Variation in transcriptional profiles of carotenoid biosynthetic genes in Indonesian yellow- and white-fleshed tuberous root cassava (Manihot esculenta Crantz) accessions

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Abstract. Root-tubers of cassava (Manihot esculenta Crantz) accumulate high starchy carbohydrates, protein, and carotenoids. Carotenoids were associated with tuber colorus, i.e., yellow-fleshed tuber accumulates more carotenoids than white-fleshed root-tubers. Carotenoids serve nutrition for health, such as an antioxidant and provitamin A. This study aimed to analyze relative expressions (RE) of carotenoid genes in Indonesian yellow and white cassava (IYC and IWC, respectively). Nine-month-old fresh root-tubers of IYC (Adira-1, Mentega-2, and Ubi Kuning) and IWC (Adira-4 and Ment) were used for the analysis. RE of five genes: PSY (phytoene synthase), CRTISO (carotenoid isomerase), LYCa (α-lycopene cyclase), LYCb (β-lycopene cyclase), and BCH1 (beta carotene hydroxylase1), and a reference gene, i.e., EF1-A (elongation factor 1-alpha), were analyzed using a qRT-PCR. Results showed that RE of all genes were detected in IYC and IWC. In IWC, all genes were expressed high, suggesting biosynthetic fluxes through yellow α-carotenoids and xanthophylls. In YWC, variations on RE levels of all genes were observed. LYCa and LYCb were expressed high in Adira-1 and Mentega-2, indicating α- and β-carotenoids accumulate in root-tubers. However, BCH1 was expressed 2-3-folds lower in both accessions than all IWC, suggesting low zeaxanthin production and accumulation of β-carotene. In Ubi Kuning, α-carotenoids accumulation may not exist due to low LYCa RE. Also, higher BCH1 expression levels were observed in Ubi Kuning, suggesting a substantial zeaxanthin accumulation. Results are potential for improving carotenoid accumulation through breeding programs in cassava.

1. Introduction

Cassava (Manihot esculenta Crantz) is an important staple food source for millions of people worldwide, particularly in developing countries. The root-tubers contain mainly 65-91% starchy carbohydrates, which is composed of around 20-30% amylose and the remaining amylopectin [1, 2]. Some minerals, such as iron, zinc, calcium, potassium and other minerals, are detected in trace amounts in cassava root-tubers [2-4]. Besides, an essential vitamin, i.e., provitamin A, is present in several cassava genotypes with yellow-fleshed root-tubers. Provitamin A content is due to carotenoids, particularly α-carotene, β-carotene and β-cryptoxanthin [5].

Provitamin A, is converted to vitamin A in the human body, is vital for tissue differentiation in the cornea, the conjunctival membranes and retina [5]. Provitamin A is also important as an antioxidant for scavenging reactive oxygen and nitrogen species resulted during metabolism in the human body. It is also claimed that provitamin A has anticarcinogenic activity. Vitamin A deficiency is widespread in tropical countries, hitting mostly children and pregnant women [2]. Efforts have been made to reduce the prevalence numbers of vitamin A deficiencies by using different strategies, such as food
fortification, dietary diversification and biofortification by improving carotenoid levels in food crops through breeding and genetic modification, such as in Golden Rice [6] and potato [7].

Colors of fleshed-root-tubers majorly found among Indonesian cassava genotypes are white and yellow colors. Yellow colors of the root-tubers have been linked to carotenoid contents and the levels vary among genotypes [8-12]. In general, conventional breeding, in parallel with the heterozygous nature of the cassava crops, creates a long step in genotypic improvement. Genetic engineering is one of the options to speed up new cultivar development. Knowledge on the molecular regulation of carotenoid biosynthetic pathway in cassava is still a handful. Therefore, it is essential to gain insights into genes encoding structural enzymes and regulators underlying the carotenoid biosynthesis in cassava.

To understand the molecular regulation underlying carotenoid accumulation in Indonesian cassava, relative expressions of five candidate genes encoding carotenoid biosynthetic enzymes were quantitatively measured in selected genotypes with yellow and white-fleshed root-tubers using quantitative real-time PCR. Previous reports showed that several genes were associated with carotenoid accumulation in cassava, such as phytoene synthase [13, 14] and phytoene desaturase [15]. In our report here, we display expression profiles of PSY (phytoene synthase), CRTISO (carotenoid isomerase), LYCa (α-lycopene cyclase), LYCB (β-lycopene cyclase), and BCHI (β-carotene 3-hydroxylase 1) in white and yellow Indonesian cassava. Results indicate two genes, LYCa and BCHI, may become the rate-limiting point in the variation of carotenoid accumulation in cassava root-tubers.

2. Materials and Methods
2.1. Plant materials
Five Indonesian cassava (Manihot esculenta Crantz) harboring white- and yellow-fleshed root-tubers were obtained from the Research Center for Biotechnology, Indonesian Institute of Sciences, germplasm collection. Accessions with yellow-fleshed root-tubers, which then were called Indonesian Yellow Cassava (IYC), were Adira-1, Mentega-2, and Ubi Kuning. Accessions with white-fleshed root-tubers, which then were called Indonesian White Cassava (IWC) were Adira-4 and Menti. Plants were cultivated in the experimental field located in Cibinong Science Center-Boetic Garden Indonesian Institute of Sciences, Cibinong, Bogor District, West Java, Indonesia. Fresh root-tubers harvested from nine-month-old plants were peeled and washed by water. Clean root-tubers were sliced into thin chips, ground into fine powder samples under nitrogen liquid condition, and stored at -80°C prior to the analysis.

2.2. Total RNA extraction and cDNA synthesis
Total RNA was extracted from freeze-powdered root-tubers using a protocol described by Cordeiro et al. 2008 [16] with modification. In a brief, about 30 mg powder samples were added to the tube containing 400 µl of pre-warmed EB solution (2% CTAB; 2% PVP; 100mM Tris/HCl, pH 8; 25mM EDTA; 2 M NaCl) and 8 µl of 2-mercaptoethanol. The mixture was incubated for 15 min and mixed in 5 min intervals using a vortex. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the mixture and vortexed for 10 min. The mixture was centrifuged 8,000 RPM for 10 min at 4°C and the aqueous phase was collected to a new tube. As much as 125 µl isopropanol was added to the aqueous phase and vortexed until homogenized. The mixture was incubated overnight at 4°C and after that, the mixture was centrifuged at 12,000 RPM for 30 min at 4°C to precipitate the total RNA. The supernatant was discarded and the precipitate was resuspended with a solution of 180 µl RNase-free water, 36 µl sodium acetate 2M (pH 5.2) dan 360 µl ethanol absolute. The resuspension was mixed and incubated for 2 hours at -20°C. After incubation, the resuspension was centrifuged at 12,000 RPM for 10 min at 4°C. The supernatant was discarded and the pellet was washed by ethanol 70%. The pellet was air-dried and it was resuspended with 30 µl RNase-free water. The quality and quantity of total RNA were measured by NanoPhotometer™ P-Class P 300 and evaluated by electrophoresis on
1.5% w/v agarose gel. cDNA was synthesized from 250 ng total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (FSQ-301; TOYOBO) according to the manufacturer’s instructions. This reaction resulted 5 ng of cDNA for gene expression analysis.

2.3. Primer design and gene expression analysis

Five structural genes of the carotenoid biosynthetic pathway, i.e., PSY, CRTISO, LYCa, LYCβ, and BCH1, were targets for gene expression analysis. Candidate genes were based on [10]. In addition, four reference genes of Rubisco, β-tubulin, EF1-A (elongation factor 1-alpha) and Zinc Finger protein (ZnF) were included in the analysis. A housekeeping gene was selected to normalize the expression levels of candidate genes due to the stability of its Ct (threshold cycle) value over all samples. Primers were designed for each candidate gene using BatchPrimer3 (https://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi) with the criteria suggested by Udvardi et al. 2008 [17]. Oligonucleotides for quantitative real-time PCR (qRT-PCR) analysis were carefully selected by re-aligning oligonucleotide candidates to cassava gene database, i.e., Phytozome v12.1, using BLAST tool. Oligonucleotides with the highest specificity were selected as described in Table 1. qRT-PCR analysis was done using SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPA Biosystem; KK4601) according to the manufacturer’s instruction. The qPCR analysis was performed with three biological replicates of each accession. Expression levels were determined by delta Ct (ΔCt) values, calculated by subtracting Ct value of each candidate gene with Ct value of α-tubulin gene, converted by 2^-ΔCt calculation, multiplied by 10000.

2.4. Statistical analysis

Statistical analysis was conducted by comparing the mean of each biological replicates (n = 3) of each accession using One-way ANOVA and continued with the Duncan’s test (α = 0.05). This was performed in IBM® SPSS® Statistics version 25.

Table 1. Carotenoid biosynthetic genes and primer sequences for gene expression analysis

| Genes             | Enzyme function in carotenoid pathway                                                                                                                                                                                                 | Gene code at Phytozone v12.1° | References | Primer sequences 5’ to 3’  |
|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|------------|-----------------------------|
| PSY (phytoene synthase) | condensation two geranylgeranyl pyrophosphate to form the colorless phytoene                                                                                                                                                           | cassava4.1_0 08121m.g           | [10]        | forward CAC AGG ATG AAT TGG CAC AG |
|                    |                                                                                                                                                                                                                                        |                               |            | reverse TTG CAG CAC TCA GCT CTG TC |
| CRTISO (carotenoid isomerase) | introduction two symmetric double bonds and formation of all-E-lycopene from phytoene                                                                                                                                                  | cassava4.1_0 03897m.g           | [10]        | forward CGA TAG CTG CTT TCC TGG AC |
|                    |                                                                                                                                                                                                                                        |                               |            | reverse CTT AAC CAG CCG AGA AGT CG |
3. Results and Discussion

3.1. Housekeeping gene selection

Four genes of Rubisco, β-tubulin, EF1-A (elongation factor 1-alpha) and Zinc Finger protein (ZnF) were evaluated for their stable expression values. These genes were previously considered as potential reference genes based on their stable expressions in different plant organs, including root-tubers and leaves, of different cassava genotypes [18, 19, 21]. Of four genes, EF1-A showed the least variation of threshold cycles (Ct) values over all candidate reference genes, including ZnF, Rubisco and β-tubulin (Figure 1). This was showed by the standard deviation of Ct values of EF1-A that had the least standard deviation (SD) value, 0.8 (Figure 1). Hence, EF1-A was selected to normalize the expression levels of candidate carotenoid biosynthetic genes in this experiment set. Variation on Ct values of reference genes may be different depending on plant organs, accessions, and treatments as well as individuals [18, 21, 22]. In other set of cassava varieties with higher cyanide contents in root-tubers, TATA box binding protein (TBP) gene showed a uniform expression values than EF1-A in both leaves and root-tubers [21]. A gene encoding ZnF protein has been reported as the superior reference for normalizing gene expression values in different organs of normally grown cassava [18].

\[ \begin{align*}
\text{LYCa} & \quad \text{(α-lycopene cyclase)} \\
& \quad \text{formation lycopene and α-ring carotenoids and xanthophylls} \\
& \quad \text{cassava4.1_0} \quad \text{[10]} \\
& \quad \text{05406m.g} \\
\text{forward} & \quad \text{GCC ACA} \\
& \quad \text{AGA AAG} \\
& \quad \text{GAA ACG} \\
& \quad \text{TC} \\
\text{reverse} & \quad \text{CAT AGT} \\
& \quad \text{TGC TCC} \\
& \quad \text{GTT TGG} \\
& \quad \text{AT} \\
\text{LYCb} & \quad \text{(β-lycopene cyclase)} \\
& \quad \text{cyclization lycopene to form β-ring carotenoids} \\
& \quad \text{cassava4.1_0} \quad \text{[10]} \\
& \quad \text{04296m.g} \\
\text{forward} & \quad \text{CAG AAG} \\
& \quad \text{CTA GGC} \\
& \quad \text{GAT CCA} \\
& \quad \text{GT} \\
\text{reverse} & \quad \text{AGT CCA} \\
& \quad \text{GAA GCA} \\
& \quad \text{CGG GAA} \\
& \quad \text{TA} \\
\text{BCH1} & \quad \text{(beta carotene hydroxylase 1)} \\
& \quad \text{introduction oxygen functions to β-carotenoid-derivative-xanthophylls} \\
& \quad \text{Manes.02G0} \quad \text{[10]} \\
& \quad \text{18300.1} \\
\text{forward} & \quad \text{GCC TAT} \\
& \quad \text{TTC CCC} \\
& \quad \text{TAC CTC} \\
& \quad \text{CA} \\
\text{reverse} & \quad \text{TGA AGC} \\
& \quad \text{TCC CTA} \\
& \quad \text{TCC AAT} \\
& \quad \text{GC} \\
\end{align*} \]

\^\text{PHYTOZOME v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html#)
3.2. Transcript profiles in five cassava genotypes

To understand the rate-limiting step in the carotenoid biosynthetic pathway in root-tubers of yellow- and white cassava, we selected five genotypes representing yellow (IYC) and white cassava groups (IWC). These genotypes are widely cultivated in West Java, Indonesia, and are valuable commercially in food market and starch industries. The yellow color of root-tuber flesh has been reported to be associated with carotenoid content [2, 9, 10, 12, 13]. Of five genotypes, Adira 1, Mentega 2, and Ubi Kuning showed yellow-fleshed tuber color (Figure 2a; [12]). In contrast, Adira 4 and Menti have no visible yellow color in the root-tuber (Figure 2a-b; [12]).

Relative expressions of PSY, CRTISO, LYα, LYβ, and BCH1 are detected in IYC and IWC (Figure 3) and the transcript levels of these genes varied among genotypes. This suggests that there is an accumulation of carotenoid pigments although there are visible color differences between root-tubers of yellow and white cassava. Adira 4 has been reported to have detected levels of β-carotene, which was around 0.009 ppm [12]. Relative expressions of PSY gene varied among IYC (Adira 1, Mentega 2 and Ubi Kuning) and IWC (Adira 4 and Menti), whereas PSY expression levels was abundant as much as those levels in other genotypes (Figure 3a). PSY expressions were significantly different (P<0.05) between IWC and yellow-fleshed cassava, Mentega 2 and Ubi Kuning. This suggested that PSY was not the rate limitation in IYC and IWC. However, an allelic polymorphism of PSY genes has been reported in cassava, which induced carotenoid biosynthesis leading to the accumulation of provitamin A carotenoids [13]. PSY catalyzes the main substrate of carotenoid biosynthesis, geranylgeranyl pyrophosphate, into phytoene and, commonly, as the limiting factor in plants with low levels of carotenoid, such as in Brassica napus [20]. The later stage of carotenoid biosynthesis involves CRTISO enzyme, which, in parallel with PDS and ZDS, catalyzes phytoene into neurosporene, a precursor of lycopene, carotenoids and xanthophylls [10]. CRTISO gene expression levels in all samples were significantly different in IWC and IYC of Mentega 2 and Ubi Kuning.
(Figure 3b). Ubi Kuning showed the lowest expression levels of CRTISO, which was around 0.3 times lower than other genotypes (Figure 3b). The low expression levels of LYCβ and LYCa, the genes encoding biosynthetic enzymes for producing β-ring carotenoids and α-ring carotenoids, respectively, were also occurred in Ubi Kuning (Figure 3c-d). LYCβ expression levels of Ubi Kuning was 0.3 times lower than its expression level in other genotypes. LYCa expression levels in Ubi Kuning was the lowest among all genotypes (Figure 3c). This suggests that LYCa is the limiting factor in carotenoid biosynthesis in Ubi Kuning, leading to fluxes of carotenoid pathway to β-carotene. However, BCH1 expression levels were higher than Adira 1 and Mentega 2, suggesting that the biosynthesis fluxes may hydrolyze β-carotene into zeaxanthin (Figure 3). In contrast, two IYC, Adira 1 and Mentega 2, contained similar levels of LYCa expressions as those in IWC, suggesting that biosynthetic pathway flux directs to both α- and β-ring carotenoids biosynthesis from lycopene. BCH1 expression levels in Adira 1 and Mentega 2 is at least two times lower than those in IWC and Ubi Kuning. This suggests that the biosynthetic flux to Zeaxanthin is very low, indicating accumulation of β-carotene in both genotypes. Correlation on low expression values of BCH1 to the accumulation of β-carotene has also been showed in an engineered potato and sweet potato plant by suppressing the expression of BCH1 [23, 24].

In IWC, Adira 4 and Menti, relative expressions of five carotenoid biosynthetic genes are in the same levels as IYC, showing that carotenoid biosynthesis still occurs in both types of cassava. The expression profile of the five genes in Adira 4 and Menti shows that the flux of the carotenoid biosynthesis directs to the formation of α and β carotenoids. LCYA expression in Adira 4 and Menti was higher than the other samples. This might influence the production of carotenoid compounds, which are light yellow to white, such as α-carotene and lutein. The β-ring carotenoid biosynthesis pathway in Adira 4 and Menti may continue to the latter pathway, i.e., the formation of zeaxanthin compounds from beta carotene precursors. This is indicated by the high level of BCH1 expression, even higher than yellow cassava.

![Figure 2](image-url)  
**Figure 2.** Fleshed root-tuber colors of yellow cassava genotypes, i.e., Adira 1, Mentega 2 and Ubi kuning, (a.) and white cassava genotypes, i.e., Adira 4 and Menti, (b.). Left figure (a) is adapted from “Karakter Umbi Dan Nutrisi Tujuh Genotip Ubi Kayu (Manihot esculenta) [Characterisation of Root-Tubers and Nutritional Content of Seven Cassava Genotypes (Manihot esculenta)], by Hartati N S, Fitriani H, Supatmi, and Sudarmonowati S 2012 Jurnal Agricola Tahun II, Nomor 2, 101-110. Adapted with permission.
Figure 3. Relative expression levels for five genes encoding for carotenoid biosynthetic pathway in cassava root-tuber. Yellow and white bars represent averages of three biological replicates and standard deviation. Yellow bars represent yellow-fleshed-tuber cassava genotypes, i.e., Adira 1 (Ad-1), Mentega 2 (Mt-2), and Ubi Kuning (UK). White bars represent white-fleshed-tuber cassava genotypes, i.e., Adira 4 (Ad-4) and Menti (Mt). The biosynthetic pathway was according to [10]. Each letter written on the top of yellow and white bars describes the statistical result of One-way ANOVA followed by Duncan’s test (α = 0.05). Abbreviations: PSY, phytoene synthase; PDS, phytoene desaturase; CRTISO, carotenoid isomerase; LYCa, α-lycopene cyclase; LYCβ, β-lycopene cyclase; BCH, β-ring hydroxylase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; NXS, neoxanthin synthase.

4. Conclusions
In this study, we showed that five carotenoid biosynthesis genes, PSY, CRTISO, LYCa, LYCβ, and BCH1 are expressed in yellow- and white-fleshed root tuber cassava. Relative expression levels of those genes varied among cassava genotypes. In white-fleshed root tuber cassava, relative expressions of five carotenoid genes were abundant, suggesting an accumulation of α-ring carotenoids and β-ring carotenoids. Low relative expression levels of BCH1 might be the rate-limiting step in the carotenoid
pathway, suggesting the accumulation of β-carotene in two yellow-fleshed root tuber cassavas, Adira 1 and Mentega 2. Ubi Kuning, another yellow-fleshed root tuber cassava showed different profile of carotenoid genes as the two-yellow cassava, i.e., the lowest expression level of \( LYCa \) and higher expression level of \( BCH1 \), indicating the accumulation of only β-carotenoids. These results provide insights into the molecular regulation of carotenoid biosynthetic gene, which may assist the new cultivar development aiming at improved carotenoid accumulation.

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6. References
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