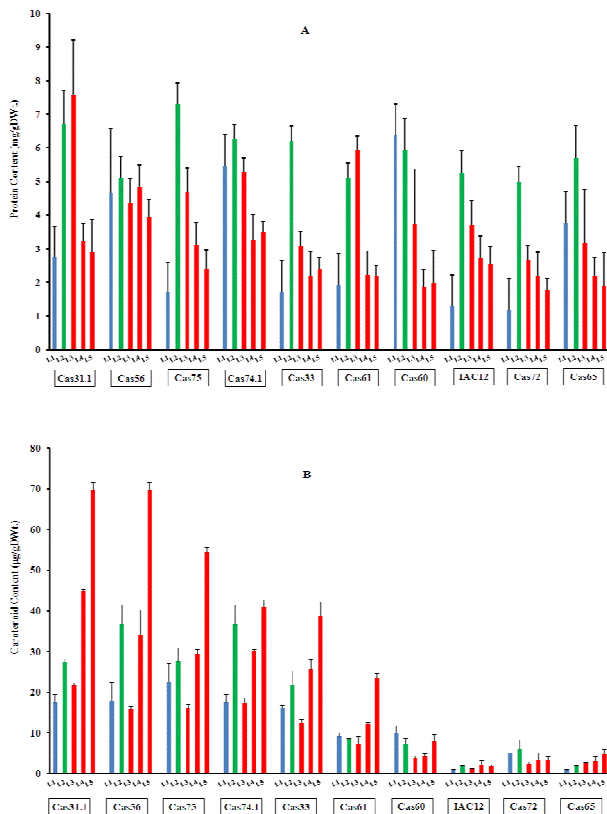| Colleting Code	Total Carotenoid Content (µg/g DW)	Total β-carotene Content (µg/g DW)	Proportion of total β-carotene over total carotenoid (%)
CAS063	0.26	NR	-
CAS037	0.26	0.07	27
CAS072	0.34	NR	-
CAS035	0.39	NR	-
CAS058	0.52	NR	-
CAS065	0.56	NR	-
IAC12.829	0.65	0.95	68
CAS059	0.88	0.44	50
CAS066	1.68	0.37	22
CAS067	1.82	NR	-
CAS061	4.40	2.93	67
CAS033	6.62	6.44	97
CAS075	10.74	4.71	44
CAS068	10.76	3.85	36
CAS074	11.71	6.30	54
CAS060	13.30	8.08	61
CAS031	13.97	5.49	39
CAS030	14.5	3.85	27
CAS064	14.84	11.43	77
CAS070	14.84	13.54	91
CAS053	17.18	11.89	69
CAS061	19.40	1.66	9
CAS052	19.45	3.10	16
CAS057	24.41	14.87	61
CAS071	24.57	20.68	84
CAS032	30.77	8.32	27
CAS034	30.77	13.96	45
CAS074.1	33.54	10.65	32
CAS062	61.66	49.91	81

Fig. 2. Total buffer extractable proteins content (A) and total carotenoid content (B) distribution in tissue layers according to advanced secondary growth model in 10 representative landraces. Concept and sampling procedures for layers [13].

Carotenoid-protein correlation

Pearson's matrix correlation analysis showed that coefficients of correlations between carotenoid and protein content were statistically significant high for data sets from landraces and F1 population (Table 2). These results provide the first reported information for cassava storage root and a guide for further characterization of particular individuals.

Carotenoid-protein association identification and characterization

One of the possibilities to explain the correlation between carotenoid and proteins was to understand the carotenoid-protein association. Here we provide results for isolation, identification and characterization of stable carotenoid-proteins association in cassava storage root by using size exclusion chromatography (SEC) of chromoplast enriched suspension. The SEC elution profile for carotenoid-proteins association was performed in three genotypes of contrasting carotenoid content - cultivar IAC 12-829 (Fig. 3(a)) with low carotenoid content, landrace-Cas31 (Fig. 3(b)) of intermediary total carotenoid content, and landrace CAS074.1 (Fig. 3(c)) of intermediary total carotenoid content – showed two major peaks. Peak 1 (common to all cultivars) was eluted within the void volume of the column as determined with blue dextran and showed carotenoids (OD readings at 461 nm) eluting together

with proteins (OD readings at 280nm). The other peaks showed no carotenoid elution together with proteins. Similar results have been reported with carrot roots [8, 16, 17]. These results indicated that stable molecular associations between proteins and carotenoids occur in chromoplast at different magnitude, depending on the genotype.
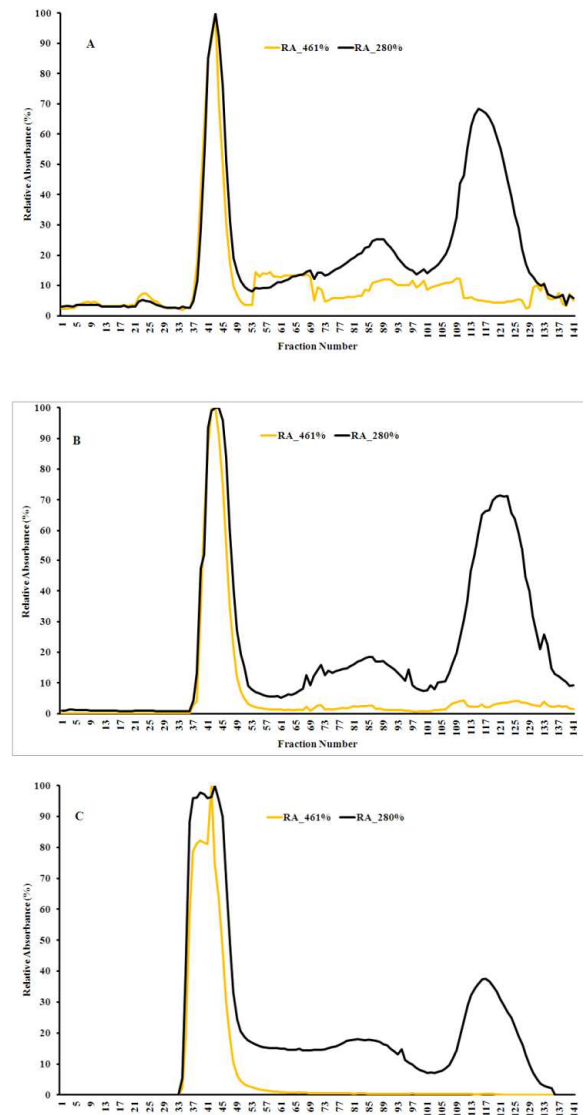

Fig. 3. Size exclusion chromatography (SEC) profile of chromoplast enriched suspension from cultivar IAC 12-829 (A), landrace CAS31 (B), and CAS074.1 (C). Fractions were monitored at both 280nm and 460nm corresponding to protein and total carotenoid. For open chromatography running specification and conditions see Material and methods. Fractions 35 to 49 were pooled and named Carotenoid-Protein Complex (CPC).

The types of carotenoids and proteins were further characterized by their HPLC profile and SDS-PAGE profile for carotenoids and proteins respectively. A specific carotenoid type was observed in the HPLC carotenoid profile (Fig. 4) that is similar to the one reported by others [14], however, with different abundance depending on the genotype. Proteins band pattern showed (Fig. 5) differences in protein band size and numbers, depending on the genotype. An 18 kDa band abundant in commercial cultivar IAC 12-829, 20 and 33 kDa bands common and abundant in landrace Cas031 and Cas74.1, and finally a 42 kDa band unique to landrace Cas31 were observed. Carotenoid-protein complexes have been documented in several plants systems at SDS-PAGE level [6, 8, 10, 16]. All of them showed similar protein profile in SDS-PAGE in terms of protein size variation ranging from 15 to 70 kDa.

Carotenoid-protein content variation in a progeny population

A progeny of 200 F1 individuals, derived from a cross of a high protein and carotenoid content landrace (CAS074.1) and a commercial variety (CPAC 753) with low content of protein and carotenoids was used to access the genetic parameters associated with those traits. Both traits (carotenoid and proteins content) showed a similar pattern of frequency of occurrence (Fig. 6) but different statistical significance level for normality distribution test (Table 3). Principal component analysis (PCA) revealed that the first principal component (carotenoid content) explained 60% (Fig. 7(a)) of the total variability, and its weight values showed a positive correlation with proteins and discriminated individuals based on this component. Two groups were established. Group I was composed of four individuals (11/12, 12/12, 14/12, and 21/12) showing high protein and high carotenoid content (Fig 7(b) and Table 4). Group II formed twenty sub-groups after identification and removal of Group I from the analysis (Fig. 8), where the width silhouette test (sj) value was highly significant at the level of 0.60. The Pearson correlation matrix showed high levels of R value as 0.68 (p-value=0.008) for correlation between total carotenoid and TBEP (Table 2). One-way ANOVA allowed us to infer on the estimated heritability of both traits indicating an h^2-value of 0,010 for TBEP and 0,339 for total carotenoid.

Fig. 4. HPLC profile of carotenoid present in peak 1 (carotenoid associated to protein complex (peak I) from SEC profile as in Figure 3. (A) Commercial cultivar IAC 12-829 with showing low total carotenoid content. (B) Landrace CAS3156 with presenting intermediary amount of total carotenoid content. (C) Landrace CAS74.1 with presenting high amount of total carotenoid content. HPLC running conditions used a monomeric 3 μm C18 waters column with a solvent mobile phase of acetonitrile:ethyl acetate:methanol (80:10:10). Peak numbers refer to 1. Neoxanthin; 2. Violaxanthin; 3. Lutein; 4. Unkown; 5. Unkown; 6. Zeinoxanthin or ß-Cryptoxanthin; 7. Trans ß-Carotene.

Fig. 5. SDS-PAGE proteins profile associated to carotenoid (peak I) separated in SEC as in Figure 3. Lane MM contains protein molecular markers. (I) proteins from peak 1 in cultivar IAC12.829; (II) proteins from peak 1 in landrace CAS31; (III) proteins from peak 1 of landrace CAS74.1. An 18 kDa band abundant in commercial cultivar IAC 12-829, 20 and 33 kDa bands common and abundant in landrace Cas 31 and Cas74.1, and finally a 42 kDa band unique to landrace Cas31 were observed.
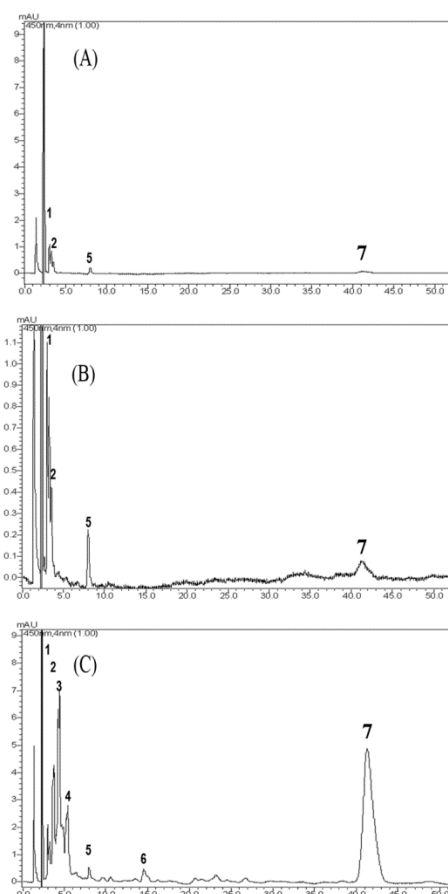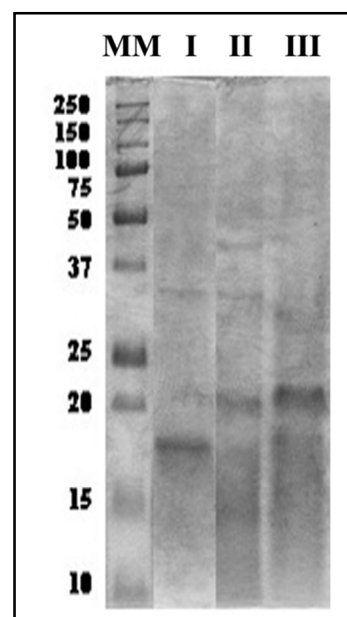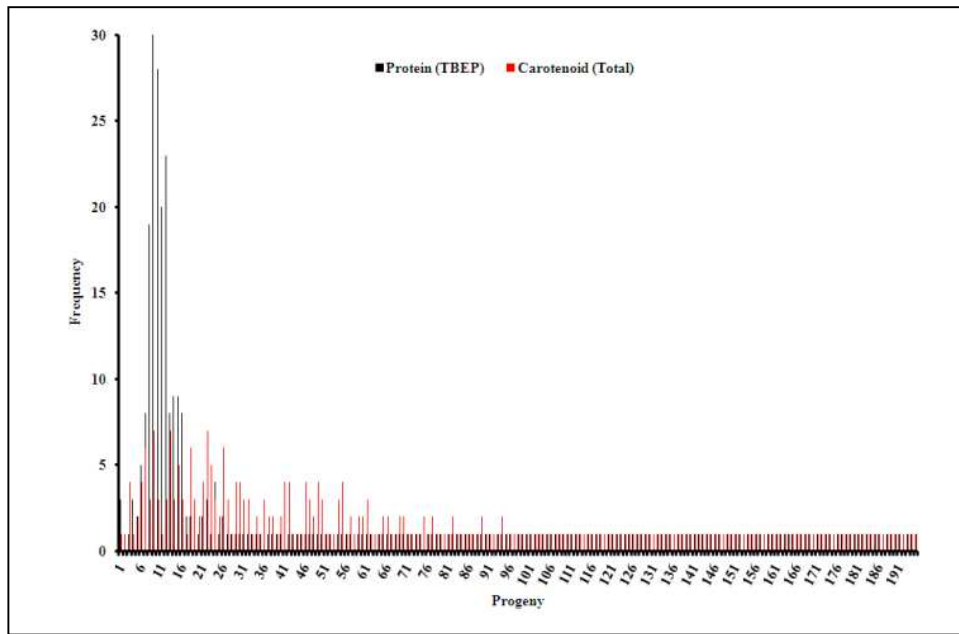
Fig. 6. Frequency (%) distribution for TBEP and total carotenoid content across 200 individuals in the progeny test. The biochemical phenotype defines a continuous distribution in this segregation population that is caused by the combined effects of allelic variation at several segregating loci (multigenic) and the environment.

Fig. 7. Scatter plot (A) of a rank-1 matrix showing entries for TBEP and total carotenoids values measured across 200 individuals from the F1 progeny and box plot for carotenoid (B) and proteins (C) group distribution.
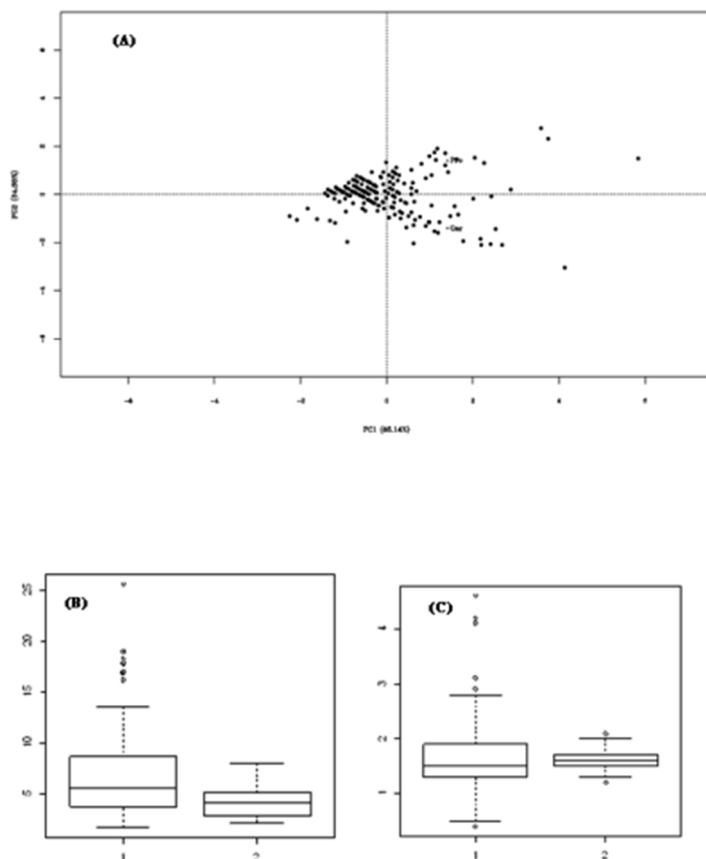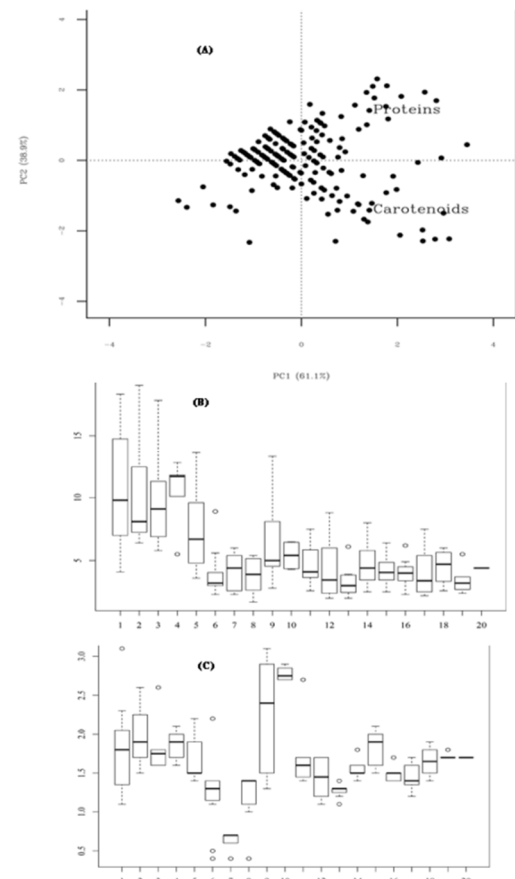

Fig. 8. Scatter plot (A) of a rank-2 matrix showing entries for TBEP and total carotenoids values measured across 196 individuals from the F1 progeny and box plot for proteins (B) and carotenoid (C) group distribution.

TABLE 2. TEST FOR CORRELATION BETWEEN BUFFER FRACTIONATED PROTEIN EXTRACT (TOTAL BUFFER EXTRACTABLE PROTEIN – TBEP, BUFFER SOLUBLE PROTEIN – BSP, AND BUFFER INSOLUBLE PROTEINS – BIP) AND TOTAL CAROTENOID CONTENT.

Proteins Ext.	R-value	R^2-value	p-value
Landraces (29)			
TBEP	0.680	0.462	0.008
BSP	0.416	0.173	0.068
BIP	0.463	0.205	0.045
Progeny (196)			
TBEP	0.299	0.089	0.0001

Note: R-value is correlation coefficient; R^2-value is the coefficient of determination and p-value level of probability of significance for R-value in the landraces and progeny study. (Pearson correlation matrix with p-values different from 0 with a significance level alpha=0.05.)

TABLE 3. TEST FOR DATA NORMALITY DISTRIBUTION FOR A PROGENY OF 200 INDIVIDUALS TESTED.

Statistics tests	Protein	Carotenoid
Progeny		
Skewness	0.04126	0.49645
s.e. Skewness	0.1226	0.0709
Kurtosis	-0.6057	-1.04936
s.e. Kurtosis	0.2447	0.1418
CI (95%)	44.19>50.4647>56.75	74.5 >77.8141> 81.13

TABLE 4. GROUPS OF INDIVIDUALS, AS DISCRIMINATED BY PRINCIPAL COMPONENT ANALYSIS FOR TBEP AND TOTAL CAROTENOID CONTENT.

Groupings	Sub-groups (#)	Group ID	Individuals ID	Protein (mg/DWt.)	Carotenoid (µg/gDWt.)
Group I	g (4)	g	11/12	4.1	9.9
		g	12/12	4.6	17.6
		g	14/12	4.2	8.3
		g	21/12	4.1	25.6
Group II	1 (n=16)	a	1/12	1.4	6.7
		a	5/12	1.3	7.3
		a	38/12	2.1	11.7
		a	42/12	1.5	5.9
		a	50/12	1.4	5.6
		a	107/12	1.4	4.7
		a	113/12	1.7	7.5
		a	141/12	1.5	4.1
		a	142/12	1.4	5.9
		a	158/12	2.1	4.6
		a	160/12	2.0	3.3
		a	167/12	1.5	3.0
		a	175/12	1.4	3.4
		a	180/12	1.6	4.8
		a	182/12	1.3	2.4
		a	193/12	1.7	3.7
	2 (n=6)	b	2/12	1.7	12.5

Groupings	Sub-groups (#)	Group ID	Individuals ID	Protein (mg/DWt.)	Carotenoid (µg/gDWt.)
		b	40/12	2.0	12.8
		b	86/12	1.3	2.8
		b	89/12	1.5	10.7
		b	93/12	2.9	13.3
		b	96/12	2.5	4.7
	3 (n=8)	c	3/12	16	16.2
		c	14/12	4.2	8.3
		c	24/12	1.7	6.8
		c	34/12	1.7	17.8
		c	36/12	1.6	11.8
		c	87/12	1.5	8.8
		c	92/12	2.9	5.0
		c	94/12	2.4	6.7
	4 (n=5)	d	4/12	1.8	18.3
		d	11/12	4.1	9.9
		d	16/12	1.9	7.5
		d	17/12	1.9	12.5
		d	22/12	1.5	7.7
	9 (n=11)	e	10/12	2.1	4.1
		e	27/12	2.4	12.0
		e	37/12	1.7	5.5
		e	39/12	1.9	10.1
		e	56/12	1.9	4.8
		e	57/12	1.8	4.7
		e	82/12	1.4	4.9
		e	108/12	1.7	6.5
		e	150/12	1.5	4.2
		e	153/12	1.5	4.4
		e	157/12	1.9	3.6
	10 (n=4)	f	15/12	2.0	7.5
		f	23/12	1.7	8.1
		f	30/12	1.6	10.7
		f	85/12	1.4	3.9

Discussion

The results from the present study showed that carotenoid-proteins content varied significantly among 29 cassava landraces indicating the existence of a notable genetic diversity. Overall, 8x more TBEP (mg/g DW) and 17x more BIP observed in the high compared to the low carotenoid landrace correspond up to 237x total carotenoid (µg/g DW.) and 713x more β-carotene respectively. Correlation coefficient values of R=0,680 for TBEP and 0,463 for BIP support the identification of carotenoid-protein complex and variation in protein content by comparing high (landrace Cas74.1) and low (cv IAC12.829) carotenoid content. Therefore, this dual concomitant accumulation of total carotenoid, β-carotene and proteins could lead to an important sink for carotenoid-protein in CSR. Detailed analysis of proteins from the carotenoid-protein complex indicated different sets of proteins related to carotenoid content present in the diverse landraces. While in the commercial variety IAC 12.829 with low total carotenoid content two protein bands of 18 and 33 kDa were detected, the high carotenoid content landrace CAS074.1 exhibited four proteins with size of 18-20-30-33 kDa. A protein band of 42 kDa was uniquely present in landrace CAS31. This diversity in carotenoid-protein content has also been observed in several non-photosynthetic tissues of many plant systems. Massive accumulation of carotenoids correlates directly to: (1) biosynthesis of components of the structures [6, 7, 8, 10, 16, 18]; (2) proliferation of

carotenoid sequestering structures rather than with changes in gene expression or enzyme abundance of the carotenoid biosynthesis pathway [19]; (3) dependence on their sequestration in specialized supramolecular structures [11, 20, 21]. Together, these studies lead to the recognition of a broad class of proteins named carotenoid associated proteins (CAP) [21]. CAP relates to cell functions such as pro-plastid and plastid differentiation and interconversion [6, 10, 21], chromoplast formation [8, 9, 22], chromoplast differentiation [11, 18], and carotenoid sequestration [10, 23]. Recently we showed that the major proteins classes present in carotenoid-protein complex of cassava belong to the small heat shock protein family and a different isoform of HSP21 may be responsible for the difference between low and high carotenoid content landrace [17]. At the same time, the carotenoid species present in carotenoid-protein complex varied according to the genotype sources (either cultivar or landrace). While in cultivar IAC 12-829 three carotenoid species were detected, landrace Cas31 and CAS74.1 showed four and seven carotenoid types respectively. Studies with carrot [8, 16], non-photosynthetic chromoplasts [8, 24, 25], algae [26] and cyanobacteria [27] showed similar results. This variability may account for the diversity of protein size observed across genotypes in the present study as well as across species in different studies.

Genetic transfer of the high carotenoid-protein character was studied in an F1 population with 200 individuals. The results suggest a high heritability of carotenoid content and low heritability for protein content. The high heritability for carotenoid content (0.33) indicates preponderance of additive gene action in the expression of this trait; therefore selection might be effective through this character in a segregating generation. The case of protein content, which showed low heritability (0.01), indicates non-additive gene action and a possible strong genotype x environment interaction playing a crucial role in the expression of this trait. Another possible explanation for the protein content diversity could be related to starch content as it has been shown to be negatively correlated [28]. Parenchyma cells in cassava storage root are packed with 85% starch (based on dry weight base), thus, for an impact of increasing carotenoid-protein content, a significant amount of starches have to be reduced. Therefore, selection for high proteins content for future breeding may not be recommended as direct measurement, but as an indirect measurement using values of carotenoid content.

Besides carotenoid-protein variation being genotype associated as demonstrated above, it also varied with root tissue type and age. The distribution of TBEP and carotenoids in five tissue layers followed a distinct pattern with variable magnitude depending on the landrace. While TBEP content decreased within tissue layers in tissue system III, total carotenoid content tended to increase. Taken together, these results indicated a strong influence of tissue age (layers in tissue system III) on the accumulation of total carotenoids and TBEP as secondary growth proceeds. The results presented in this study, represent the first reporting of a storage organ like the one in cassava storage root (CRS). Together, the genetic diversity observed in the landraces and progeny studies as well as the high throughput screening (HTS) for a direct (landraces access) and indirect (progeny access) quantitative assay for both traits revealed a possibility of using this concept for selection of cassava storage root enriched with carotenoid and proteins in a conventional breeding programme, or using induced mutations for further trait enhancement.

Conclusions and forward look

Results from this study allow us to conclude that: natural carotenoid-protein content varies due to genetic background of landraces and storage root tissue age; carotenoid-protein association is stable across landraces and clones, and showed variation in protein and carotenoid types; estimated heritability of proteins and carotenoids traits showed different values. The role of proteins associated with massive carotenoid accumulation and the mechanisms of carotenoid sequestration in CSR are on going research activities in our laboratory.

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