Molecular analysis of the expression of a crtB transgene and the endogenous psy2-y1 and psy2-y2 genes of cassava and their effect on root carotenoid content.

Citation:

Chavarriaga-Aguirre, Paul; Prías, Mónica; López, Danilo; Ortiz, Darwin; Toro-Perea, Nelson; Tohme, Joseph M.. 2017. Molecular analysis of the expression of a crtB transgene and the endogenous psy2-y1 and psy2-y2 genes of cassava and their effect on root carotenoid content. Transgenic Research 26(5): 639-651.

Publisher's DOI:
https://doi.org/10.1007/s11248-017-0037-y

Access through CIAT Research Online:
http://hdl.handle.net/10568/88045

Molecular analysis of the expression of a *crt*B transgene and the endogenous *psy*2-y_1 and *psy*2-y_2 genes of cassava and their effect on root carotenoid content.

Paul Chavarriaga-Aguirre, Mónica Prías, Danilo López, Darwin Ortiz, Nelson Toro-Perea, Joe Tohme.

P. Chavarriaga-Aguirre, M. Prías & J. Tohme: Agrobiodiversity Research Area, International Center for Tropical Agriculture–CIAT, AA 6713 Cali, Colombia. e-mail: p.chavarriaga@cgiar.org

D. López: Syngenta S.A., Colombia, Calle 64 N #5BN-146, Local 104C, Edificio Centroempresa, Cali, Colombia

D. Ortiz: Department of Food Science, Purdue University,West Lafayette, IN, USA

N. Toro-Perea: Department of Biology, Universidad del Valle, Cali, Colombia

Abstract. A conventional breeding program was established to transfer the bacterial phytoene synthase transgene–crtB– from a transgenic, white-rooted cassava to yellow-rooted cassava plants carrying the endogenous phytoene synthase alleles named psy2-y_1 and/or psy2-y_2. Combining endogenous phytoene synthase enzymes (PSYs) with CRTB in a single cassava plant would allow the molecular dissection of individual allele contributions to carotenoid synthesis and/or accumulation in cassava roots. The simultaneous expression of the crtB transgene and psy2-y_2 in individuals planted in the field coincided with higher total, HPLC-quantified carotenoid content in roots, although the variability among replications (plants) precluded the detection of statistically significant differences. Nevertheless, the highest total carotenoid content in roots within a family coincided with one individual of the F1 progeny carrying both psy2-y_2 and crtB genes. The results also indicated the presence of at least one more key gene–different from psy or crtB—which too is necessary for the synthesis and/or accumulation of Pro-Vitamin A carotenoids in cassava roots.

Keywords. Micronutrients; Root biofortification; Transgenic cassava; Pro-Vitamin A; β-carotene; Transgenic field testing; Carotenoids

Introduction.

Cassava is considered the second source of starch globally, after maize (FAO 2013), but it is a poor source of micronutrients like carotenoids and proteins, especially white roots that are the most consumed fresh and/or for starch production. Regarding carotenoid synthesis, cassava has three Phytoene Synthase (psy) genes (Arango et al. 2010) of which two, psy1 and psy2, synthesize the first committed step of the carotenoid synthesis pathway, Phytoene (Fig. 1; Welsch et al. 2010). Gene psy2 has two alleles named psy2-y1 and psy2-y2; the former is homozygous in white cassava roots while yellow roots always carry at least one psy2-y2 allele. Several researchers have used genetic transformation to introduce genes to biofortify cassava, that is, to increase the content of macro- and micro-nutrients in roots (reviewed by Chavarriaga et al. 2016). Expressing genes like crtB, the bacterial version of plants' phytoene synthases (PSYs), in white cassava roots of cv. 60444 increased total carotenoid content (TCC) up to[30 times, raising the levels of bcarotene (BC) up to almost 7 lg/g (dry weight; DW) making former white roots appear orange (Welsch et al. 2010; Failla et al. 2012). These transgenic cassava plants carried two copies of the exogenous crtB transgene together with unfavorable alleles for the synthesis of phytoene, the phytoene synthase (psy) endogenous cassava gene psy2-y1 in homozygous state (Welsch et al. 2010). Even though, the crtB transgene alone, or together with phytoene desaturase (crtI) and lycopene b-cyclase (crtY; Bonilla 2010), or with the upstream gene deoxy-xylulose 5-phosphate synthase (dxs; Failla et al. 2012) helped the white-rooted cassava cultivar 60444 produce and accumulate carotenoids in roots. Although the increases in TCC were substantial in the best transgenic lines, they were not enough to render transgenic cassava as a pro- Vitamin A carotenoids (PVAC) source. Besides, they

were in a cultivar of limited use in, i.e., Africa, where biofortification efforts through biotechnology have focused (Sayre et al. 2011). Furthermore, for plants with only the crtB transgene, TCC levels apparently fell as the roots matured (Chavarriaga 2013). Also, cassava cultivars with white roots more efficiently catabolize b-carotene, producing derived molecules such as b-ionone (Maldonado, unpublished), which reduce the accumulation of the key carotenoids important for human health.

In breeding for increase carotenoids in roots of cassava, the variability contributed by transgenesis has not been taken into account. The reasons for not including genetically modified organisms are manifold, e.g., acceptance, delivery costs and/or intellectual property, to name a few. However, on the one hand, high-potential genes from transgenic parents are transferred to progeny for the production/accumulation of carotenoids, to genotypes better suited for conventional breeding. On the other hand, bottlenecks emerge along the carotenoid biosynthetic pathway that expose the importance of genes involved in it, or new and unexpected allele combinations occur, resulting in carotenoid content relevant to human health. Therefore, we decided to test the hypothesis that by bringing together the crtB transgene and psy2-y_2 in a single individual it would be possible to discover synergisms between crtB and other beneficial alleles contributed by yellow-rooted parents for the synthesis/accumulation of carotenoids in cassava roots. Thus, a breeding program was so established to produce progeny carrying both phytoene synthases and analyze their field performance in terms of gene expression and HPLC quantified root carotenoid content. The findings of this breeding effort are presented further down in this publication.

Materials and methods

Crosses. Three male parents, GM905-21, GM905-57 and GM905-60 were selected from CIAT's breeding program, with high fresh-weight TCC (ranging from 6.65 a–20.64 mg/g), for crossing with the transgenic female parent pCasPhyt-12, derived from the white genotype 60444. All the male parents were derived from the cross between yellow and rooted landraces MCR 87 9 MPER 297, obtained from CIAT's germplasm bank. Only one-way crosses were done by pollinating the transgenic mother with pollen from the non-transgenic male parent. Every day for one week, the pollen donors were inspected for bagging (i.e., covering with nylon mesh bags) of the male flowers approaching maturity and releasing pollen. The same was done for the flowers of female progenitor to prevent uncontrolled pollination by insects or other agents. Early in the morning on the day of the crossing, mature male flowers would be collected and transported in Petri dishes lined with filter paper moistened with water to the plot where the female receptor for the pollination had been planted. A single male flower would fertilize several female flowers, with the identity of the crosses recorded. The fertilization process would be repeated for three weeks until there was a minimum of 40 crosses for each pair of parents.

Once the crosses were established, the fruits grew and were left to mature and dry on the plant (approximately three months). Each fruit was duly labeled and bagged in nylon-mesh bags to prevent seed dispersal. Once the seeds were obtained, they were thereafter germinated in vitro to multiply theprogeny and maintain copies in our in vitro, transgenic gene bank. Seeds germinated for approximately one month at 28^{o}C with a 12 h photo-period in growth rooms.

Establishment in the greenhouse and in the field. The seedlings obtained in vitro were propagated indefinitely in the same culture medium every three to four months. All the progeny were transferred to greenhouses and then to the field where they were planted with at least two replicates for each individual,

91 together with the parents (Table 1 in results section). Recommended agronomic practices for cassava
92 were followed, i.e., fertilization and weed and pest control (Calle 2002; Cadavid 2002), and removal of
93 flower buds to prevent pollen dispersion, until the harvest when the plants were 12–13 months of age.
94 Once the plants reached 12–13 months of age, they were harvested in accordance with the following
95 protocol:

96 1. Selection of harvested individuals was based on the presence or absence of the hpt and crtB genes,
97 detected using conventional PCR (Supplementary Material; Chavarriaga 2013), and by plant vigor,
98 indicated by plant height generally exceeding 1.5 m. Selection was not based on root color. At least two
99 biological replicates were harvested for each selected individual.

100 2. For each plant, all the roots that were in good condition were gathered, i.e., roots were confirmed free
101 of any disease symptoms (i.e., rot, or cassava frogskin disease), and generally of good market value (i.e.,
102 400–800 g, 20–30 cm long and 5–10 cm in diameter; Ortiz et al. 2011). Once gathered, the roots were
103 packed in craft paper bags to protect them from light while the harvesting for the day continued, after
104 which the roots were taken to the laboratory for processing.

105 3. Once in the laboratory, all the roots of each plant were quickly peeled, washed, dried and machine
106 ground. Next, the ground roots of each plant were combined and samples taken to determine dry matter
107 and carotenoid content using HPLC (see below). Extraction and quantification of carotenoids were done
108 between two and six hours after harvest. Dry matter was determined by taking a 20–30 g sample of fresh
109 ground roots, evaporating the water in the sample for 48 h at 60^{o}C, and was expressed as a percentage of
110 the final weight (after drying) relative to the initial fresh weight.

111

112 ***Detection of the hptII and crtB transgenes.*** The detection of transgenes inserted in the genome of the
113 progeny of selected individuals was done using the non-radioactive Southern blot method, following the

instructions of the DIG kit (Roche, Cat# 11636090910), and by conventional PCR (Supplementary Material; Chavarriaga 2013).

Carotenoids extraction. All sample preparations and extractions were performed in a laboratory equipped with lights with UV filters to minimize photoisomerization reaction, following the procedure described by Ortiz et al. 2011. Briefly, cassava root tissue (5.0 g) were homogenized for 1 min with 10 ml acetone:petroleum ether (1:1) using a Polytron homogenizer (IKA T18, Staufen, Germany), followed by centrifugation (Eppendorf 5804R, Hamburg, Germany), at 3000 rpm, for 10 min, at 4^{o}C. The liquid phase was collected and extraction of the residue, followed by centrifugation, was repeated until it turned colourless (usually 3 times). The extracts were then combined with 10 ml of 0.1 M NaCl solution and the petroleum ether phase containing the carotenoids separated from the lower aqueous-acetone phase. Extracts were dried under a stream of nitrogen, resolubilized in methanol/methyl tert-butyl ether (1:1) HPLC-grade and filtered through a 0.22 lm PTFE syringe filter and then analyzed by HPLC.

Carotenoid quantification. Carotenoids were separated using a YMC C30 3 lm 2.0 mm 9 150 mm column, with a YMC carotenoid guard column (2.0 9 23 mm) in a HPLC system (Agilent Technologies 1200 series, Waldbronn, Germany) coupled with diode array detector. Carotenoids were identified by comparing spectral information with the literature (Britton 2004) and retention times with authentic all-trans carotenoid standards. Quantification was based on seven-point calibration curves prepared spectrophotometrically with authentic all trans standards. All the isomers of b-carotene were quantified with the standard curve for b-carotene, as were also violaxanthin, antheraxanthin and unknown carotenoids. α-carotene and lutein were quantified with their respective standard curves. Phytoene and phytofluene were quantified with the phytoene curve. Data were expressed as lg/g dry weight (lg/g DW).

***DNA sequencing for detection of SNPs in parents and progeny*.** DNA samples from the parents and progeny were sequenced using primers specific for the psy2-y2 gene to detect the SNP which is associated with increased carotenoid content (Welsch et al. 2010; Chavarriaga 2013; Suppplementary Material). The sequencing was conducted by the Macrogen Company.

qPCR to quantify the expression of PSY1, PSY2, crtB and 18S genes. RNA was extracted using Trizol Invitrogen method, in accordance with manufacturer's instructions. To purify the RNA, equal amounts of RNA were passed through the DNAse-I column to remove DNA (Qiagen RNeasy mini kit). The cDNA was synthesized with TaqMan (Applied Biosystems) reverse transcription reagents, following the supplier's instructions. The equipment used for qPCR was a fluorometric thermocycler, Mx3005p, from Stratagene (Agilent Technologies) and the DynamoTM SYBR Green qPCR kit from Finnzymes (Finnzymes Oy, Espoo, Finland). To calculate the amplification efficiency of each gene (psy2-y1, psy2-y2, crtB and 18S), amplification curves from a randomly selected sample were established, with serial dilutions of cDNA of 0.5X for each. A linear Ct (cycle threshold) regression was done (see below) against the fluorescence in order to derive the measure of negative slope that would make it possible to calculate the amplification efficiency thus: Amplification efficiency (E) = $10^{(-1/\text{slope})}$.

The amplification cycles were one melting cycle of 10 min at 95°C, followed by 30 amplification cycles of 30 s at 95°C each, 45 s at the annealing temperature for each primer pair, and a final extension cycle of 45 s at 72°C. Dissociation curves (Tm; Temperature of Melting) were determined for each amplified gene, including 18S, to verify the purity (absence of contaminants) amplified by comparison with a standard,

159 which, in this case, was the same gene expressed in the transgenic plant. This curve was established by
160 defining a temperature gradient of 65–95°C in the thermal cycler.

161

162 The relative expression level R was quantified using the equation formulated by Livak and Schmittgen
163 (2001) and Pfafll (2001), using the Ct values for each sample normalized to the Ct values of the reference
164 gene, 18S, and calibrated against the line of least expression. The equation

165 $R = [(E_{goi})^{(\Delta Ct\text{-}goi)}] \text{ X } 1/[(E_{gor})^{(\Delta Ct\text{-}gor)}]$, quantifies the expression as a function of: Egoi, the amplification
166 efficiency of the gene of interest (psy2-y_1, psy2-y_2 or crtB); Δ*Ctgoi*, the difference in Ct average between
167 th eamplified gene of interest in the control and the sample; Egor, the amplification efficiency of the
168 reference gene, 18S; ΔCt*gor*, the difference between the Ct averages of the amplified reference gene,
169 18S, in the control, and in the sample.

170

171 **Results.**

172 ***F1 seed germination and establishment in greenhouse and field***. Once the seeds were collected from all
173 the crosses, they were next germinated in vitro, followed by planting them in the greenhouse, taking leaf
174 samples, and confirming the presence of the crtB and hpt genes by PCR and Southern blot hybridization.
175 An unexpected characteristic in some of the seedlings germinated in vitro was yellow cotyledons (not
176 shown). The progeny with yellow cotyledons consistently showed the presence of crtB gene detected
177 using PCR and Southern hybridization. Plants were finally established in the greenhouse and field.

178

179 ***Estimated carotenoid content using the HPLC method***. Based on the ability of the crtB transgene to
180 increase TCC in white cassava (Welsch et al. 2010), we decided to challenge the hypothesis that this effect

would be magnified in an individual whose genetic background derived from yellow-rooted parents that were providing beneficial alleles for the production/accumulation of carotenoids. To this end, crosses were made between the transgenic line pCasPhyt-12, which flowered prolifically, and three yellow fathers. The results of the TCC analysis, and the composition of carotenoids in the progeny, estimated using the HPLC, are shown in Table 1.

Analysis of expression of phytoene synthases PSY1 and PSY2 in family 1221. The individual carrying the crtB gene with the highest TCC was in the 1221 family. It was therefore decided to analyze the expression of three phytoene synthase genes—PSY1, PSY2 and crtB in all members of this family. Thus, Fig. 2 shows that both the transgenic mother, pCasPhyt-12, and its progeny, 1221–55, expressed the crtB transgene at similar levels, as would be expected for carriers of the transgene. However, taking the same dry-matter content (33%) from both individuals, only 1221–55 showed an increase in TCC (Table 1), i.e., 33.5 lg/g in DW. The TCC of 1221–55 was 9.46 units more than that in the yellow father GM905-21 (24.09 lg/g DW). This increase in TCC is noticeable considering that with each annual selection cycle through conventional breeding, TCC increases between 1 and 2 units/cycle (DW; Ceballos et al. 2013). These results suggested that expression of the crtB transgene coincides with the highest content of carotenoids in the line 1221–55 and also remained constant with sexual transmission.

Discussion

Crossing. The male parents were selected for crossing based on tuber color which was a good indicator of carotenoid content (Chavez et al. 2005), and on the basis of different ability to produce offspring. As will be shown further below, the progeny showed different ability to produce and/or accumulate carotenoids

in the tuber (Table 1), most likely the product of the aggregate different abilities of each parent to transmit their genes to their progeny for additive effect and/or dominance (Ceballos et al. 2004), which, in turn, influenced the synthesis and/or accumulation of carotenoids.

Carotenoid content. Just as with TCC analysis conducted previously, for some individuals, the variance was considerable, with standard deviations exceeding 30% from the mean, e.g., for the TCC in genotypes GM905-57 and 1221–84, or the trans-b-carotene (trans-BC) for the individual 1257-7. This variance is normal in cassava when the carotenoid content is estimated using the HPLC method: indeed, according to Ortiz et al. (2011), this positive correlation, although moderate (R2 = 0.3794), with dry-matter content, is a characteristic that varies with the environment. The TCC in the cassava root, when expressed in fresh weight (FW), increases between 8 and 10 months (after planting), and then decreases a little at 12 months. When TCC is expressed to dry-matter content (dry weight), it steadily increases at 8–12 months after planting (Ortiz et al. 2011). Despite what has been posited by Ortiz et al. (2011), where the relationship between dry matter and TCC in 26 genotypes was analyzed, the latest evidence, after analyzing more than 4000 cassava genotypes, indicates that there is no correlation between dry-matter content and the level of carotenoids in the roots, at least for market varieties whose dry-matter content ranged between 25 and 45% (Ceballos et al. 2013; Belalcazar et al. 2016).

Of the individuals carrying the crtB gene confirmed by Southern blot (Fig. 3), in terms of TCC and the content of individual carotenoids, 1221–55 surpassed all the other individuals, including its own parent. Its roots contained more antheraxanthin, lutein, 13-cis-bcarotene and a-carotene–amongst other carotenoids– than the yellow parent, GM905-21. This observation suggested that carrying and expressing the crtB transgene substantially increases TCC, which could be explained by the possible synergistic effect

of the presence and expression of the transgene, added to the combination of favorable alleles contributed by both parents, for the synthesis and/or accumulation of carotenoid in the root. However, as shown in Table 1, the positive effect of the crtB gene depended on genotype, since not all individuals carrying the crtB gene showed increased TCC. The positive effect was comparable, within proportions, to that reported by Naqvi et al. (2011) in maize, where the transfer of three transgenes (psy1, crtI, lcyB) in the carotenoid biosynthetic pathway to yellow maize varieties through conventional breeding resulted in exceptional increases in carotenoids in the grain, and in an altered accumulation pattern in at least one individual in the progeny. The authors demonstrated that, on the one hand, genes were transferred with high potential for the production of carotenoids, to genotypes more able to store carotenoids in the grain. On the other hand, bottlenecks were found in the carotenoid biosynthetic pathway, and new and unexpected genotypes were obtained, with carotenoid content relevant for human health: for example, genotypes with more lutein and zeaxanthin. The hypothesis for the case of maize was that the observed effect was due to the synergistic action of the transgenes and the endogenous genes. Thus, in the case of cassava, transfer and overexpression of the crtB gene in the progeny might have affected the balance of the pathway, for example, the accumulation of lutein (3.5 times more than the father, see Table 1), or the duplication of 13-cis and 9-cis-bcarotene, through possible synergies with products of the endogenous genes. These changes were ultimately reflected in an overall increase of about three units in fresh weight in the TCC. The synergistic interactions between the enzymes of the carotenoid synthesis pathway should be common given its location in complex multiproteins in the chloroplast membrane. For example, recent models of the interaction of enzymes in the chloroplast to synthesize carotenoids suggest that PSY could be free in the stroma and/or associated with two other enzymes (GGDS and IDI; Ruiz-Sola and Rodriguez-Concepcion 2012). This mechanism requires, at a minimum, physical proximity of the enzymes to efficiently channel the substrate necessary for the synthesis of more complex compounds such as phytoene. Furthermore, phytoene desaturase is located in a large complex of membrane proteins (Lopez

et al. 2008) but how it interacts with other enzymes in the same complex to desaturate phytoene has not yet been described. Recently, the synergistic interaction between P450 hydroxylases (CYP97A and CYP97C) that hydroxylate rings b and e, respectively, from a-carotene to form lutein has been described (Quinlan et al. 2012). This work demonstrated that to increase lutein production, coexpression, and physical and synergistic interaction between the two enzymes, are required. Although in this research there was no physical evidence of the interaction of endogenous PSY enzymes and the transgenic CRTB, it is reasonable to suggest that these two enzymes might have acted synergistically in channeling the GGPP substrate necessary in order to increase the production of phytoene. In fact, an examination of Table 1 shows that the individual 1221–55 produced more phytoene/phytofluene than its full siblings. This may be due to the presence and expression of the crtB transgene in a heterozygous genetic background containing one favorable psy2-y2 allele for phytoene synthesis (see below). In other words, hypothetically, the transcriptional regulation of CRTB, PSY2-Y2 and PSY2-Y1 enzymes may be different in cassava. The former comes from a transgene with its own root-specific, strong promoter (Beltra´n et al. 2010), while the latter two are subjected to the intrinsic regulatory mechanism of the psy genes in cassava roots (Arango et al. 2010). Then, the overproduction of phytoene by the three enzymes possibly pushed the pathway to higher carotenoid production resulting in higher TCC. In the 1221 family, the individual carrying the crtB gene was found to have the highest average in the whole population for four of the ten carotenoids analyzed. The individual 1221–55 also had more carotenoids and more TCC than its yellow parent, except for phytoene, phytofluene and trans-b-carotene. The latter carotenoid was highest in the individual 1260-1 (4.37 ± 0.66 lg/g FW), which was not a crtB gene carrier but was a descendant of the parent with the highest content of trans-b-carotene (GM905-60; 5.98 ± 0.51 lg/g FW). This suggested that other paternal alleles were highly instrumental in increasing trans-b-carotene in this individual.

Expression of crtB and PSY genes and its relationship with carotenoid content in roots. Figure 2 reveals an interesting aspect of the expression in roots of two of the three psy genes existing in cassava: usually, the psy1 gene was the one with the highest transcriptional activity. The work by Arango et al. (2010) showed that for in vitro cassava plants, subjected to stress from lack of water simulated using polyethylene glycol (PEG 6000), there was increased transcription in both psy1 and psy2 genes, with transcription stronger in psy1. According to Fig. 2, for most 1221 family members sown in the field and harvested at 12–13 months of age, psy1 transcription was higher, suggesting that they were under drought stress. To verify whether this was the case, rainfall data around harvest-time were reviewed. The accumulated rainfall between the months of harvest was 30 mm3/mm2; it was during this interval that the roots of parents and progeny were harvested. This would indicate that at the time of harvest and molecular and biochemical analysis, the roots were under water deficiency stress. Comparatively, during a rainy period at the CIAT–Palmira experimental station, the accumulated rainfall exceeded 250 mm3/mm2 and could be in an excess of 300 mm3/mm2. This dry period of 21 days probably activated the transcription of both psy genes, but transcription was more noticeable for psy1. The relative expression of the psy3 gene was not measured in this work, because this gene does not seem to contribute to the formation of carotenoids in tubers, leaves or flowers; neither does it seems to respond to stresses such as drought and salinity (Arango et al. 2010), despite being the most homologous to the psy3 genes in rice and maize, which respond to these stresses (Li et al. 2008; Welsch et al. 2008).

The increase in psy1 gene transcription does not necessarily lead to greater TCC in the root, as shown by the individual 1221–7 (TCC = 6.38 lg/g DW), despite 1221–7 being a carrier of the beneficial allele, psy2-y2, and expressing it at a higher level than its yellow father (Fig. 2). Is there another level of variation and/or control of TCC that we do not know? Most likely, yes, and it is important to decipher it to learn more about the genetic control of the synthesis and accumulation of carotenoids in cassava. However, it

297 should be mentioned again that the amount of carotenoids accumulated in the root does not exclusively
298 depend on the transcription of psy genes. The type–or activity–of the PSY enzyme plays an important role
299 (Welsch et al. 2010). For example, the father GM905-21 has a lower relative level of psy2-y2 transcription
300 compared to its progeny 1221–4, –6 and –7. But the father has higher phytoene production and TCC. This
301 phenomenon could be explained by the fact that the father is homozygous for the psy2-y2 allele, which
302 would double the PSY2-Y2 enzyme content, thus conferring to the father the ability to synthesize more
303 phytoene (Table 1). However, the individuals GM 905-57 and GM905-60 –both siblings of GM905-21– are
304 heterozygous for PSY2-Y2 enzyme, and have higher TCC than the latter. This indicates that increase in
305 phytoene does not necessarily translate into an increase of TCC. On the other hand, the individual 1221–
306 55, although heterozygous psy2-y1/psy2-y2, and expressing both psy genes, also carries and expresses
307 the crtB gene, which, apparently, would confer an advantage to produce more phytoene than its siblings,
308 and more carotenoids than any of the individuals analyzed in this study. In terms of messenger quantity,
309 that of crtB is what makes the difference between 1221–55 and its siblings, because the level of psy2-y2
310 expression is very similar in all of them, and identical to that of the yellow father, GM905-21. To support
311 the idea that psy genes are not solely responsible for the TCC in cassava, the work by Morillo (2012) on
312 heredity of TCC, and that of Ovalle et al. (2016) on SNP-based mapping analysis indicates that, based on
313 TCC trait segregation, the synthesis/accumulation of carotenoids in cassava roots involves at least two
314 major genes–some recessive– and that there are also minor genes involved that affect TCC. A similar
315 situation exists with the heredity, in carrots, of the content of phytoene, lycopene, a-, b- and f-carotene,
316 and of total carotenoids (Santos and Philipp 2006). After establishing a segregating population from a
317 cross between orange- or white-rooted parents, it was determined that at least four genes were involved
318 in a-carotene content, of which one or two were involved in total carotene and lycopene content, and
319 one for the content of the other carotenoids analyzed. As can be inferred, TCC heredity, and heredity of
320 the content of individual carotenoids, is complex for accumulation in roots. What would have been

expected in a segregating population such as the cassava one analyzed in this study, would have been to obtain individuals with multiple combinations of the multiple alleles involved. Not all individuals carry as many favorable alleles for, for example, increase in TCC. If we consider the crtB gene as ‘‘another favorable allele’’, which, when present, increases TCC, the line 1221–55 would be exceptional. This is because the line would have a greater number of beneficial alleles, which, added to the action of the crtB gene, would favor the increase of TCC.

The presence of the psy2-y2 allele, concurrently with the crtB gene, influence the phytoene content and TCC. As mentioned above, it seemed that the presence of the crtB gene positively influenced TCC, and the content of specific carotenoids in some individuals. With the evidence accumulated from this investigation, it cannot, however, be assumed that this positive effect was due solely to the action of crtB gene. It should also be considered that TCC increase was also influenced by the combined action of the crtB gene and other favorable alleles of the carotenoid pathway contributed by the yellow parents. For example, the single nucleotide polymorphism (SNP) of the PSY2 gene, played an important role. In the case of the 1221 family, the father was homozygous for the psy2-y2 allele, while the mother was for the psy2-y1 allele. Thus, all the progeny were heterozygous for PSY2 (psy2-y2/psy2-y1), and hemizygous for crtB. The psy2-y2 allele expresses a phytoene synthase more effective for producing phytoene and for boosting the carotene synthesis pathway, in such a way that psy2-y2/psy2-y2 homozygous individuals such as GM905-21, reached higher levels of phytoene production, the heterozygous psy2-y2/psy2-y1 were at intermediate level (e.g., 1221–6 and 1221–84), while the heterozygous psy2- y2/psy2-y1, crtB gene carriers, outperformed the intermediate group in phytoene production (e.g., 1221–55 and 1257–10). In summary, for the production of phytoene, the homozygous psy2-y2/psy2-y2 was more effective than the heterozygous psy2-y2/psy2- y1 + crtB (crtB carrier), and the latter more effective than the heterozygous psy2-y2/psy2-y1. It should be mentioned that increased phytoene production does not

always translate into higher TCC. In this case the individual 1221–55 was exceptional, because it had the highest TCC (fresh weight), probably due to a combination of favorable alleles for phytoene synthesis (psy2-y2 and crtB) with other pathway alleles that favor the accumulation of carotenoids. This did not materialize for 1257–10, although also a carrier of psy2-y2 and crtB, and which outperformed its yellow parent, GM905-57, in the production of phytoene (both with high standard deviation), but not in TCC. This supports the argument that the effect of overexpressing a Phytoene Synthase such as CRTB, driven by a strong, root-specific cassava promoter (Beltrán et al. 2010), would also depend on the combination of alleles in other pathway genes present in each individual (it would be dependent on the genotype).

The 1257 family had an individual (1257–13) with the highest amount of phytoene registered using the HPLC method. This is probably because the individual did not inherit all the beneficial alleles to desaturate/isomerize phytoene. Indeed, its TCC was the same as its father, GM905-57, despite having triple its father's phytoene content, and despite being genetically identical in terms of alleles for the PSY2 gene (both heterozygous G/T). This favors the argument that, after crossing, the inheritance pathway for 1257–13 must have had deficiencies in the conversion of phytoene and other carotenoids, reconfirming that the level and type of carotenoid in cassava depends on the genotype, whose alleles are then transmitted to the progeny. This result is predictable given the heterozygosity of cassava, and that both parents are presumed to be heterozygous for an unknown number of alleles of genes involved in the synthesis and accumulation of carotenoids. Unfortunately, for most of the genes involved in the synthesis of carotenoids in cassava, the sequence of the alleles with positive effects (for increased carotene) is unknown, so it is impossible to discern the contributions of each allele towards TCC. This becomes the most critical perspective arising from this research to increase TCC in the roots of cassava.

In general terms with regard to TCC, phytoene, and its relationship with the Phytoene Synthases (CRTB and PSY), the ideal situation for cassava to become a rich source of pro-vitamin A, and of other carotenoids important for human and animal health, is that cassava accumulates carotenoids (high TCC), and that the phytoene/TCC ratio be low. This research has shown that this is possible, and individuals such as the GM905-60 parent, or 1221–55, which carries and expresses crtB, reach similar levels of TCC, with good production and low accumulation of phytoene. The question is if, between these two genotypes, one had to be selected for commercialization, which one would it be? The answer is obvious: GM905-60 because it would not be subject to the strict biosafety regulations that almost forbid the use of GMOs through the high costs of analysis involved. However, if we had more transgenes available to increase the TCC in GM905-60 by 20 times, would it be worth trying? The answer is definitely yes.

To discern whether there was synergism in expressing the psy2andcrtB alleles, itwas necessary to quantify the mRNA of both using real-time PCR, as explained above. However, it should be remembered that the difference between a cassava root with more carotenoids and one with less cannot be explained solely by the level of expression (mRNA) of the pathway genes (Arango 2010; Welsch et al. 2010). It can also be explained by single nucleotide polymorphisms in genes such as psy2-y2 (Welsch et al. 2010), by the post-transcriptional control that has been observed in transcription factors that interact with pathway genes (Welsch et al. 2007), or by epigenetic control factors that have been described as potential checkpoints for carotenoid synthesis (Xu et al. 2010; Ruiz-Sola and Rodriguez-Concepción 2012). Nevertheless, the SNPs of the PSY gene's coding region are not the only source of variation in the genes for carotenoid synthesis. For example, for maize, it is known that the lycopene gene for e-cyclase (LYC-e), with alleles that have insertions in the promoter regions or in the 30coding sequence, balance the pathway for the formation of beta rings in lycopene, to make more bcarotene (Ha¨rje et al. 2008), demonstrating that genes other than PSY also influence the synthesis and accumulation of pro-vitamin A carotenoids. As was

392 the case for maize, it would be necessary to know the sequence of the genes that are beneficial for
393 increasing the TCC in the cassava root. This will enable the development of markers to speed up
394 conventional breeding programs, or to explore new combinations of genes for breeding using
395 biotechnology.

396

397 ***Other genes with beneficial alleles may be involved in the increase of b-carotene and TCC in cassava***.
398 The research published by Welsch et al. (2010) showed that when the gene for bacterial phytoene
399 synthase, crtB, was inserted into a white cassava plant whose genotype for the psy2 gene was psy2-y1/y1
400 (TT), this resulted in the accumulation of b-carotene, a carotenoid further down phytoene in the
401 carotenoid pathway, corresponding to&30–80% TCC (published in Welsch et al. 2010), suggesting that it
402 is not only the synthesis of phytoene that is essential for increasing the TCC in the cassava root. Thus,
403 despite its color, white cassava provided other beneficial alleles, other than psy2-y1/y1, to increase b-
404 carotene and TCC. The work by Morillo (2009) provides further proof that there are probably other crucial
405 genes involved in increasing the TCC in the cassava root. Very recent SNP-based mapping analysis
406 indicates that there are at least two sites in the cassava genome, in chromosome 2 and 7, involved in b-
407 carotene accumulation (Ovalle et al. 2016). If these loci are related to carotenoid synthesis, catabolism or
408 storage is still unknown, which constitutes an excellent opportunity to use genome-editing tools to find
409 out the true role of this and other genes of the carotenoid pathway in cassava. This reinforces the
410 hypothesis that it is not only the PSY gene that is vital in the synthesis and accumulation of carotenoids in
411 cassava. Indeed, this current research shows that individuals carrying the psy2-y2 allele (heterozygous
412 psy2-y1/y2; all GT), and non transgenics, such as 1257–14, synthesize and/or accumulate low levels of
413 total carotenoids, indicating that psy2-y2 is not the only gene involved in increasing the TCC in cassava
414 roots. It could be argued that the heterozygosity of psy2 is what causes the decline of TCC in these
415 individuals, which is refuted by the GM905-57 and GM905-60 parents, both heterozygous GT, and that

have similar–or higher–total carotenoid content than their siblings, GM905-52 and GM905-21, that are TT homozygotes. Therefore, to achieve substantial increases in b-carotene and TCC in the cassava root, it is necessary that the other crucial synthesis pathway genes be characterized at the molecular level, as was done for the PSY gene (Welsch et al. 2010).

About significant differences in b-carotene and TCC between individuals. For all the carotenoid analyzed, the ANOVA showed significant differences between genotypes within and between families. For example, in the 1221 family, the individual 1221–55 was the only one that carried and expressed the crtB transgene and that surpassed its siblings and parents in TCC, except for the 1221–74 line, with which there was no difference (t = 0. 4904), which could be explained by lower dry-matter content of the latter line. Low dry-matter content ‘‘artificially’’ increases TCC.

One interpretation of these results is that whereas there had indeed been a crtB gene effect, statistically, this effect was however confused with the effect of other favorable alleles that increase the TCC. Work published by Ortiz et al. (2011) and Morillo et al. (2012) confirms that the TCC in the roots of the same cassava plant varies, and depends on, among other factors, the stage of root development and water availability (rain) which, in turn, affect the dry matter content. Furthermore, insufficient replicates (small n) and imbalance (different replicates for each genotype) could also contribute to variability in this study.

Another interpretation is that, in the case of the 1221 family, all the progeny were heterozygous for the gene psy2 (psy2-y2/y1) so their production of carotenoids was assured because it carried a favorable psy2 allele. In the individual 1221–55, which carried and expressed the crtB transgene, the synergistic effect of

having two favorable enzymes for the synthesis of carotenoids (PSY2-Y2 and CRTB) could not be statistically differentiated, difficulting the differentiation between CRTB and PSY2-Y2 contributions towards TCC. This result contrasts with that observed in the transgenic lines pCasPhyt-12 and 309, where the presence of the CRTB enzyme–alone or in combination with the CRTI and CRTY enzymes–resulted in TCC increase in genotypes lacking PSY2-Y2 (i.e., that were homozygous for psy2-y1/y1; Bonilla 2010).

Conclusions. The data presented in this research provides grounds to suggest that combining the crtB transgene with beneficial alleles for the synthesis and accumulation of carotenoids derived from yellow-rooted cassava parents results in a synergism that leads to an increase in the total root carotenoid content of the progeny. This synergism may have varied with genotypes, which was also reflected in the altered accumulation pattern of carotenoids in some individuals.

Cassava remains an important source of provitamin A, despite having a high content (&48%) of cis isomers of b-carotene (Howe et al. 2009), therefore the increase in cis isomers for b-carotene through transgenics reported here is encouraging. As has been demonstrated in this research, by transferring a single transgene, TCC can be increased by up to three units (from 8.08 to 11.11 lg/g FW), equivalent to 9.46 units in dry weight (24.09–33.55 lg/g DW). The increase from this transfer is equivalent to an increase of between &25% (FW) or 39% (DW) in one cycle of crossing. This is a good reason to combine conventional breeding and biotechnology to keep increasing the nutritional value of cassava. Finally, the results obtained in this research support the hypothesis that there are at least two key genes crucial for increasing TCC in cassava: one is the psy gene, the other is yet to be discovered.

Acknowledgements. The authors wish to knowledge the support received from HarvestPlus' (www.harvestplus.org) to develop research on cassava bio-fortification.

Compliance with ethical standard Conflict of interest. The authors declare that they have no conflict of interest.

Tables & Figures.

Table 1 Composition of carotenoids in lg/g (fresh weight—FW or dry weight—DW), TCC and dry matter (DM %) in a subsample of segregating progeny and parents.

Genotype	FW										DW	DM (%)
	Violaxanthin	Antheraxanthin	Lutein	13-cis-β-Carotene	α-Carotene	trans-β-Carotene	9-cis-β-Carotene	Phytoene	Phytofluene	TCC	TCC	
pCasPhyt12	0,05 +/- 0,02	0,05 +/- 0,01	0,11 +/- 0,05	0,03 +/- 0,01	0,01 +/- 0,00	0,21 +/- 0,05	0,08 +/- 0,02	0,21 +/- 0,09	0,03 +/- 0,03	0,60 +/- 0,09	1,97 +/- 0,58	31,64 +/- 6,36
GM905-21	*0,33 +/- 0,08	0,56 +/- 0,34	0,40 +/- 0,16	0,64 +/- 0,12	0,04 +/- 0,00	*4,76 +/- 0,40	0,63 +/- 0,11	*3,10 +/- 1,98	*1,53 +/- 0,97	8,08 +/- 0,71	24,09 +/- 1,26	33,51 +/- 1,71
1221-4	0,09 +/- 0,02	0,15 +/- 0,03	0,10 +/- 0,02	0,33 +/- 0,03	0,03 +/- 0,00	1,40 +/- 0,20	0,49 +/ -0,04	0,55 +/- 0,11	0,14 +/- 0,08	2,82 +/- 0,23	8,73 +/- 1,02	32,45 +/- 1,63
1221-6	0,34 +/- 0,02	0,40 +/- 0,03	0,38 +/- 0,07	0,69 +/- 0,09	0,03 +/- 0,01	3,21 +/- 0,67	0,73 +/- 0,08	1,92 +/- 0,95	*0,73 +/- 0,47	6,53 +/- 1,04	21,99 +/- 3,69	29,78 +/- 2,75
1221-7	0,07 +/- 0,02	0,10 +/- 0,01	0,07 +/- 0,00	0,21 +/- 0,02	0,02 +/- 0,00	0,83 +/- 0,22	0,28 +/- 0,04	0,56 +/- 0,12	0,21 +/- 0,07	1,74 +/- 0,32	6,38 +/- 1,35	27,77 +/- 4,34
1221-55	*0,42 +/- 0,04	***0,92 +/- 0,32**	***1,40 +/-0,50**	***1,33 +/- 0,01**	***0,09 +/-0,01**	*4,34 +/- 0,57	*1,52 +/- 0,03	*2,67 +/- 0,87	*1,00 +/- 0,20	***11,11+/-0,27**	*33,55 +/- 4,45	33,34 +/- 3,61
1221-74	*0,32 +/- 0,01	*0,79 +/- 0,11	0,63 +/- 0,09	*1,24 +/- 0,08	*0,08 +/- 0,00	*4,18 +/- 0,09	***1,61 +/-0,20**	*1,51 +/- 0,07	*0,49 +/- 0,21	*9,70 +/- 0,37	***37,1 +/- 4,04**	26,24 +/- 1,85
1221-84	*0,34 +/- 0,13	0,51 +/- 0,19	0,51 +/- 0,19	0,42 +/- 0,26	0,02 +/- 0,01	2,01 +/- 0,80	0,45 +/- 0,30	0,86 +/- 0,45	*0,30 +/- 0,12	4,79 +/- 2,27	21,66 +/- 17,11	25,75 +/- 6,96
1221-88	0,09 +/- 0,03	0,06 +/- 0,01	0,03 +/- 0,02	0,22 +/- 0,11	0,02 +/- 0,01	0,91 +/- 0,03	0,37 +/- 0,21	0,73 +/- 0,36	*0,31 +/- 0,17	1,84 +/- 0,28	6,40 +/- 1,04	28,76 +/- 0,29
GM905-57	0,21 +/- 0,11	0,16 +/- 0,12	0,21 +/- 0,21	0,72 +/- 0,25	0,03 +/- 0,01	5,85 +/- 1,45	0,73 +/- 0,22	*1,92 +/- 1,68	*1,13 +/- 1,09	8,57 +/- 2,66	*25,6 +/- 4,57	33,09 +/- 4,48
1257-2	0,10 +/- 0,01	0,08 +/- 0,02	0,22 +/- 0,12	0,21 +/- 0,02	0,02 +/- 0,00	0,80 +/- 0,13	0,33 +/- 0,06	*1,76 +/- 0,31	*0,86 +/- 0,21	2,23 +/- 0,43	6,71 +/- 1,44	33,29 +/- 0,78
1257-7	**0,46 +/- 0,10**	*0,91 +/- 0,56	*1,34 +/- 0,41	1,00 +/- 0,21	0,04 +/- 0,00	*4,35 +/- 1,78	0,93 +/- 0,08	*1,35 +/- 0,40	*0,66 +/- 0,31	*9,92 +/- 1,20	*25,72 +/- 0,3	38,54 +/- 4,21
1257-10	*0,34 +/- 0,09	0,15 +/- 0,01	0,26 +/- 0,02	0,71 +/- 0,09	0,04 +/- 0,00	*3,96 +/- 0,17	0,80 +/- 0,09	*2,65 +/- 1,45	*1,17 +/- 0,59	7,08 +/- 0,43	22,74 +/- 0,74	31,13 +/- 0,86
1257-13	0,23 +/- 0,03	0,24 +/- 0,10	0,38 +/- 0,24	0,78 +/- 0,12	0,03 +/- 0,01	*3,98 +/- 0,80	0,65 +/- 0,16	**5,64 +/- 1,60**	**1,88 +/- 0,91**	7,48 +/- 1,11	22,05 +/- 2,99	33,93 +/- 2,69
1257-14	0,02 +/- 0,00	0,01 +/- 0,00	0,02 +/- 0,02	0,05 +/- 0,01	0,01 +/- 0,00	0,23 +/- 0,05	0,08 +/- 0,02	0,66 +/- 0,37	0,14 +/- 0,10	0,50 +/- 0,11	1,37 +/- 0,25	36,17 +/- 3,15
GM905-60	0,46 +/- 0,21	0,47 +/- 0,17	0,45 +/- 0,28	*1,29 +/- 0,25	0,06 +/- 0,01	5,98 +/- 0,51	1,21 +/- 0,25	*1,51 +/- 0,39	*0,96 +/- 0,22	*11,24 +/- 1,64	*28,41 +/- 3,89	39,57 +/- 1,70
1260-1	0,28 +/- 0,03	0,47 +/- 0,12	0,61 +/- 0,22	0,94 +/- 0,07	0,05 +/- 0,00	***4,37 +/-0,66**	0,91 +/- 0,07	*2,02 +/- 0,05	*1,04 +/- 0,14	8,50 +/- 0,73	*27,15 +/- 3,01	31,41 +/- 1,97
1260-6	0,05 +/- 0,03	0,09 +/- 0,04	0,12 +/- 0,07	0,31 +/- 0,06	0,02 +/- 0,01	1,20 +/- 0,18	0,51 +/- 0,09	0,76 +/- 0,18	*0,25 +/- 0,05	2,54 +/- 0,41	7,78 +/- 0,40	32,53 +/- 3,63
60444	0,02 +/- 0,00	0,01 +/- 0,00	0,02 +/- 0,02	0,06 +/- 0,02	0,01 +/- 0,00	0,23 +/- 0,08	0,09 +/- 0,02	0,16 +/- 0,15	0,00 +/- 0,00	0,55 +/- 0,11	1,68 +/- 0,29	32,55 +/- 1,25

The transgenic line pCasPhyt12, derived from genotype 60444, was the mother of all the crosses with yellow-rooted parents of the GM905 series (-21, -57 and -60). At least two biological replicates of each genotype planted in the field were analyzed. Italics indicate individuals carrying the crtB gene. Bold are the highest averages for each carotenoid and the TCC in the progeny. The asterisk (*) within each column indicates no significant difference (a = 0.05) in carotenoid content between the individuals labeled and line 1221–55. 60444 is wild cassava (control)

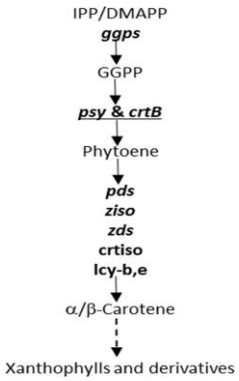


Fig. 1 Carotenoid biosynthesis pathway in plants (enzymes are in bold). PSY plays a pivotal role since it catalyzes the first committed biosynthetic step in carotenogenesis. PSY together with the bacterial Phytoene Synthase CRTB (underlined) can enhance the production of Phytoene to increase carotenoid content in white cassava roots (Welsch et al. 2010). IPP, isopentenyl-diphosphate; DMAPP, dimethylallyl-diphosphate; GGPP, geranylgeranyl-diphosphate; GGPS, geranylgeranyldiphosphate synthase; PDS, phytoene desaturase; ZISO, z-carotene isomerase; ZDS, z-carotene desaturase; CRTISO, carotene isomerase; LCY, lycopene b and e-cyclase. The dashed arrow represents multiple enzymatic steps.

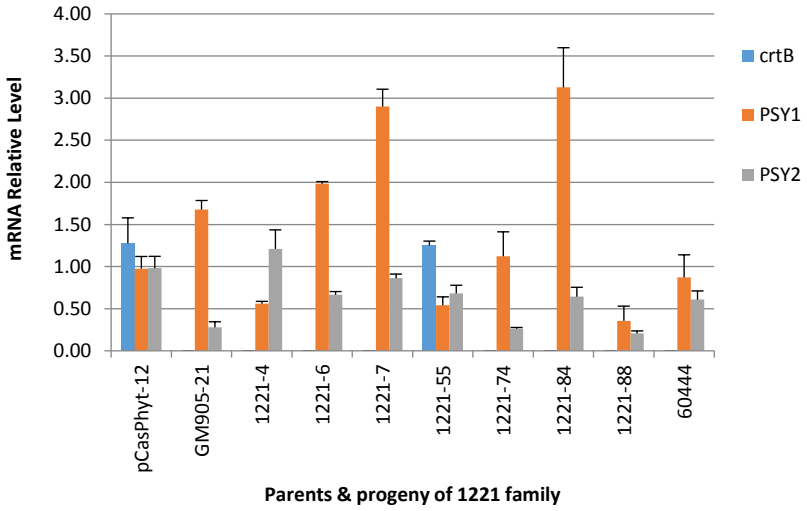


Fig. 2 Relative expression of PSY1, PSY2 and crtB genes in the 1221family. The level of mRNA for each individual, and for each gene, is expressed as relative units normalized to the pCasPhyt-12. The data represent the average of at least three biological replicates with three technical replicates each, and the respective standard deviation. The primers used to amplify each gene did not discriminate alleles, e.g., expression of PSY2 measured co-expression of psy2-y2-and psy2-y1 alleles in the progeny. GM905-21 is the male parent, and 60444 corresponds to the white cassava variety, not the transgenic one, from which pCasPhyt-12 was derived

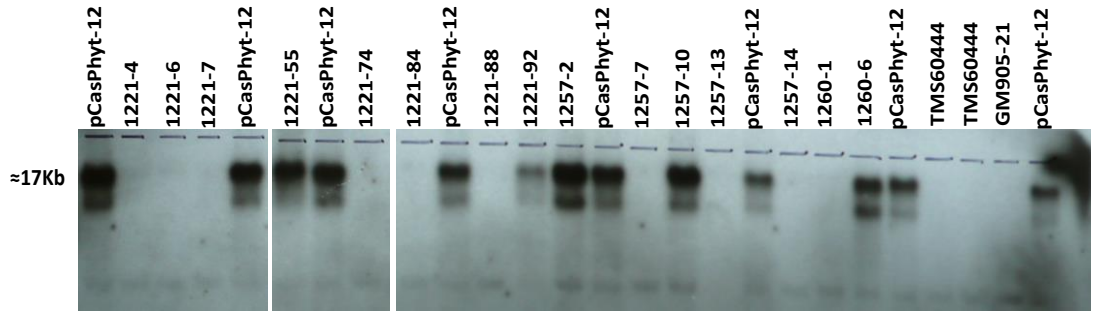


Fig. 3 CrtB gene detection, using Southern blot, in the parents and progeny of crosses. Only parents pCasPhyt-12 and GM905- 21 are shown. 60444 corresponds to the non-transgenic version pCasPhyt-12. The presence of two bands at 17 Kb in the all progeny that received the crtB transgene suggests that the two insertions are linked and segregate as a single locus.

References

Arango J (2010) Towards pro-vitamin A accumulation in cassava roots through genetic modification: Phytoene Synthase catalyzes the rate-limiting step. Dissertation. Albert- Ludwigs-Universitat, Freiburg.

Arango J, Wust F, Beyer P, Welsch R (2010) Characterization of phytoene synthases from cassava and their involvement in abiotic stress-mediated responses. Planta 232(5):1251–1262.

Belalcazar J, Dufour D, Pizarro M, Luna J, London˜o L, Morante N, Calle F, Jaramillo AM, Pino L, Becerra LA, Davrieux F, Ceballos H (2016) High-throughput phenotyping and improvements in breeding cassava for increased carotenoids in the roots. Crop Sci 56:2916–2925.

Beltran J, Prias M, Al-Babili S, Ladino Y, Lopez D, Beyer P, Chavarriaga P, Tohme J (2010) Expression pattern conferred by a glutamic acid-rich protein gene promoter in field-grown transgenic cassava (Manihot esculenta Crantz). Planta 231:1413–1424

Bonilla A (2010) Análisis de expresión de una miniruta de síntesis de carotenos en yuca (Manihot esculenta Crantz) transgénica por medio de qRT-PCR (reacción en cadena de la polimerasa en tiempo real). Universidad del Quindío, Armenia.

Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoid handbook. Basel, Birkhau¨ser

Cadavid LF (2002) Suelo y fertilizacio´n para la yuca. In: Ospina B, Ceballos H (eds) La yuca en el tercer milenio: sistemas modernos de produccio´n, procesamiento, utilizacio´n y comercializacio´n, vol 327. CIAT, Cali, pp 76–103

Calle F (2002) Control de malezas en el cultivo de la yuca. In: Ospina B, Ceballos H (eds) La yuca en el tercer milenio: Sistemas modernos de produccio´n, procesamiento, utilizacion y comercializacio´n, vol 327. CIAT, Cali, pp 126–128

Ceballos H, Iglesias C, Pe´rez JC, Dixon AGO (2004) Cassava breeding: opportunities and challenges. Plant Mol Biol 56:503–516

Ceballos H, Morante N, Sa´nchez T, Ortiz D, Arago´n I, Cha´vez AL, Pizarro M, Calle F, Dufour D (2013) Rapid cycling recurrent selection for increased carotenoids content in cassava root. Crop Sci 53:2342–2351

Chavarriaga P (2013) Biotecnologı´a y mejoramiento convencional para incrementar el valor nutricional de la raı´z de yuca (Manihot esculenta Crantz). Ph.D. dissertation. Doctorado en Ciencias-Biologı´a, Facultad de Ciencias Naturales y Exactas, Universidad del Valle, Cali, Colombia. (330 p)

Chavarriaga-Aguirre P, Brand A, Medina A, Prı´as A, Escobar R, Martinez JF, Dı´az P, Lo´pez C, Roca WM, Tohme J (2016) The potential of using biotechnology to improve cassava: a review. In Vitro Cell Dev-Pl 52(5):461–478. doi:10.1007/ s11627-016-9776-3

Cha´vez AL, Sa´nchez T, Jaramillo G, Bedoya JM, Echeverri J, Bolan˜os E, Ceballos H, Iglesias CA (2005) Variation of quality traits in cassava roots evaluated in landraces and improved clones. Euphytica 143:125–133

Failla ML, Chitchumroonchokchai C, Siritunga D, De Moura FF, Fregene M, Sayre RT (2012) Retention during processing and bioaccessibility of b-carotene in high b-carotene transgenic cassava root. J Agric Food Chem 60:3861–3866

FAO (2013) Save and grow cassava: a guide to sustainable production and identification. E-ISBN 978-92-5-107642-2

Harjes CE, Rocheford TR, Bai L, Brutnel TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET, Yan J, Buckler ES (2008) Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319:330–333

Howe JA, Maziya-Dixon B, Tanumihardjo SA (2009) Cassava with enhanced b-carotene maintains adequate vitamin A status in Mongolian gerbils (Meriones unguiculatus) despite substantial cis-isomer content. Brit J Nutr 102:342–349

Li F, Vallabhaneni R, Wurtzel ET (2008) Psy3, a new member of the Phytoene Synthase gene family conserved in the poaceae and regulator of abiotic stress-induced root carotenogenesis. Plant Phys 146:1333–1345

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2DDCT method. Methods 25:402–408

Lopez AB, Yang Y, Tannhauser TW, Li L (2008) Phytoene desaturase is present in a large protein complex in the plastid membrane. Physiol Plantarum 133:190–198

Morillo Y, Sanchez T, Morante N, Chavez AL, Morillo AC, Bolan˜os A, Ceballos H (2012) Estudio preliminar de herencia del contenido de carotenoides en raı´ces de poblaciones segregantes de yuca (Manihot esculenta Crantz). Acta Agrono´mica 61(3):253–264

Morillo-Coronado Y (2009) Herencia del contenido de carotenos en raı´ces de yuca Manihot esculenta Crantz. Universidad Nacional de Colombia. Palmira, Colombia, Tesis de doctorado, p 225

Naqvi S, Zhu C, Farre G, Sandmann G, Capell C, Christou P (2011) Synergistic metabolism in hybrid corn indicatesbottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid. Zeaxanthin Plant Biotechnol J 9:384–393

Ortiz D, Sa´nchez T, Morante N, Ceballos H, Pacho´n H, Duque MC, Cha´vez AL, Escobar AF (2011) Sampling strategies for proper quantification of carotenoid content in cassava breeding. J Plant Breed Crop Sci 3(1):14–23

Ovalle T, Perea C, Pizarro M, Morante N, Ceballos H, Dufour D, Meike A, Becerra LA (2016) Elucidating high beta-carotene accumulation in cassava based on next-generation sequencing. In: world congress on root and tuber crops, Abstract No. SP06-16, Jan 18–22, 2016, Nanning, Guangxi, China

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res 29(900):2002–2007

Quinlan RF, Shumskaya M, Bradbury LMT, Beltran J, Ma C, Kennelly EJ, Wurtzel E (2012) Synergistic interactions between carotene ring hydroxylases drive Lutein formation in plant carotenoid biosynthesis. Plant Phys 160(1):204–214

Ruiz-Sola MA´ , Rodrı´guez-Concepcio´n M (2012) Carotenoid biosynthesis in Arabidopsis: a colorful pathway. The Arabidopsis Book/American Society of Plant Biologists 10:e0158. doi:10.1199/tab.0158

Santos CA, Philipp WS (2006) Heritabilities and minimum gene number estimates of carrot carotenoids. Euphytica 151:79–86

Sayre R, Beeching JR, Cahoon EB, Egesi C, Fauquet C, Fellman J, Fregene M, Gruissem W, Mallowa S, Manary M, Maziya-Dixon B, Mbanaso A, Schachtman DP, Siritunga D, Taylor N, Vanderschuren H, Zhang P (2011) The biocassava plus program: bio fortification of cassava for sub-Saharan Africa. Annu Rev Plant Biol 62:251–272

Welsch R, Maass D, Voegel T, Dellapenna D, Beyer P (2007) Transcription factor RAP2.2 and its interacting partner SINAT2: stable elements in the carotenogenesis of Arabidopsis leaves. Plant Phys 145:1073–1085

Welsch R, Wu¨st F, Ba¨r C, Al-Babili S, Beyer P (2008) A third Phytoene Synthase is devoted to abiotic stress-induced ABA formation in rice and defines functional diversification of PSYs. Plant Phys 147:367–380

Welsch R, Arango R, Ba¨r C, Salazar B, Al-Babili S, Beltra´n J, Chavarriaga P, Ceballos H, Tohme J, Beyer P (2010) Provitamin A accumulation in cassava (Manihot esculenta) roots driven by a single nucleotide polymorphism in a Phytoene Synthase gene. Plant Cell 22:3348–3356

Xu L, Ye R, Zheng Y, Wang Z, Zhou P, Lin Y, Li D (2010) Isolation of the endosperm-specific LPAAT gene promoter from coconut (Cocos nucifera L.) and its functional analysis in transgenic rice plants. Plant Cell Rep 29(9):1061–1068

Supplementary Material

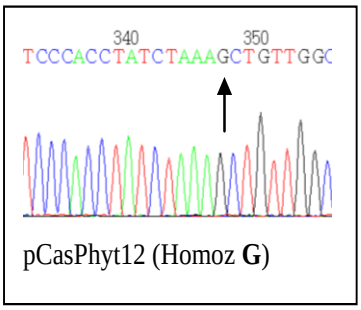
Molecular analysis of the expression of a crtB transgene and the endogenous psy2-y1 and psy2–y2 genes of cassava and their effect on root carotenoid content.

Transgenic Research

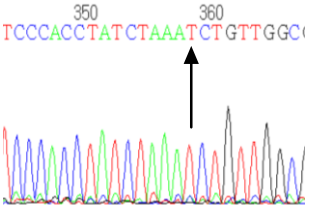
Paul Chavarriaga-Aguirre[1], Mónica Prías, Danilo López, Darwin Ortiz, Nelson Toro-Perea, Joe Tohme

[1]Author for correspondence; tel: (57-2) 445-0000 extension 3258; fax (57-2) 445-0073; e-mail p.chavarriaga@cgiar.org

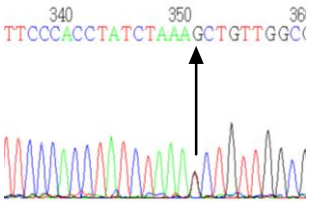
SNPs of PSY2 gene of cassava. Allele PSY2-Y1 is represented by the homozygous (GG/CC) mother pCasPhyt-12, and the PSY2-Y2 allele, associated with higher carotenoid content in roots, is represented by the homozygous (TT/AA) father GM905-21. The F1, represented by line 1221-55, is heterozygous (GT/CA). Allele nomenclature is according to Welsch et al (2010).



pCasPhyt12 (Homoz **G**)



GM905-21 (Homoz **T**)



1221-55 (Hetero **GT**)

Primer sequences used in this research designed with Primer Express® v2.0 of Applied Biosystems.

Primer name (gene)	Secuece (5´-3´)	Used for	Product (bp)	Location on crtB sequence
crtB F2 crtB R2	TAGCGTGCTGATGCTCTACACCTG ACCTCCTGAAAGGCAGCGAA	Southern	192	Underlined (light gray shade)
CrtBFor1 CrtBRev1	GGGCGTGGTGGGTCTGATGA ACTCGGCGGGCAGATAGCAG	qPCR with SYBR Green	158	Underlined (dark gray shade)
CrtB-1-Fw CrtB-1-Rv	*CGTAGCGTGCTGATGCTCTA* **tcacctttgctggtgtgct**	PCR for crtB	731	Bold
CrtB-2-Fw CrtB-2-Rv	*CGTAGCGTGCTGATGCTCTA* **TGCCAAATGTTTGAACG**TCA	PCR for crtB	912	Underlined, bold and Capitalized
HPT-RKfw HPT-RKRv	CTATTTCTTTGCCCTCGGACG CTCCGCATTGGTCTTGACCA	PCR for hpt II	228	---
18 S rRNA F 18 S rRNA R	ATGATAACTCGACGGATCGC CTTGGATGTGGTAGCCGTTT	qPCR with SYBR green for 18S	169	---
SNP-asen SNP-seq	ATTCAGGTGCAATGCCCATC AGAATCCATTCCTCTTGAAT	PCR for SNP-PSY2	---	---
PSY1Fw-Exp PSY1Rv-Exp	CCGACGAGACGGCCATT CATAGGATTAGGTAGTGGAAGCAATT	qPCR with SYBR green for PSY1*	---	---
PSY2 Fw-Exp PSY2 Rv-Exp	GCAGCATCAAGCATATCAAAGG TGGGAAGCAAGGTTGGAAGA	qPCR with SYBR green for PSY2*	---	---

(*) reference: Welsch et al. (2010).

Sequence of the *crt*B gene introduced into cassava. Highlighted in light gray, PCR probe for Southern blots; highlighted in dark gray, qPCR product for crtB expression; Bold and gray are the start (ATG) and stop codon (TGA), the latter followed by the NOS polyadenylation signal. Primers are underlined and/or highlighted according to the table with Primer sequences.

ATGagccaaccgccgctgcttgaccacgccacgcagaccatggccaacggctcgaaaagttttgccaccgc
tgcgaagctgttcgacccggccacccgc*CGTAGCGTGCTGATGCTCTA*ACAcctggtgccgccactgcgatg
acgtcattgacgaccagacccacggcttcgccagcgaggccgcggcggaggaggaggccacccagcgcctg
gcccggctgcgcacgctgaccctggcggcgtttgaaggggccgagatgcaggatccggccttcgctgcctt
tcaggaggtggcgctgacccacggtattacgccccgcatggcgctcgatcacctcgacggctttgcgatgg
acgtggctcagacccgctatgtcacctttgaggatacgctgcgctactgctatcacgtggcgggcgtggtg
ggtctgatgatggccagggtgatgggcgtgcgggatgagcgggtgctggatcgcgcctgcgatctggggct
ggccttccagctgacgaatatcgcccgggatattattgacgatgcggctattgaccgctgctatctgcccg
ccgagtggctgcaggatgccgggctgaccccggagaactatgccgcgcgggagaatcgggccgcgctggcg
cgggtggcggagcggcttattgatgccgcagagccgtactacatctcctcccaggccgggctacacgatct
gccgccgcgctgcgcctgggcgatcgccaccgcccgcagcgtctaccgggagatcggtattaaggtaaaag
cggcgggaggcagcgcctgggatcgccgcc**agcacaccagcaaaggtga**aaaaattgccatgctgatggcg
gcaccggggcaggttattcgggcgaagacgacgagggtgacgccgcgtccggccggtctttggcagcgtcc
cgtt**TGA**CGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTA
TCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGA
TGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAA
CTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGG