Identification of QTLs for carotene content in the genome of cassava (*Manihot esculenta* Crantz) and S1 population validation

Identificación de QTLs para carotenos en el genoma de yuca, (*Manihot esculenta* Crantz), y validación en poblaciones S1

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Abstract

The β-carotene content in cassava is important to supply vitamin deficiencies in regions where this is the main source of food. The families used for molecular analysis of carotenoid content were: GM708, GM734 and CM9816. We used Bulked Segregant Analysis (BSA) to evaluate 800 microsatellite markers. To determine the association with de color of the root parenchyma we used a correlation analysis and simple regression between phenotypic and genotypic data from each marker. Additionally, an analysis of QTLs with these families was made and its presence was validated in S1 progenies. We found that SRRY313, NS7171, SSRY251 were strongly associated with the high carotene content. The mapping analysis showed that five QTLs are controlling the expression of β-carotene content and three control the color of the root pulp, which was validated in populations S1. In general, knowledge of inheritance and action of the accumulation of β-carotene gene in cassava, can be used to efficiently guide improvement processes aimed at improving the nutritional quality of cassava.

Key words: Carotenes, *Manihot esculenta* Crantz, genetic mapping, QTLs, SSR.

Resumen

El contenido de β-caroteno en yuca (*Manihot esculenta* Crantz) es importante para suplir las deficiencias de vitamina A en regiones donde esta raíz es la principal fuente de alimento. Las familias de esta especie utilizadas para el análisis molecular del contenido de carotenos fueron: GM 708, GM 734 y CM 9816. Se utilizó el Análisis de Grupos Segregantes (Bulked Segregant Analysis, BSA por su sigla en inglés) para la evaluación de 800 marcadores microsatélites. Para determinar la asociación con el color del parénquima de la raíz se hizo un análisis de correlación y regresión simple entre los datos fenotípicos y los genotípicos de cada marcador. Se hizo, además, un análisis de QTLs con estas familias y se validó su presencia en descendencias S1. Se encontró que los marcadores SSRY313, NS717, SRRY251 están fuertemente asociados con los altos contenidos de carotenos. Los análisis de mapeo mostraron que existen cinco QTLs que controlan la expresión del contenido de β-caroteno y tres el color de pulpa de la raíz, lo cual fue validado en poblaciones S1. En general, el conocimiento de la herencia y la acción del gen de acumulación del β- caroteno en yuca puede ser usado para guiar eficientemente los procesos que buscan mejorar la calidad nutricional de la yuca.

Palabras clave: Carotenos, *Manihot esculenta* Crantz, mapeo genético, QTLs, SSR.

Introduction

Cassava, *Manihot esculenta* Crantz, is one of the highly consumed carbohydrate sources by the majority of the population in under development countries (Ceballos and De la Cruz, 2002). Vitamin A deficiency is an important problem for public health in under development countries since it causes preventable blindness at an estimate of 20,000 to 100,000 kids or teenagers (Tanumihardjo and Yang 2005).

Carotenoids synthesis is an important metabolic process to understand the molecular biology of carotenogenesis (Cunnigham and Gantt, 1998). The pathway starts with the formation of phytoene from the condensation of two molecules of trans GGPP (Geranyl-geranylpyrophosphate). The reaction is catalyzed by the phytoene synthase enzyme and the PSY gene suffers four desaturations to form lycopene (Fraser and Bramley, 2004).

Lycopene β-cyclase (LCY-β) catalyzes the formation of β-carotene and of a α-carotene ring from lycopene (Cunningham, 2002). the genes (PSY, PDS, ZDS, CRTISO) and enzymes involved in the biosynthesis of carotenoid pigments have been widely studied (Tian and Dellapenna 2004a; Tian *et al.*, 2004b; Park *et al.*, 2002; Tucker, 2003; Laule *et al.*, 2003).

At the International Center for Tropical Agriculture (CIAT) studies related with map construction and searching of QTLs associated with traits of interest of cassava have been developed (Jorge *et al.*, 2000; Ferguson *et al.*, 2012; Okogbenin and Fregene, 2002; 2003). Many of the characteristics of economic importance are governed by these loci of quantitative heredity, therefore, the objective of this study was to identify those genomic regions of the cassava map associated with carotene content and validate them in S1 populations.

Materials and methods

Molecular analysis

This work was done at the Cassava genetic lab at CIAT, Palmira. The families GM 708 (MBRA1A * MMAL66), GM 734 (MTAI2 *CM3750-7) and CM9816 (MCOL 2295 *SM980-4) from the Cassava Breeding Program were selected. For the molecular analysis were used 800 microsatellites (Mba *et al.*, 2001) that were analyzed using the Bulk Segregant Analysis (BSA) methodology described by Mba *et al.* (2001). DNA extraction was performed with the protocol by Dellaporta *et al.* (1983) and was quantified using a spectrophotometer (Shimadzu UV-VIS 160). Microsatellite amplification was carried on in a thermocycler MJ research PTC-100™ Programmable Thermal Controller with hot bonnet (MJ Research, Inc – USA), temperatures and PCR mix were done as described by Mba *et al.* (2001). For microsatellite detection and visualization a vertical electrophoresis in 4% polyacrylamide gels was done (McCouch *et al.*, 1997) using a sequencing machine Sequi-Gen GT Nucleic Acid Electrophoresis Inc U.S.A. Bio-Rad 2001.

For the simple regression analysis between the carotene content, root pulp color and number of alleles at the marker locus, it was used the command Single Point in the MapMarker software. The mapping analysis by intervals was done using the interval regression command and to visualize the results of the simple regression the multiplot command of Mapmaker was used.

QTLs analysis

To create a QTLs map and do the mapping by intervals the software Mapmaker/QTL was used, using as reference the cassava map (Fregene *et al.*, 1997) and the one developed for the carotene trait (Marín *et al.*, 2009). A QTL was found significant $\alpha < 0.05$ and explained a large part of the phenotypic

variation given by the R^2 value. For the QTLs mapping by intervals associated to traits such as carotene content and cassava root pulp color, it was used a LOD 3.0 in order to state a QTL, which was confirmed using a multiple QTL by interval mapping model (Mapmaker / QTL) and multiple regressions (Zeng, 1993) using 10,000 iterations.

QTLs validation in S1 families

The S1 families used (Table 1) to evaluate the polymorphic microsatellites associated with carotene content in cassava roots that were found in evaluations with segregant populations (Morillo *et al.*, 2011), were obtained from a population of over 50 full sibling families and a group of five selected families (Morillo *et al.*, 2012).

Analysis of total carotene frequencies in S1 families

Total carotene and β-carotene contents were analyzed using the estimates of mean, correlation, regression and standard deviation (Microsoft Excel, SAS (SAS, 2005)). Histograms of frequency and dispersion graphs were built. The association between total carotene content and the intensity in parenchyma color were analyzed by lineal regression.

Evaluation of polymorphic microsatellite markers in the evaluated S1 families

56 polymorphic microsatellite markers (SSRY) were used (Morillo *et al.*, 2011) to do the correlation analysis in order to determine the association degree between carotene content and the molecular marker. To determine the relative positions in the map and validate the QTLs found, the software Mapmaker and QTL-Cartographer were used.

Results

QTLs analysis

With the information of the genetic and molecular map of cassava (Fregene *et al.*, 1997) and the one for carotene QTLs (Marín *et al.*, 2009) the microsatellites associated with QTLs in the linkage groups formed were placed using the Single Marker Analysis, Interval Mapping Analysis (SIM) and Composite Interval Mapping (CIM). For the GM708 family, with the regression analysis between the evaluated microsatellites and the β–carotene content, correlation values between 23% (SSRY-226) and 44% (SSRY-313) and regression values between 0.05 and 0.19, were respectively obtained (Table 2).

For the GM734 family the β-carotene correlations varied between 0.27 (SSRY-66)

Table 1. S1 families to study the inheritance of the carotene content trait in cassava roots.

Parent	Content	S1 family	Genotypes (no.)
CM 9816-1 (4.48)	Intermediate	AM 689	71
CM 9816-2 (10.98)	High	AM 690	90
CM 9816-5 (1.70)	Low	AM 691	73
CM 9816-6 (6.86)	Intermediate	AM 692	48
GM 893-5 (9.17)	High	AM 710	29
GM 893-8 (6.80)	Intermediate	AM 712	57
GM 893-16 (6.94)	Intermediate	AM 718	38
GM 893-18 (2.62)	Low	AM 720	40
GM 708 - 20 (0.43)	Low	AM 697	38
GM 708 - 27 (0.46)	Low	AM 698	34
GM 708 - 47 (12.04)	High	AM 700	2
GM 708 - 63 (12.75)	High	AM 702	30
Total	–	–	**550**

Table 2. Association between SSRs markers and the β-carotene content in the GM708, GM734 and CM9816 families according to the results of the analysis of single marker (simple regression).

Family	Microsatellite	Correlation	Regression	Linkage group
GM708	SSRY-226	0.23	0.05	G
	NS-267	0.26	0.07	R
	SSRY-9	0.27	0.07	F
	SSRY-242	0.28	0.08	A
	SSRY-178	0.31	0.10	H
	SSRY-88	0.31	0.10	K
	NS-717	0.32	0.12	D
	SSRY-251	0.42	0.18	D
	SSRY-313	0.44	0.19	D
GM734	SSRY-66	0.27	0.08	
	SSRY-21	0.28	0.08	D
	SSRY-242	0.31	0.10	A
	SSRY-313	0.35	0.13	D
	NS-717	0.41	0.17	D
	SSRY-251	0.51	0.26	D
CM9816	SSRY-324	0.23	0.05	D
	SSRY-242	0.30	0.09	A
	SSRY-172	0.33	0.11	J
	SSRY-251	0.35	0.13	D
	SSRY-330	0.37	0.14	N/A
	NS-717	0.41	0.17	D
	SSRY-49	0.42	0.18	C
	SSRY-195	0.42	0.18	F
	NS-158	0.43	0.18	G
	SSRY-313	0.47	0.22	D

and 0.51 (SSRY-251) with regression values between 0.08 and 0.26 (Table 2). In the CM9816 family, the SSRY-313 located in the linkage group D, showed the highest values for correlation and regression. For the three families were identified five QTLs at the linkage group D, suggesting that possibly the major QTLs, that increases β-carotene content, are placed in this linkage group (Table 2).

The GM708 and CM9816 families presented QTLs at the linkage group G (Table 3), whereas the other six QTLs were unique between the studied families. With the regression analysis using the QGENE, mapdisto and WinQTLcart softwares were identified five linkage groups which showed markers associated with possible QTLs for total carotenes, they have a significant effect ($\alpha = 0.005$). Also, seven microsatellites located on the linkage group 1 of the genetic map for the carotene content trait, with a probability P ($\alpha < 0.001$) were identified, which explains of the observed phenotypic variation from 7.3% till 37.2% for the microsatellite markers that showed the best performance (Table 3).

In Figure 1 is observed that the markers with red color, among them NS-717, SSRY-9, SSRY-251 and SSRY-313, have a highly significant effect in the linkage group 1 with a probability $\alpha < 0.001$. In the linkage group 22 is located the SSRY-242 marker, which has shown consistency when the polymorphism has been evaluated in different populations of cassava with yellow roots (Marín *et al.*, 2009).

In the interval mapping was found that the SSRY-313, SSRY-251, SSRY66, SSRY-242, NS-717 and SSRY-9 markers explained between 20 and 40% of the phenotypic variation ($\alpha < 0.001$) (Table 3), with five possible QTLs, which are found at the intervals QCCT1 (SSRY60-SSRY66), QCCT2 (SSRY66-SSRY313), QCCT3 (SSRY313-NS109),

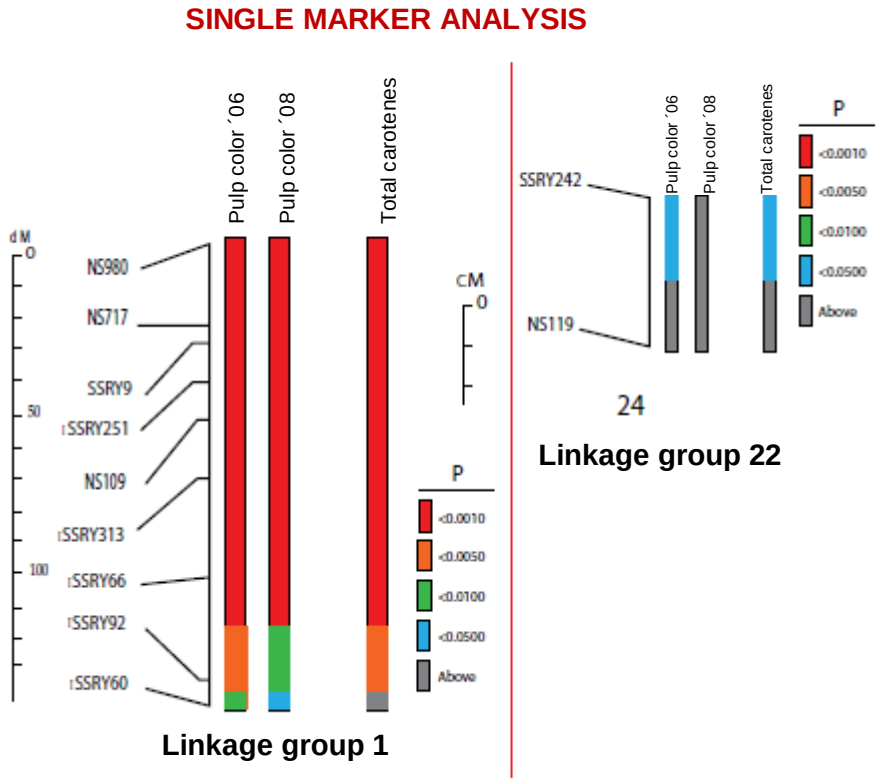

Figure 1. Microsatellite markers associated with possible QTLs for the characteristic of total carotene content (2008), root pulp color (2006-2008). Analysis done with QGENE. Figure 1a. = Linkage group 1. Figure 1b. = Linkage group 22 (Marín et al., 2009).

QCCT4 (NS109-SSRY251), QCCT5 (SSRY251-NS717) and on the linkage group 1 of the genetic map available for this characteristic (Figure 1). For the root pulp color trait, using a LOD score > 3.0 five possible QTLs were found that were located at the linkage group 1 between the intervals QCPR8-1 (SSRY60-SSRY66), QCPR8-2 (SSRY66-SSRY313), QCPR8-3 (SSRY313-NS109), QCPR8-4 (NS109-SSRY-251) and QCPR (SSRY251-NS717).

Table 3. SSR markers associated with total carotene (2008) by analysis of simple regression using QGENE.

Markers	L.G	P <	V.E (%)
SSRY313	1	0.0000	27.7
SSRY251	1	0.0000	32.0
NS109	1	0.0000	37.2
SSRY66	1	0.0000	16.4
NS717	1	0.0000	16.9
SSRY9	1	0.0000	12.7
NS980	1	0.0010	7.3
SSRY92	1	0.0031	6.5
SSRY60	1	0.0540	3.3
SSRY95	6	0.0359	3.4
SSRY223	11	0.0424	3.3
SSRY177	11	0.0275	4.4
SSRY272	18	0.0347	3.5
SSRY242	24	0.0109	4.7

L.G= Linkage group, P = Probability value; V.E = Explained variance. *Statistical significant at levels <0.01 by Duncan (P ≤ 0.05).

The compound interval mapping analysis show that three QTLs, located between the intervals QCCT1 (SSRY66-SSRY313), QCCT2 (SSRY313-NS109) and QCCT3 (NS109- SSRY251), control the total carotene content and explain 40% of the phenotypic variation. For root color pulp four QTLs were found at the intervals QCPR8-1 (SSRY66-SSRY313), QCPR8-2 (SSRY313- NS109), QCPR8-3 (NS109-SSRY251), QCPR8- 4 (SSRY251-NS717) and located at the linkage group 1.

QTLs validation in S1 families

In the analysis of frequency of total carotene content in the AM-691, AM-697, AM-698 and AM-720 families, several S1 genotypes from parents with low total carotene content show total carotene contents that are higher than the ones of their respective parents (Figure 2). In the AM-689, AM-692, AM-712 and AM-718 families more symmetrical distributions were observed, in which most of the individuals present intermediate carotene contents (Figure 3).

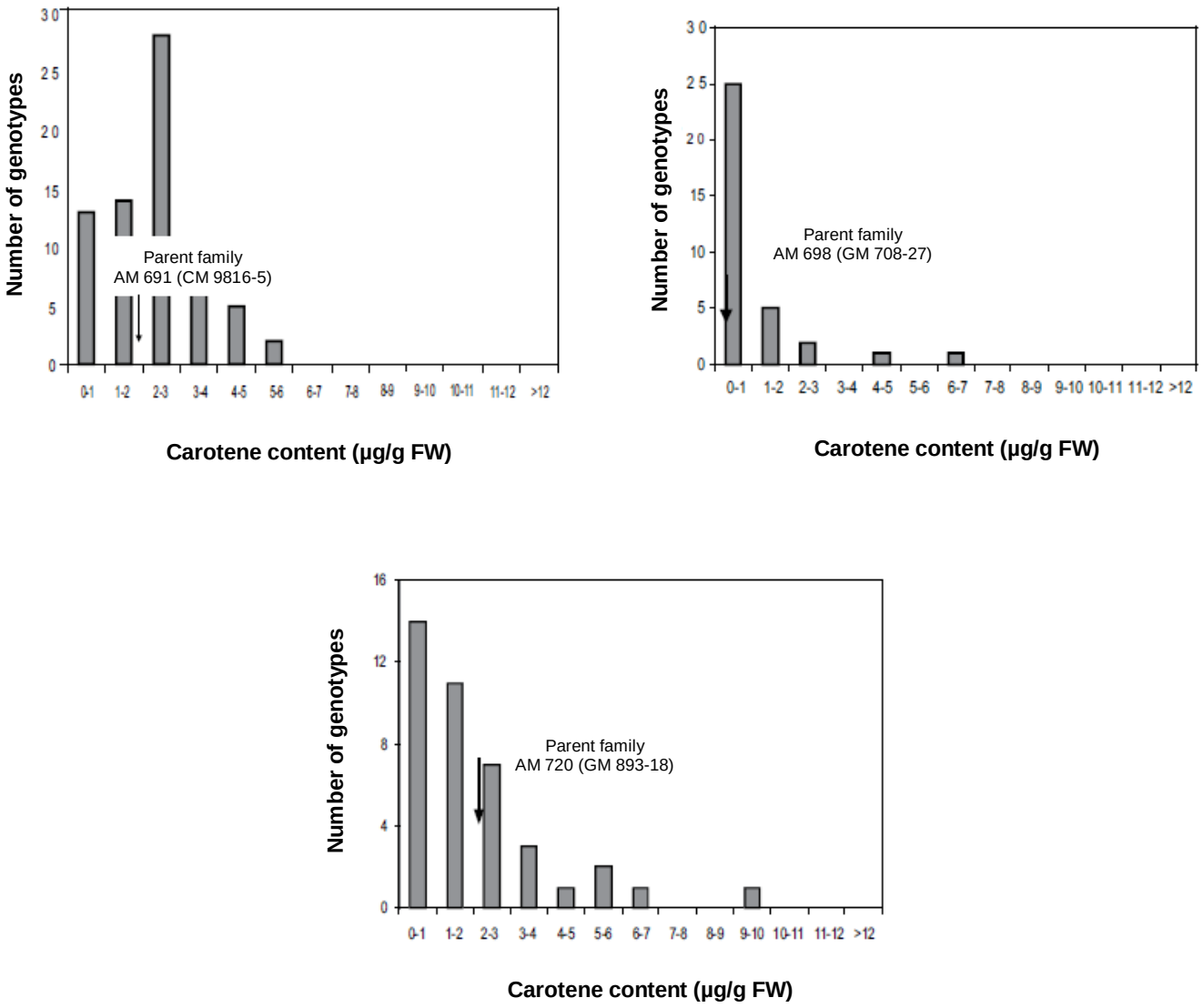

Figure 2. Analysis of frequency distribution of carotene content in S1 families: AM-691, AM-698 and AM-720.

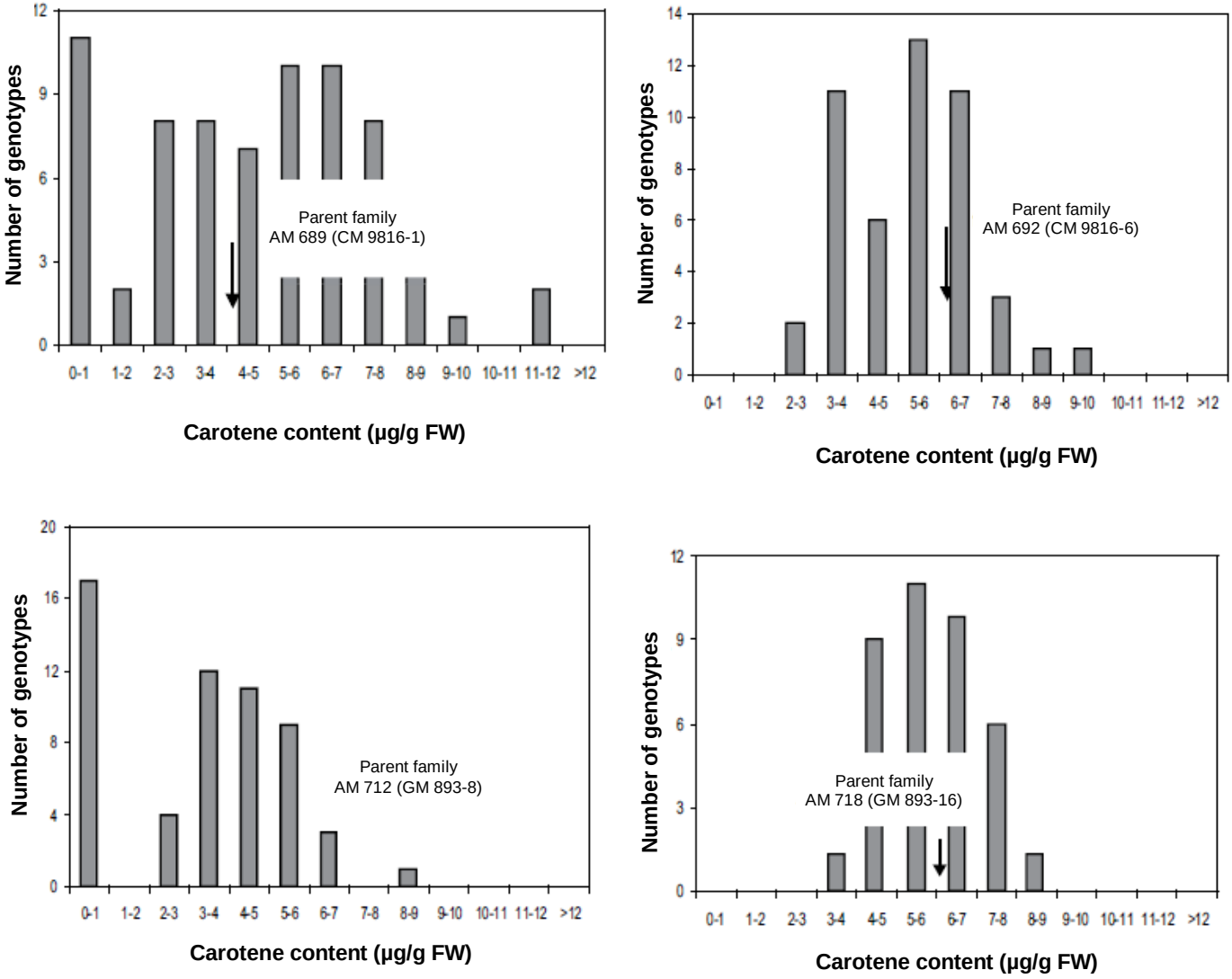

Figure 3. Analysis of frequency distribution of carotene content in S1 families: AM-689, AM-692 AM-712 and AM-718.

Microsatellite marker analysis in S1 families

The analysis of correlation and regression of the β-carotene content and the evaluated microsatellites proved the presence of Quantitative Trait Loci (QTLs), which allowed the identification of microsatellites that are associated to low, intermediate and high β-carotene contents. In the S1 families: AM-691, AM-697, AM-698 and AM-720 of parents with low β-carotene content, were found highly correlated microsatellites such as SSRY-49 , SSRY-88 and SSRY-178 located in the cassava map at the linkage groups F, K and H (Table 4). Regions in the genome associated to intermediate β-carotene contents were found in the S1 families: AM-689, AM692, AM-712 and AM718 with the microsatellites SSRY-172, SSRY-195 and SSRY-49, which are located at the linkage groups J, F and F, respectively (Table 5). The markers SSRY-313, SSRY-251, NS717, SSRY-21, SSRY-242, NS-158 have shown, not only in this study but, in evaluations of other populations that they correlate with high carotene contents (Table 6).

Table 4. Analysis of correlation of the microsatellites associated with low β-carotene content in S1 families (AM-691, AM697, AM-698 and AM720) and its relative location at the linkage groups in the cassava molecular-genetic map (Fregene *et al.*, 1997).

Microsatellite	Family				
	AM-691	AM-697	AM-698	AM-720	Linkage group
NS-717	0.32	–	–	0.36	D
NS-158	0.33	0.34	0.32	0.36	G
SSRY-195	0.33	–	–	–	F
SSRY-324	0.34	–	–	–	D
SSRY-21	0.36	–	–	0.36	D
SSRY-330	0.36	–	–	–	D
SSRY-226	–	0.37	0.33	0.34	G
SSRY-172	–	–	–	0.40	J
SSRY-49	0.45	0,44	0.49	–	F
SSRY-88	0.46	0,42	0.42	–	K
SSRY-178	0.48	0.45	0.44	0.36	H

Table 5. Analysis of correlation of microsatellite markers associated with intermediate β-carotene content in S1 families (AM-689, AM692, AM-712 and AM718) its relative location at the linkage groups in the cassava molecular-genetic map (Fregene *et al.*, 1997).

Microsatellite	Family				
	AM-689	AM-692	AM-712	AM-718	Linkage group
SSRY-195	0.34	0.46	0.35	0.33	F
SSRY-9	0.35	–	–	–	D
SSRY-31	–	–	0.35	–	F
SSRY-172	0.41	0.47	0.55	–	J
SSRY-330	0.44	0.37	–	–	D
NS-158	0.44	0.46	0.49	0.48	G
SSRY-226	–	–	0.45	0.49	G
SSRY-49	0.46	–	–	–	F
SSRY-324	0.47	0.34	–	–	D
SSRY-242	0.47	0.45	–	–	A
NS-717	0.48	0.40	0.54	0.51	D
SSRY-313	0.48	–	0.49	0.51	D
SSRY-251	0.48	–	–	–	D
SSRY-21	0.49	0.36	0.48	0.57	D

Discussion

In the GM708 and CM9816 families were found QTLs at the linkage group G, whereas other six were unique among the three evaluated families, thus, the regions in the genome that control the β-carotene content in the cassava root are common for different sources that increase the contents but, also they can be unique for the evaluated source. The gene action for all the QTLs mentioned

Table 6. Analysis of correlation of microsatellite markers associated with high β-carotene content in S1 families (AM-689, AM692, AM-712 and AM718) its relative location at the linkage groups in the cassava molecular-genetic map (Fregene *et al.*, 1997).

Microsatellite	Family			
	AM-690	AM-702	AM-710	Linkage group
SSRY-31	–	–	0.32	F
NS-717	0.34	0.47	–	D
SSRY-21	0.36	0.46	0.33	D
SSRY-313	0.38	–	–	D
SSRY-178	–	0.38	–	H
SSRY-324	0.38	–	–	D
NS-158	0.39	0.46	0.42	G
SSRY-49	0.39	0.37	–	F
SSRY-226	–	–	0.40	G
SSRY-251	0.42	0.48	–	D
SSRY-330	0.43	–	0.46	D
SSRY-195	0.44	–	0.48	F
SSRY-172	0.47	–	0.45	J
SSRY-242	0.48	0.47	0.47	A

above occurs by additive effects (Marín *et al.*, 2009).

There are yet unidentified factors associated with the carotene content but, the QTL studies have significantly contributed to the discovery of new factors that are modifying this metabolic pathway in different species (Pérez *et al.*, 2012). In tomato (*L. esculentum*), for instance, introgression lines presented a higher content of carotenoids than their respective parents. The same has been observed in cassava where the additive genetic effects of this trait are expressed and, QTLs for the analysis of resistance in the field to *Xanthomonas axonopodis* pv. *manihotis* (Jorge *et al.*, 2001), morphological traits (Boonchanawiwat *et al.*, 2011), for production (Okobenin *et al.*, 2002), quality (Akinbo e*t al.*, 2012; Marín *et al.*, 2009), disease resistance (Marín *et al.*, 2004; Lokko *et al.*, 2005), cyanide content (Whankaew *et al.*, 2011) and the values for phenotypical variance observed are in general lower than 40% due to the nature of these characteristics and the effect of the environment.

A second possible QTL for carotene was reported at the linkage group A of the cassava map (Fregene *et al.*, 1997) and explains 7% of the phenotypic variance observed. The results found in this study corroborated the ones obtained by Fregene *et al.* (1997) and Marín *et al.* (2009) which show that the genetic factors that govern the carotenoid content in cassava are found at the linkage group D.

The mapping by intervals showed that: SSRY-313, SSRY-251, SSRY-66, SSRY-242, NS-717 and SSRY-9 explained between 20 and 40% of the phenotypic variation. Marin (2002) found that the same markers explained between 13 and 37% of the phenotypic variance and that were localized at the linkage group 1 of the map for total carotene content (Marín *et al.*, 2009) correspond to the linkage group D of the cassava map (Fregene *et al.*,1997). Additionally, in several studies

show that they control both the total carotene content and the pulp color of the cassava roots, which can be clearly seen in Figure 1, where each color represents the probability of a microsatellite linked or not linked to a QTL.

In the last years, in yellow cassava has been identified root specific promoters (Zhang *et al.*, 2003, Arango *et al.*, 2010a; Beltrán *et al.*, 2010) and it has been proved the presence of three PSY genes (PSY1, PSY2 and PSY3) from which two (PSY1 and PSY2) are expressed in normal conditions and under salinity and drought stress (Arango *et al.*, 2010b). The analysis of quantitative expression have demonstrated that the expression of PSY2 in cassava roots is approximately ten times higher than the one of PSY1 (Arango *et al.*, 2010b). Welsch *et al.* (2010) demonstrated that an allelic variation of the PSY gene allows a better enzymatic activity and that a single nucleotide polymorphism (SNP) in the PSY2 gene, produced by a change on a non-conserved amino acid, markedly shows a formation and accumulation of carotenoids in cassava roots.

The genetic control of the traits for total carotene and β-carotene contents in cassava roots is complex, since there has been found from multiple alleles interacting with dominant genes and numerous genes with additive effects, till epistatic effects between the different identified QTLs (Marín *et al.*, 2009). The genetic mapping and QTLs analysis used to study the content of total carotene and the color of the pulp of cassava roots, are essential to understand the genetics behind this characteristic, the interaction genotype x environment, the heritability and the identification of the associated genome regions, using microsatellite markers in order to improve the nutritional quality of cassava.

Conclusions

- Three QTLs were found that control the content of carotenoids and four QTLs that control the color of cassava roots pulp, which explains a large part of the phenotypic variation observed and confirms that the inheritance of this trait is not simple.
- The presence of these QTLs was validated in S1 populations, which allowed the identification of markers associated with low, intermediate and high carotenoids content, which can be incorporated on a marker assisted selection program to identify the superior genotypes with good nutritional content.
- The genomic region associated with carotene content in cassava roots is located at the linkage group D of the genetic map, which should be studied in more detail in order to identify the responsible genes for synthesis, transport and accumulation of these pigments in cassava roots.

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