Protein content in cassava storage root is associated with total abundance of carotenoids

Luiz JCB Carvalho[1,2*], James V Anderson[2], J Padilha da Silva[1], S Chen[3], Claudia RB de Souza[4]

[1]EMBRAPA Genetic Resources and Biotechnology, Brasilia, DF Brazil

[2]USDA-ARS Red River Valley Agricultural Research Center, Fargo, ND, USA

[3]Tropical Crop Genetic Resources Institute, CATAS, Danzhou, Haikou, China

[4]UFPA, Belem, PA, Brazil

Abstract

Protein content in Cassava (*Manihot esculenta Crantz*) Storage Root (CSR) is low but variable. Detailed characterization of this variability is missing due to past inappropriate protein quantification procedures. Here we used conventional protein analytical procedures to access variation in Total Buffer Extractable Proteins (TBEPs) associated with Cassava Storage Root (CSR) color in landraces from the Amazon (Brazil). TBEPs values varied according to storage root color from 7.5 (mg/g DWt) in intense yellow landraces to less than 2 (mg/g DWt) in white CSR, an enhancement of up to 4x were detected in intense yellow compared to white CSR, represent up to 55% more protein in the bulk of CSR. Correlations of total buffer extractable proteins and total carotenoid content produced R^2 values of 0.4757, 0.6849 and 0.8958 depending on the extraction procedure. Protein polymorphisms were accessed by buffer fraction, carotenoid-protein complex separation in size exclusion chromatography, and protein size separation in SDS-PAGE profile analysis. Total buffer extractable protein molecular weight varied from 10 to 260 Kda in chromoplast enriched suspensions, while purified chromoplast carotenoid-protein complex had specific protein molecular weights of 18 kDa in white CSR, 38 and 43 kDa for yellow CSR, and 23 and 58 kDa in pink CSR phenotypes. Furthermore, of the 143 protein spots observed from purified chromoplasts using 2DE, up to 30 protein spot variations were identified among the four CSR color categories. Furthermore, -carotene and lycopene were the major carotenoid types present in the carotenoid-protein complexes, which was dependent on the CSR color phenotype.

Keywords: Cassava, Storage root, Protein, Carotenoid, Lycopene, β-carotene

INTRODUCTION

Among the five tubers and root crops considered staple foods (i.e. potato, sweet potato, cassava, taro and yam); Cassava Storage Root (CSR) represents 20% of the world production in tropical regions of the globe. The bulk of dietary intake for human nutrition provided by cassava (*Manihot esculenta Crantz*) includes carbohydrate (85% dry weight based as starch), moderate provisions of proVit A carotenoid with large variation (Guimaraes and Barros, 1971; Ortega Flores, 1991; Marinho et al., 1996; Carvalho et al., 2016) and very low protein content with ranging from 1% to 2.5% (dry weight base) depending on cultivars (Yeoh and Chew, 1977). Detailed characterization of this protein content variation is missing; mainly due to the lack of critical information that could be genetically manipulated. Reliable measurements of true protein content variation in cassava root spans a range of 1% to 3.5% on dry weight base (Stupak et al., 2006). However, none of these observed variations provided the knowledge needed for genetic manipulation and enhancement of protein content in CSR. Thus, recognition of classical storage protein presence in CSR is needed (Shewry, 2003). Since CSR did not evolve to be a reproductive organ like grains, cereals and tubers, this organ does not remobilize nitrogen

from storage proteins for plant propagation. The occurrence of vegetative storage proteins in CSR, as described in leaves of soybean (Wittenbach, 1982), in tubers and storage roots of potato and sweet potato (Witshire and Cobb, 1996), and bark of poplar (Zhu and Coleman, 2001) could be possible. However, attempts to find storage proteins, of any kind, in CSR have not been conclusive (Shewry, 2003). A major prolamin type protein (Pt2L4) from the bulk of the central cylinder of storage roots has been associated with CSR formation (de Souza et al., 2006), was identified by antibody that reacts to allergenic Hev b 5 protein from rubber tree (de Souza et al., 2008) and allergenic reactions when CSR is fed to humans (Galvao et al., 2003; Gaspar et al., 2003; Souza et al., 2008; Souza et al., 2011a; 2011b). However, Pt2L4 has not been tested as a source of remobilized nitrogen. A major protein of about 22 kDa has also been found in the peel of the storage root but no detailed characterization about its potential role as a store protein has been reported (Souza et al., 1998). Variation of protein content in germplasm collections, carried out by several groups, provided no evidence of relevant variation of reliable genetic confidence, mainly due to technical quantification procedures used for obtaining estimates (Yeoh and Truong, 1996). Consequently, conventional breeding techniques have not presented evidence for successful (Bolhuis, 1953) genetic manipulation of protein content in cassava storage root, which is mainly due to a lack of variability in the germplasm collections (CIAT, 2008). The pool of free amino acids could offer the possibility for genetic manipulation of protein synthesis and stabilization in roots, but not as a food nutrient supply. Genetic engineering through transgenic technology to express heterologous genes coding for storage proteins from other crops, or artificial storage proteins, have not produced stable proteins in cassava storage root (Zhang et al., 2003, Stupak et al., 2006; Stupak, 2008). This was mainly resultant from the need for a suitable sub-cellular localization for the expression and stores the target proteins (Zhang et al., 2003; Stupak et al., 2006; Stupak, 2008). Cumulatively, the above observations dictate the need for a revisionary approach to all these initiatives. In our group, we are focusing on a naturally occurring sub-cellular sink suitable for protein accumulation. As such, we propose to exploit root color diversity and target chromoplast as a sink for storage of extra protein, similar to its cellular function for massive accumulation of carotenoids in non-green tissue such as CSR (Carvalho et al., 2012, Carvalho et al., 2013).

Massive accumulation of intermediary carotenoid types in the chromoplast membranous system of non-green tissue is a balance of cell specific organelle mechanisms related to carotenoid biosynthesis, degradation, and sequestration. The process is dependent on a broad class of proteins named Carotenoid-Associated Proteins (CAP), which are related to several cell functions such as pro-plastid and plastid differentiation and interconversion (Pozueta-Romero et al., 1997; Vishnevetsky et al., 1999a), chromoplast formation (Bryant et al., 1992; Smirra et al., 1993; Deruere et al., 1994; Lopez et al., 2008), chromoplast differentiation (Camara et al., 1995; Vishnevetsky et al., 1997; Li et al., 2001), and carotenoid sequestration (Zagalsky, 1976; Vishnevetsky et al., 1999b) that could provide a cellular sink for functional proteins leading to stable protein accumulation. This reasoning has led us to exploit the causal effect of carotenoid and protein accumulation in pigmented cassava storage root cells that have naturally occurring variation in color (Carvalho et al., 2013). The carotenoid-protein sink is of three fold importance in cassava: (1) For the recognition of chromoplasts as a natural sub-cellular stable sink of functional proteins that are required for massive accumulation of carotenoids; (2) To bring together high amounts of accumulated carotenoids that can offer additional nutritional value with pro-VitA carotenoid in the storage roots; and (3) To identify proteins/genes that may be manipulated genetically in a breeding program to increase the carotenoid-protein sink of cassava storage root. In this study, we provide evidence for enhancing protein content by showing that total buffer extractable protein content variation in cassava storage root is associated with CSR color being positively correlated with total carotenoid contents, and are differentially distributed in the storage root tissue compartments due to tissue age as the secondary growth proceeds.

MATERIALS AND METHODS

Plant Material

Plant Diversity study used twenty-six accessions of pigmented cassava landraces from our GENEBANK collection (Silva and Espindola, 2011; Ferreira and Clementino, 2010). The plants were cultivated in field plots at Embrapa Genetic Resources and Biotechnology and used for quantification of extractable proteins, total carotenoid and β-carotene from freeze dried root tissue to study protein profiles of carotenoid-protein complexes characterization. Rehydrated powdered tissue was processed with 2 ml Extraction Buffer (EB) composed of Tris 80 mM pH 6.8, NaCl 0.2M, Pefabloc SC 0.1% and DTT 25 mM, vigorously vortexed and sonication for extraction of total protein and carotenoid by phase separation with the addition of 4 volume acetone, incubation at -20°C for 1 hour, and centrifugation (30000 rpm/4°C/20 min). The supernatant was first collected and pooled after wash the pellet twice with pure acetone with centrifugation (30000 rpm/4°C/20 min). Acetone was

evaporated under N2 flush, and the formed yellow color phase was solubilized in anhydrous ether for total carotenoid quantification as above. The remain pellet was added with 3 ml Extraction Buffer, vortexed, sonicated and heated at 60°C for one hour and centrifuged (30000 rpm/4°C/20 min) again to measure total proteins (TBEP) in the supernatant with Bradford assay. For the diversity landrace experiment, protein from tissue powder was buffer fractionated as follow. For total extracted proteins (TBEP), the tissue powder was added with 2 ml of EB, 4 volume of acetone, vortexed, sonicated, incubated at -20°C, (30000 rpm/4°C/20 min) and the supernatant used for protein assayed as above. A new tissue powder sample was treated with EB as above without acetone treatment, centrifuged (30000 rpm/4°C/20 min) and the supernatant used for protein quantification by protein Bradford assay and treated as Buffer Soluble Protein (BSP). The pellet was resuspended with 2 ml EB added with 4 volume of acetone, vortexed, heated at 60°C, cooled at room temperature, incubated at -20° for 1 hour, centrifuged (30000 rpm/4°C/20 min). The pellet was collected and resuspended with 2 ml EB for quantification of protein in the supernatant after centrifugation (30000 rpm/4°C/20 min). Protein assay used were BCA (PIERCE micro kit assay) or Bradford (BioRad micro assay) according with the manufacturer.

Data Analysis

Comprehensive and standard statistical analyses were performed using R Statistics package, SISA package and XLSTAT package and ChemImagen 4400 (Alpha Innotech, San Leandro, CA).

Tissue Preparation

Cylinders of storage roots 30-40 cm long and 4-6 cm in diameter were manually sliced as bulk root or tissue layers as previously described (Carvalho et al., 2018) and immediately frozen in liquid nitrogen, freeze-dried and stored in -80°C until used for protein content and analysis, and for total carotenoid extraction and quantification. For the carotenoid-protein complexes studies, fresh intact storage roots were peeled off and freshly processed immediately after harvest.

Total Buffer Extractable Proteins and Quantification

One gram of dried tissue of CSR from 29 cassava clones was rehydrated with extraction buffer (EB), addition of 20 ml of acetone (100%), vigorously vortexed and incubated in a water bath at 50°C for 30 min, followed by the addition of 2 ml of extraction buffer (SDS 5%, Glycerol 10%, Tris 80 mM pH 6.8, and DTT 25 mM). After cooled to room temperature, samples were incubated at -20°C for a minimum of 1 h to obtain a protein precipitate free of pigments. After centrifugation (30000 rpm/4°C/20 min) the supernatant was discarded, and the pellet was dried by blowing N^2 air over the pellet and then re-suspending with 5 ml of EB. The re-suspended solution was centrifuged again (30000 rpm/4°C/10 min) to collect 1 ml aliquot of the supernatant that was stored in -80°C until use. Aliquots of 100 treated with DOC (0.15%) and TCA (12%) to precipitate Aqueous Soluble Proteins (ASP). After centrifugation (13000 rpm/4°C/20 min) the pellet was suspended in 100 µl of EB, and 50 µl was used for tissue protein estimation by the BCA method using the Micro Kit of PIERCE according to the manufacturer.

SDS-PAGE Analysis

Total extractable proteins, fractionated proteins and pooled protein fractions from carotenoid-protein complexes and non-carotenoid-protein complexes were separated by one-dimensional SDS-PAGE as previously described by de Souza et al. 2004.

Carotenoid Extraction and Quantification

Approximately 5-20 g of powder (dependent on the intensity of color) was transferred to a 50 ml tube for total carotenoid extraction, and total and specific carotenoid quantification as previously described at Carvalho et al. 2016.

Chromoplast Enriched Suspension Preparation and Chromoplast Separation

Intensely colored layers of fresh storage roots were separated, sliced, and treated as previously described by Carvalho et al. 2012 for chromoplast enriched suspension preparation.

Chromoplast Associated Protein Characterization by 2DE-PAGE

Purified chromoplasts were washed with HB and lyzed in suspension buffer by sonication. An aliquot of CrAP was precipitated using the DOC/TCA procedure. The pellet was washed twice with cold acetone and suspended in rehydrating IPG buffer. IPG dry strips (pH 3-10 linear and 18 cm long) were rehydrated with protein samples and focused according to the manufacturers (Pharmacia) protocol and are shown in Figure 1. Protein molecular weight standards and pI standard (2-DE standard molecular marker from BioRad) were also treated and focalized on separate strips. The second dimension protein separation used an ISODALT gel system from Pharmacia according to the manufacturer protocol using 13% SDS-PAGE. Two-dimensional gel images were generated using a high resolution (1024 x 1024 pixel) Dual Scan system (AGFA T2000) and transferred to a high capacity computer. Images were analyzed with the GELLAB II + software (Scanalytics). Protein molecular weight and pI

standards were calibrated, and relative OD reads within and across gels of a particular experiment using white cassava (cv. IAC 12-829) was used as reference. Experiments were repeated twice and high quality gels with reproducibility were used to identify individual spots within samples and to estimate pI, MW and %OD reads. Pair wise comparison of protein spot regulation (Intensity %) across genotypes representatives' storage root color categories (Table 6).

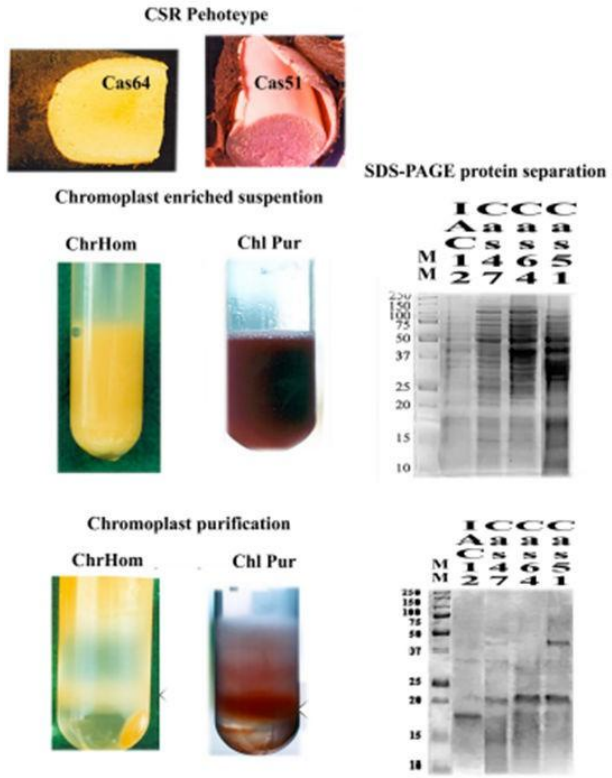

Figure 1. Illustrative diagram of the laboratory procedures addressed with storage root phenotype color representative (Cas64 intense yellow, and Cas51 pink), fractionated chromoplast enriched homogenate (ChrHom) as TBEP, sucrose gradient Purified Chromoplast (Chl Pur) and corresponding protein size separated in SDS-PAGE for genotypes representative for four color variants of CSR (i.e. white, IAC12; yellow, Cas47; intense yellow, Cas64; and pink, Cas51)

RESULTS

Protein and Total Carotenoid Content Variation Cassava Storage Root

Twenty-six landraces representing five color categories of cassava storage roots were assessed for their Total Buffer Extractable Protein (TBEP) and total carotenoid content. Results in Table 1 indicate that up to 2.4x and 7.9x more TBEP was observed in intense yellow CSR when compared to white CSR. Heat map analysis for these landraces genetic diversity (Figure 1A) and a sampler of 198 progeny (Figure 1B) for measured protein and carotenoid. These results suggest that diversity and intensity of CSR color contributes significantly to differential carotenoid/protein sink capacity. This is likely due to the naturally occurring genetic background of landraces, which could open a new avenue of research for improving cassava nutritional quality. For the sake of simplification, only phenotypes with highly contrasting CSR color (intense yellow Cas64, and pink-Cas51) were further used in the following experimental work, which was compared to a white CSR color phenotype (cv. IAC12.829) as a control in a conventional breeding program (Carvalho et al. 2013; Carvalho et al. 2011).

Chromoplast Fractionation and Protein-Carotenoid Complex Separation

Chromoplast enriched suspension in extraction buffer, and their constituents separated by size exclusion chromatography for cassava storage root of landrace Cas64 (Figure 2; intense yellow) and Cas51 (Figure 3; pink color) (Table 2).

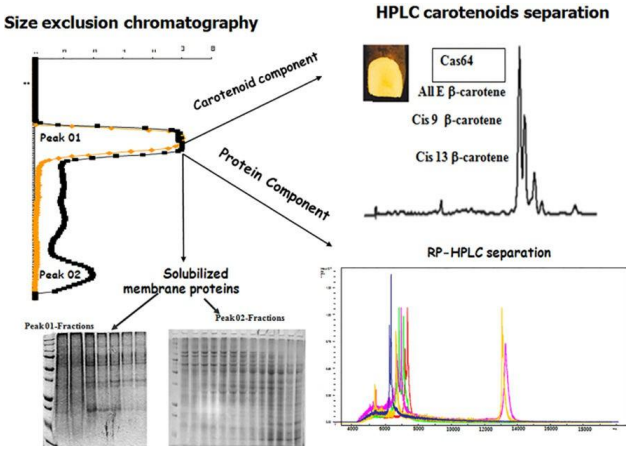

Figure 2. Illustrative diagram of the laboratory procedures addressed with storage root to separate proteins-carotenoid complex (Size exclusion chromatography, Peak01), major carotenoids identification components (HPLC separation), major proteins component separation (RP-HPLC separation), membrane solubilized proteins patterns for Peak 01 (proteins-carotenoids complex) and Peak 02 (non-carotenoid associated proteins) separated by SDS-PAGE. Fresh Storage roots of intense yellow landrace Cas64 (mainly β-carotene) was used as source of material

Table 1. Total Buffer Soluble Protein (TBEP) and total carotenoid content in storage root of cassava landraces representing five

classes of color. One gram of dried tissues from 25 genotypes of cassava storage root was treated as described in material and methods

Storage Root Color categories	Landraces ID	Total β-carotene (µg/g DWt.)	Total carotenoid (µg/g DWt.)	Protein (mg/g DWt.)
Intense Yellow	Cas62	3.9	10.76	3.85
	Cas64	20.68	24.57	7.51
	Cas56	10.65	33.54	6.33
	Cas31	5.49	13.97	4.33
	Cas61	1.66	19.4	2.94
	Cas32	11.43	19.4	6.12
	Cas47	8.32	14.87	4.75
	Cas34	14.87	24.41	4.36
	Cas63	13.54	14.84	3.17
	Cas68	13.96	30.77	3.3
	Cas35	3.1	19.45	-
Yellow	Cas53	4.71	10.74	3.04
	Cas33	6.62	6.44	2.98
	Cas60	8.08	13.3	3.07
	Cas66	11.89	17.18	-
	Cas71	2.93	4.4	2.39
	Cas70	6.3	11.71	2.39
	Cas52	0.37	1.68	2.52
	MC027	-	0.52	2.11
	MC028	-	1.82	2.07
Pink	Cas51	-	14.8	6.82
White	IAC12.829	0.27	0.65	0.95

Table 2. Quantitative analysis of the total buffer extractable proteins from intense yellow (landrace. Cas64) and white (cv. IAC 12.829 as reference) storage roots of cassava. One kg of fresh harvested storage root was fractionated by size exclusion chromatography as showed in Figure 1-3

Extractable proteins fractionation steps	Extraction Yield		Recovered fraction	
	IAC12.829	Cas64	IAC12.829	Cas64
Total Buffer Extractable Proteins (mg/50 ml)	81.2	255.5	100	100
Chromoplast Enriched Suspention (mg/5 ml)	8.2	20.9	10.1	8.2
Purified Chromoplast (mg/0.1 ml)	0.2	0.6	2.5	2.9
Peak 1 (mg/0.1 ml)	0.1	0.1	1.4	0.7
Peak 2 (mg/0.1 ml)	0.1	0.2	1.7	0.9

Table 3. Protein-carotenoid content informative data were used for correlations study across the fractionation steps as Figure 1 and Figure 2. Total buffer extractable proteins (TBEP) and chromoplast suspension protein content (ChlHom) were as detailed in materials and methods

Fractionation steps	R2 value
TBEP	0.48
ChlHomSP	0.68

Carotenoid Protein Correlation Studies

The content of carotenoids and proteins associations as accessed above was further characterized by correlation analysis as shown in Table 3-5.

Protein Content Distribution Associated with CSR Tissue Age

Protein accumulation in storage organs are usually related to their development and maturation, like in seeds and tubers. For the case of cassava storage root, that has indeterminate secondary growth, tissue age effects confirms (Figure 5) previous report (Carvalho et al., 2012), indicating that protein content varying according to storage root tissue age as secondary growth proceeds as illustrated elsewhere (Carvalho et al., 2018). Taken together, these results indicated a strong effect of tissue age in the accumulation of total carotenoid and protein contents as secondary growth proceeds in different color classes of CSR.

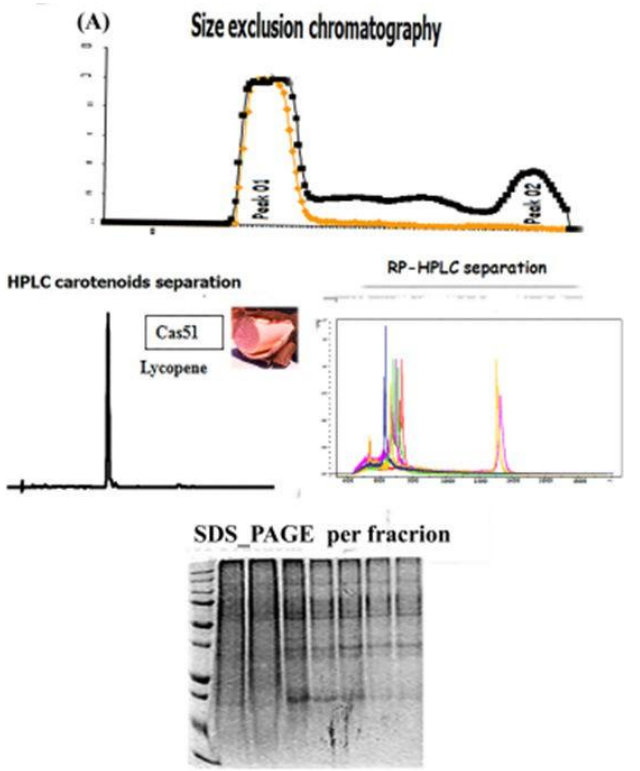

Figure 3. Illustrative diagram of the laboratory procedures addressed with storage root to separate proteins-carotenoid complex (Size exclusion chromatography, Peak01), major carotenoids identification components (HPLC separation), major proteins component separation (RP-HPLC separation), membrane solubilized proteins patterns for Peak 01 (proteins-carotenoids complex) and Peak 02 (non-carotenoid associated proteins) separated by SDS-PAGE. Fresh Storage roots of pink colour landrace Cas51 (solely lycopene) was used as source of materials

In addition, further compartmentalization of carotenoid and protein content variations was accessed by separating Chromoplast Enriched Buffer Suspensions (CEBS) by Size Exclusion Chromatography (SEC) and HPLC to partially purify carotenoid-protein complexes and their components in contrasting color phenotypes.

Carotenoid-protein complexes have been documented in several plants systems by SDS-PAGE (Bryant et al., 1992; Deruere et al., 1994; Zhou et al., 1994; Vishnevetsky et al., 1999a; Pozueta-Romero et al., 1997;), including cassava storage roots (Carvalho et al., 2012). All of them had similar protein polymorphisms in terms of protein size in gels with specific protein bands varying from 15 to 70 kDa.

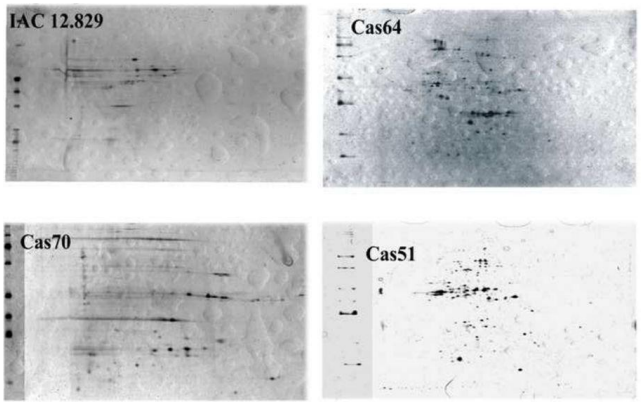

Figure 4. Membrane proteins from sucrose gradient purified chromoplast (Chl Pur) as in Figure 1 and Figure 2, were solubilized, separated and characterized by using in 2DE-PAGE. Fresh storage roots of white (cv. IAC12), intense yellow landrace (Cas64), yellow landrace (Cas70) and pink color landrace (Cas51) were used

Table 4. SDS-PAGE image analysis: Estimated protein size (kDa), band abundance (non-dimensional density value) revealed by SDS-PAGE analysis of the carotenoid-protein complex fractionated as in Figure 1-3 using software ChemImagen 4400 (Alpha Innotech, San Leandro, CA) to observe differences in unique protein obtained from fresh white (cv. IAC12.829) yellow (Cas70) intense yellow (Cas64), and pink (Cas51) cassava storage roots protein extracts as described in materials and methods

Protein Size (kDa)	Root color phenotype			
	IAC12.829	Cas70	Cas64	Cas51
58	3192	6584	4661	13253
43	6080	6474	7940	7091
38	4190	4914	6173	3798
23	6140	9638	9450	12988
18	7361	6211	7177	4781

Table 5. Pair wise comparison of unique protein spot numbers (numerator) for representative storage root color categories, and their total number of spots (denominator) by each storage root color category in 2DE-PAGE of the carotenoid-protein complex fractionated as in Figure 1 and Figure 4. Image analysis was performed by using software ChemImagen 4400 (Alpha Innotech, San Leandro, CA). Fresh cassava storage roots were used as described in materials and methods

Genotype	Number of spots		Pair wise comparisons			
	Total	Unique	IAC12	Cas70	Cas64	Cas51
IAC12.829	50	24	50/50	Jun-83	21/138	9/142
Cas70	83	52	Jun-50	87/83	19/138	16/142
Cas64	138	83	21/50	19/83	138/138	30/142
Cas51	142	97	Sep-50	16/83	30/138	142/142

Table 6. Pair wise comparison of protein spot regulation (Intensity %) across genotypes representatives storage root color categories. 2DE-PAGE of the carotenoid-protein complex fractionated as in Figure 1 and Figure 4. Image analysis was performed by using software ChemImagen 4400 (Alpha Innotech, San Leandro, CA).

Protein spot regulation (% intensity in relation to IAC12)			
Ratio genotype	**Unchanged**	**Up-regulated**	**Down regulated**
Cas70	33.33	16.67	50
Cas64	11.11	55.56	33.33
Cas51	19.05	71.43	9.52
Protein spot regulation (% intensity in relation to Cas70)			
Ratio genotype	Unchanged	Up-regulated	Down regulated
IAC12	33.33	50	11.11
Cas64	31.58	57.89	10.53
Cas51	50	25	25
Protein spot regulation (% intensity in relation to Cas51)			
Ratio genotype	Unchanged	Up-regulated	Down regulated
IAC12	71.43	9.53	19.05
Cas64	53.33	36.67	10
Cas70	50	25	25
Protein spot regulation (% intensity in relation to Cas64)			
Ratio genotype	Unchanged	Up-regulated	Down regulated
IAC12	55.56	33.33	11.11
Cas70	31.58	10.53	36.67
Cas51	53.33	10	36.67

Carotenoid Types Present in Carotenoid-Protein Complex

Overall the carotenoid profile in peak 1 from Figure 2, correspondent to carotenoid profiles of carotenoid-protein complexes, is similar to the full carotenoid profile observed for each phenotype when obtained directly from the plant tissue (Carvalho et al., 2012; Carvalho et al., 2016). Similarly, carotenoid profiles in peak 1 from Figure 3 are identical.

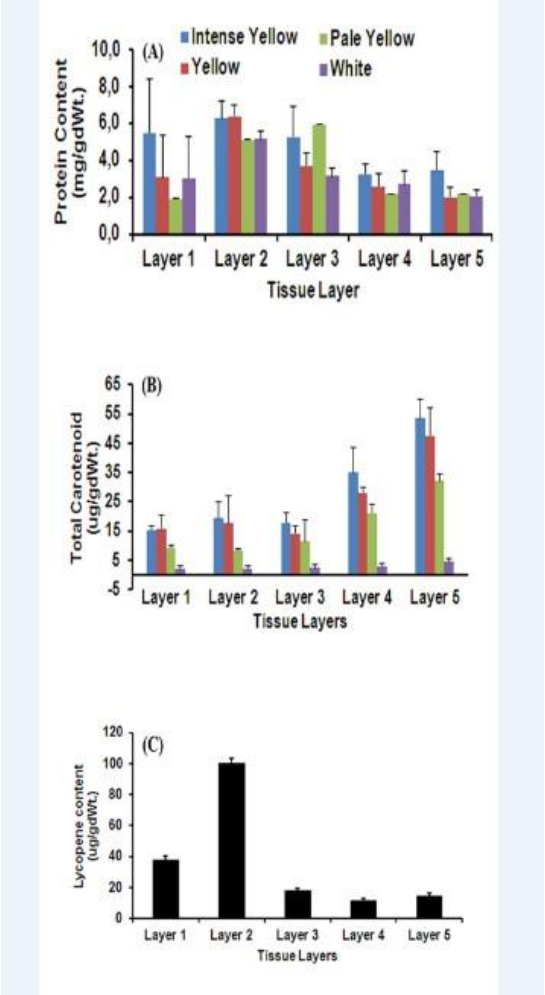

Figure 5. Total buffer extractable Proteins (TBEP, mg protein/g DWt.) and carotenoid (µg/g DWt.) content distribution in tissue layers according to the sampling regime described elsewere. (A) refers to total buffer extractable proteins content distribution. (B) refers to total carotenoid content distribution. (C) Refers to lycopene content distribution. A minimum of 4 landraces for color variants were used for each error bars

Protein and Total Carotenoids Content Grouping and Correlation Observation

Cluster analysis (Figure 6, Panel A) and Pearson Correlation analysis (Figure 6, Panel B) indicate the groups formed among the 26 tested landraces for TBEP content (mg/g DWt.) and total carotenoid content (µg/g DWt.), Correlation between those traits as Pearson r value reach level of 0.74.This results confirm the high correlation values of those two traits at the level the level of Chromoplast and carotenoid-protein complex as showed elsewhere (Carvalho et al. 2012; Carvalho et al. 2013; Carvalho et al. 2016).

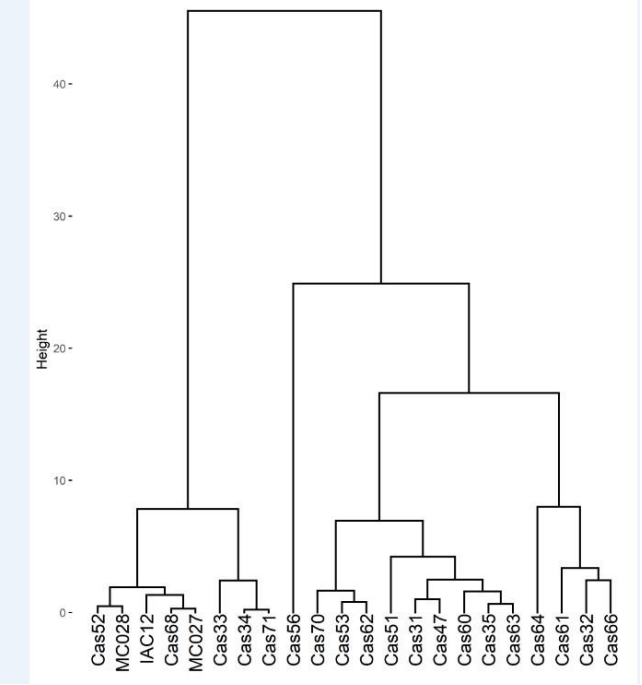

Figure 6. Cluster analysis for protein content (mg/gDWt) and total carotenoids content (mg/gDWt) among the 22 landraces studied

DISCUSSION

Massive accumulation of carotenoid in non-green tissue has been correlated directly with biosynthesis of the components of accumulating structures, and the actual model for carotenoid sequestration includes carotenoids, proteins and lipids (Deruere et al., 1994; Pozueta-Romero et al., 1997; Vishnevetsky et al., 1997). This corroborates with proliferation of carotenoid sequestering structures rather than with changes in gene expression or enzyme abundance of the carotenoid biosynthesis pathway (Al-Babili et al., 1996; Del Villar-Martínez et al., 2005), as well as being dependent on their sequestration in specialized supramolecular structures (Camara et al., 1991; Bartley and Scolnik, 1995; Vishnevetsky et al., 1999a). Together, these studies led to the recognition of a broad class of Carotenoid-Associated Proteins (CAP) (Vishnevetsky et al., 1999b) that are related to several cell functions such as pro-plastid and plastid differentiation and interconversion (Pozueta-Romero et al., 1997; Vishnevetsky et al., 1999a), chromoplast formation (Bryant et al., 1992; Smirra et al., 1993; Deruere et al., 1994; Lopez et al., 2008), chromoplast differentiation (Camara et al., 1995; Vishnevetsky et al., 1997; Li et al., 2001), and carotenoid sequestration (Zagalsky, 1976; Vishnevetsky et al., 1999b), especially in non-green tissue such as roots (Hagenimana et al., 1999), seeds (Kirk and Tiliney-Bassett, 1978) and flowers (Libal-Weksler et al., 1995). These observations provide a cellular sink for functional protein accumulation in non-green tissue like cassava storage root. Results in the present document provide evidence that protein content in cassava storage root varies with color and color intensity in the representative yellow group of cassava landraces. Enhancement of up to 55% more protein content in the intense yellow cassava storage root was observed in relation to white CSR. Similar results have been observed in different plant species in which CAPs were accumulated in extremely high levels (up to 80%) in the fibril's external half-membrane of the fibrillar chromoplast with variability due to structural proteins, enzymes of the carotenoid biosynthesis pathway, organelle differentiation processes, protein-protein as well as protein-molecules interactions (Smirra et al., 1993; Deruere et al., 1994). These proteins accumulated in parallel to carotenoid deposition in the stroma, which is organized in several types of supramolecular structures (Camara et al., 1991), including fibrillar-type composed of protein, carotenoid and lipids (Deruere et al, 1994). Similarly, less abundant proteins, also accumulates with identical patterns to that of CAP in fibrillar chromoplast (Libal Weksler et al., 1997). These CAPs, in non-green tissues, have been shown to respond to heat stress (Parsell and Lindquist, 1993), drought stress, wounding (Chen et al., 1998), changes in fruit development (Lawrence et al., 1997), and in transgenic manipulated tuber of potato (Lopez et al., 2008). In the case of cassava storage root, our results indicate that it is mainly dependent on the sequestration of carotenoids of a particular landrace, root tissue age and the carotenoid-protein complexes association with the color categories studied. Comparative studies between intense yellow and white CSR indicates that the carotenoid biosynthesis pathway is active in both, but intense yellow landraces accumulate 100x more total carotenoid (Carvalho et al., 2016) than white cassava. Protein polymorphisms observed in CAPs of cassava storage root varied in terms of size and abundance according to the color category of the landraces, depending on the major carotenoid type accumulated. Four major proteins, revealed in this study, a 18KDa in the white root, a 23 kDa and a 33 kDa in the yellow and a 59 kDa specific to pink root were associated with two major-carotene and lycopene), depending on the genotype. This observation provides evidence that these proteins in CSR form causal associations with the high carotenoid content in intense yellow CSR.

CONCLUSION

In conclusion, this study provided evidence that total buffer extractable protein content variation in cassava storage root was associated with storage root color, was correlated with total carotenoid contents, and was differentially distributed in the storage root tissue

compartments due to tissue age as the secondary growth proceeded. In addition, protein polymorphisms were also observed to be associated with storage root color diversity and distinct carotenoid-protein complex variants associated with root carotenoid type. In order to further elucidate the process of massive accumulation of carotenoid content in CSR and the mechanisms of carotenoid sequestration in non-green tissue like CSR, the issue of carotenoid biosynthesis and identification of the proteins involved in the carotenoid-protein complexes need further study. In particular, a conventional breeding program was started by preparing a single segregating population with promising landraces showing high protein and carotenoid content. The results from this research program are expected to provide significant contribution to our understanding of the molecular machinery to improve pro-Vit-A and protein content in cassava.

ACKNOWLEDGEMENT

Special acknowledgements are extended for financial and technical support provided by The Rockefeller Foundation (RF96010#25 and RF9707#26 for the landraces search in Amazon (Brazil); Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq, (Grant #480410.2001-1 for the functional genomics work); Programa Nacional de Pesquisa em Biotecnologia-CENARGEN (Project N° 060302058 for carotenoid analysis and EMBRAPA_LABEX_USA collaborative program); USDA_ARS for providing lab facilities in Fargo, ND (USA) and providing access to Euphorbiaceae microarray cDNA chip technology; IAEA (contract # 13188 for funds supplied for gene expression analysis); National Nature Science Foundation of China (NSFC grant number 31271776); and NSFC-CGIAR International (Regional) Cooperation and Exchange Programs (grant number 31361140366). The germplasm derived from this study was deposited in CIAT facilities and donated to the Harvest-Plus Initiative for dissemination worldwide, which is also acknowledged.

REFERENCES

Al-Babili S, Von Lintig J, Haugruck H, Beyer P (1996). A novel, soluble form of phytoene desaturase from Narcissus pseudonarcissus chromoplasts is Hsp70-complexed and competent for flavinylation, membrane association and enzymatic activation. Plant J. 9(5): 601-612.

Bartley G, Scolnik PA (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell. 7(7): 1027-1038.

Bolhuis GG (1953). A survey of some attempts to breed cassava varieties with high content of protein in the roots. Euphytica. 2(2): 107-112.

Britton G (1998). Carotenoids. In G. Britton, S. Liaaen-Jensen, H. Pfander, Eds. Carotenoids: Biosynthesis and Metabolism;, Eds.; Basel, Switzerlalnd. 3:13-147.

Bryant JD, McCord JD, Unlu LK, Erdman JW (1992). Isolation and partial characterization of alpha and beta-Carotene-containing carotene-protein from carrot (Daucus carota L.) root chromoplast. J. Agric. Food Chem. 40(4): 545-549.

Bullerjahn GS, Sherman LA (1986). Identification of a carotenoid-binding protein in the cytoplasmic membrane from the heterotrophic cyanobacterium Synechocystis sp. Strain PCC 6714. J. Bacteriol. 167(1): 396.

Camara B, Hugueney P, Bouvier F, Kuntz M, Moneger R (1995). Biochemistry and molecular Biology of chromoplast development. Int. Rev. Cytol. 163: 175-235.

Carvalho LJCB, Agustini MA, Anderson JV, Vieira EA, De Souza CRB, Chen S, Schaal BA, Silva JP (2016). Natural variation in expression of genes associated with carotenoid biosynthesis and accumulation in cassava (Manihot esculenta Crantz) storage root. BMC Plant Biol. 16(1): 133.

Carvalho LJCB, De Almeida JD, Da Silva P, Anderson JV, Vieira EA, Fuhrmann E, De Souza CRB, Chen S (2013). Studies on Variation of Carotenoid-Proteins Content in Cassava (Manihot esculenta Crantz) Storage Root Reveal Implications for Breeding and the Use of Induced Mutations. Plant Mutation Reports. 3(1): 25.

Carvalho LJCB, Filho JF, Anderson JV, Figueiredo PG, Chen S (2018). Storage Root of Cassava: Morphological Types, Anatomy, Formation, Growth, Development and Harvest Time. Chapter 4: 54-68.

Carvalho LJCB, Lippolis J, Chen S, De Souza CRB, Vieira EA, Anderson JV (2012). Characterization of Carotenoid-protein Complexes and Gene Expression Analysis Associated with Carotenoid Sequestration in Pigmented Cassava (Manihot Esculenta Crantz) Storage Root. Open Biochem. J. 6: 116-130.

Cervantes-Cervantes M, Hadjeb N, Newman JA, Price CA (1990). ChrA is a carotenoid-binding protein in chromoplasts of Capsicum annuum. Plant Physiol. 92: 1241.

Chen HC, Klein A, Xiang MH, Backhaus RA, Kuntz M (1998). Drought and wound induced expression in leaves of a gene encoding a chromoplast-associated protein. Plant J. 14(3): 317-326.

Clark RJH, Rodley GA, Drake AF, Church RA, Zagalsky PF (1990). The carotenoproteins of the starfich Linckia

laevigata (Echinodermata: asteroidean): a resonance raman and circular dichroism study. Comp. Biochem. Physiol. 95B:847.

De Souza CR, Carvalho LJCB, De Almeida ER, Gander ES (2002). Towards the identification of cassava root protein genes. Plant Foods Hum. Nutr. 57(3-4): 353-363.

De Souza CR, Carvalho LJCB, De Almeida ER, Gander ES (2006). A cDNA sequence coding for a glutamic acid-rich protein is differentially expressed in cassava storage roots. Protein Pept. Lett. 13(7): 653-657.

De Souza, CRB, Beezhold D, Carvalho LJCB (2008). Pt2L4 protein, an homologue to Hev b 5 from rubber tree, may not be responsible for the cross-reactions to cassava shown by people allergic to latex. Protein Pept. Lett. 15(9): 900-902.

Del Villar-Martinez AA, García-Saucedo PA, Carabez-Trejo A, Cruz-Hernández A, Paredes-Lópeza O (2005). Carotenogenic gene expression and ultrastructural changes during development in marigold. J. Plant Physiol. 162(9): 1046-1056.

Deruere J, Romer S, D`Harlingue A, Backhouse RA, Kuntz M, Camara B (1994). Fibril assembly and carotenoid over accumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell. 6(1): 119-133.

Ferreira SN, Clementino ANR (2010). Lesgilação de Acesso a Recursos Geneticos e Conhecimento Tradicionais Associados e Repartição de Benefícios. Brasilia, DF. EMBRAPA. Departamento de Pesquisa e Desenvolvimento. Pp: 334.

Galvao CES, Iwai LK, Andrade MEB, Kalil J, Morato FF (2003). Latex allergy and crossreactivity to manioc: report of 2 cases [abstract] . J. Allergy Clin. Immunol. 113 S61.

Gaspar A, Neto-Braga C, Pires G, Murta R, Morais-Almeida M, Rosado-Pinto J (2003). Anaphylactic reaction to manioc: cross-reactivity to latex. Allergy. 58(7): 683-684.

Guimaraes ML, Barros MSC (1971). Sobre a ocorrência de caroteno em variedades de mandioca amarela. Bol. Tec. Div. Tecnol. Agri. Aliment. 4:1-4.

Hadjeb N, Gounaris I, Price CA (1988). Chromoplast-specific proteins in Capsicum annuum. Plant Physiol. 88(1): 42.

Hagenimana V, Carey EE, Gichuki ST, Oyunga MA, Imungi JK (1999). Carotenoid content in fresh, dried and processed sweetpotato products. Ecology of Food and Nutrition. 37(5): 455-473.

Kimura M, Kobori CN, Rodriguez-Amaya DB, Nestel P (2007). Screening and HPLC methods for carotenoids in sweet potato, cassava and maize for plant breeding trials. Food Chemistry. 100(4): 1734-1746.

Kirk JT, Tiliney-Bassett RA (1978). Proplastids, etioplasts, amyloplasts, chromoplasts and other plastids. In The Plastids: Their chemistry, Structure, Growth and Inheritance 9EDS st Kirk and Tiliney-Bassett. Elsevier/North Holland Biomedical Press, Amsterdam, Holand. Pp: 227-239.

Lawrence SD, Cline K, Moore GA (1997). Chromoplast development in ripening tomato fruit: Identification of cDNAs for chromoplast-targeted proteins and characterization of a cDNA encoding a plastid-localized low-molecular-weight heat shock protein. Plant Mol. Biol. 33(3): 483-492.

Li L, Paolillo DJ, Parthasarathy MV, DiMuzio EM, Garvin DF (2001). A novel gene mutation that confers abnormal patterns of ß-carotene accumulation in cauliflower (Brassica olera- cea var. botrytis). Plant J. 26(1): 59-67.

Li L, Yuan H (2013). Chromoplast biogenesis and carotenoid accumulation. Arch. Biochem. Biophys. 539(2): 102-109.

Libal-Weksler Y, Vishnevetsky M, Ovadis M, Ithizaki H, Vainstein A (1995). Flower-specific carotenoid accumulation in chromoplasts: Molecular control of carotenoid-associated proteins. Acta Hortic. 420: 32-34.

Libal-Weksler Y, Vishnevetsky M, Ovadis M, Vainstein A (1997). Isolation and regulation of accumulation of a minor chromoplast-specific protein from cucumber corollas. Plant Physiol. 113(1): 59-63.

Lopez AB, Eck JV, Conlin BJ, Paolillo DJ, O'Neill J, Li L (2008). Effect of the cauliflower Or transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. J. Exp. Bot. 59(2): 213-223.

Mansfield MA, Key JL (1987). Synthesis of the low molecular weight heat shock proteins in plants. Plant Physiol. 84(4): 1007-1017.

Marinho HA, Xavier JJBN, Miranda RM, Castro JS (1996). Estudos sobre carotenóides com atividade de pro-vitamina "A" em cultivares de mandioca (Manihot esculenta Crantz) em ecossistema de terra firme de Manaus, Amazonas, Brasil. Acta Amazônica. 1(3): 127-136.

Markwell J, Bruce BD, Keegstra K (1992). Isolation of a carotenoid-containing sub-membrane particle from the chromoplast Envelope outer membrane of pea (Pisum sativum) J. Biol. Chem. 267(20): 13933.

Milicus JCG, Juarros LJ, De las Rivas J, Ibarrondo J, Gomez R (1991). Isolation of a yellow carotenoprotein from carrot. Phytochemistry. 30(5): 1535.

Ortega-Flores CI (1991). Carotenoides com atividade pro-vitamica A e teores de cianeto em diferentes cultivar de mandioca no Estado de São Paulo. MSc. Thesis. Universidade de São Paulo, Brazil. Pp: 77.

Parsell DA, Lindquist S (1993). The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins. Annu. Rev. Genet. 27: 437-496.

Pozueta-Romero J, Rafia F, Houlne G, Cheniclet C, Carde JP, Schantz ML, Schantz R (1997). A ubiquitous plant housekeeping gene, PAP, encodes a major protein component of bell pepper chromoplasts. Plant Physiol. 115(3): 1185-1194.

Rateaver B (1951). In: Anatomy and regeneration in the stem and root of Manihot utilíssima Pohl. Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan, USA.

Santos KS, Galvao CE, Gadermaier G, Resende VMF, Martins CO, Misumi DS, Yang AC, Ferreira F, Palma MS, Kalil J, Morato-Castro FF (2011). Allergic reactions to manioc (Manihot esculenta Crantz): Identification of novel allergens with potential involvement in latex-fruit syndrome. J. Allergy Clin. Immunol. 128(6): 1367-1369.

Shewry PR (2003). Tuber Storage root. Ann. Bot. 91(7): 755-769.

Silva FA, Espindola LS (2011). Access legislation on genetic resources patrimony and traditional knowledge. Rev. bras. farmacogn. 21(1): 1-2.

Smirra I, Halevy AH, Vainstein A (1993). Isolation and Characterization of a Chromoplast-Specific Carotenoid-Associated Protein Cucumis sativus Corollas. Plant Physiol. 102: 491-496.

Souza KS, Galvao CE, Ferreira R, Virginia M, Martins C, Vevjar E, Gadermaier G, Ferreira F, Kalil J, Morato-Castro FF (2011). Identification of new Brazilian allergens from manioc (Manihot esculenta). Clinical and Translational Allergy 1(Suppl): 07-08.

Souza PAS, Gomes E, Campos FAP (1998). Tissue distribution and deposition pattern of a cellulosic parenchyma-specific protein from cassava roots. Braz. Arch. Biol. Technol. 41(1): 1-9

Stupak M (2008). Improving protein content in cassava storage roots. Dissertation. ETH No. 17557. Pp: 95.

Stupak M, Vanderschuren H, Gruissem W, Zhang P (2006). Biotechnological approaches to cassava protein improvement. Trends Food Sci. Technol. 17(12): 634-641.

Vishnevetsky M, Ovadis M, Itzhaki H, Vainstein A (1997). CHRC encoding a chromoplast-specific carotenoid-associated protein, is an early gibberellic acid-responsive gene. J. Biol. Chem. 272(40): 24747-24750.

Vishnevetsky M, Ovadis M, Vainstein A (1999). Carotenoid sequestration in plants: the role of carotenoid- associated proteins. Trends Plant Sci. 4(6): 232-235.

Vishnevetsky M, Ovadis M, Zuker A, Vainstein A (1999). Molecular mechanisms underlying carotenogenesis in the chromoplast: multilevel regulation of carotenoid-associated genes. Plant J. 20(4): 423-431.

Wiltshire JJJ, Cobb AH (1996). A review of the physiology of potato tuber dormancy. Ann. Appl. Biol. 129(3): 553-569.

Wittenbach VA (1982). Effect of pod removal on leaf senescence in soybeans. Plant Physiol. 70(5): 1544-1548.

Yeoh HH, Truong VD (1996). Protein contents, amino acid compositions and nitrogen-to-protein conversion factors for cassava roots. J. Sci. Food Agric. 70(1): 51-54.

Zagalsky PF (1976). Carotenoid-protein complexes. Pure Appl. Chemistry. 47(2-3): 103.

Zhang P, Jaynes JM, Potrykus I, Gruissem W, Puonti-Kaerlas J (2003). Transfer and expression of an artificial storage protein (ASP1) gene in cassava (Manihot esculenta Crantz). Transgenic Res. 12(2): 243-250.

Zhou JR, Gugger ET, Erdman Jr JW (1994). Isolation and partical characterization of an 18 kDa carotenoid-protein complex from carrot roots. J. Agric. Food Chem. 42(11): 2386-2390.

Zhu B, Coleman GD (2001). The poplar bark storage protein gene (Bspa promoter is responsive to photoperiod and nitrogen in transgenic poplar and active in floral tissues, immature seeds and germinating seeds of transgenic tobacco. Plant Mol. Biol. 46(4): 383-394.