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The ripening influence of two papaya cultivars on carotenoid biosynthesis and radical scavenging capacity

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ABSTRACT

Carotenoid biosynthesis in papaya fruits from the cultivars (cv.) 'Golden' and 'Sunrise Solo' was studied throughout three different ripening stages. The content of these secondary metabolites was assessed using HPLC–PDA–MS^n. Carotenoid levels increased during ripening, with all-*trans*-lycopene varying from 0.73 to 1.58 μg/g in the cv. 'Golden' and from 0.68 to 1.67 μg/g in the cv. 'Sunrise Solo'. The all-*trans*-β-cryptoxanthin content varied from 1.29 to 3.0 μg/g in the cv. 'Golden' and from 0.28 to 5.13 μg/g in the cv. 'Sunrise Solo'. The *Zds* gene showed a different pattern of expression during the ripening and between cultivars, while the *Lcy*β gene expression was up-regulated in the two cultivars. The capacity to scavenge peroxyl radicals did not show a significant difference among the ripening stages and between the different cultivars. This study describes, for the first time, a tentative correlation between carotenoid biosynthesis in papaya pulp and the gene expression of the enzymes related to this pathway.

1. Introduction

Papaya (*Carica papaya* L.) is a climacteric fruit worldwide consumed with per capita consumption of 6.4 g/day in Brazil (Instituto Brasileiro de Geografia e Estatística (IBGE), 2011). The fruit is much appreciated for its sweetness and soft pulp (Fabi et al., 2007) and is mostly consumed ripened and *in natura*. Although its origin location is not well defined, evidence suggests the American tropics as the main source of this fruit (Garret, 1995). Its adaptation to the Brazilian climate has allowed Brazil to become one of the largest producers of papaya in the world, producing 1.9 million tons of fruit in 2008 (Instituto Brasileiro de Geografia e Estatística (IBGE), 2010).

The main papaya cultivars are 'Sunrise Solo', also known as *Hawaí* in Brazil, and 'Formosa'. Recently, the cv. 'Golden', a mutant form of the cv. 'Sunrise Solo', that emerged in Brazilian farms has reached international markets due to its attractive flavor and taste, and also because of its abundant, sweet, soft, and red pulp (Fabi et al., 2007; Fabi et al., 2014). In addition to the pleasant flavor, papaya is a known dietary source of bioactive compounds such as carotenoids (Fabi et al., 2007), mainly all-*trans*-lycopene, all-*trans*-β-cryptoxanthin and all-*trans*-β-carotene (Barreto et al., 2011).

The beneficial effects of eating carotenoid-rich foods include nutritional assistance in human recovery from various types of cancer, cardiovascular diseases, and diseases caused by sensitivity to UV radiation (Fiedor & Burda, 2014). Along with other factors, one of the hypotheses is that this effect is due to the antioxidant properties of carotenoids, which minimize the oxidative damage induced by the reactive species of oxygen and nitrogen in vivo (Rock, 2009).

The accumulation of carotenoids occurs during fruit ripening, resulting in alterations in tissue pigmentation. Generally, in higher plants, the carotenoid biosynthesis pathway begins with the condensation of isopentenyl diphosphate (IPP) (C5) and its isomer, dimethylallyl diphosphate (DMADP), generating a molecule of geranyldiphosphate C10 (GPP) (Britton, Pfander, & Liaaen-Jensen, 1998). The condensation of 2 geranylgeranyldiphosphate (GGDP) molecules and the elimination of diphosphate by the enzyme phytoene synthase (PSY) result in the synthesis of phytoene (C_{40}), a colorless carotenoid containing 3 conjugated double bonds. The phytoene is in turn converted to ζ-carotene by phytoene desaturase (PDS) and subsequently converted into colored carotenoids such as lycopene (Britton et al., 1998; Fraser & Bramley, 2004) through the action of ζ-carotene desaturase (ZDS). In cultivars such as the yellow papaya, lycopene is rapidly converted into β-carotene by the action of lycopene beta-cyclase (LCY-β), which is in turn converted into xanthophylls (β-cryptoxanthin and zeaxanthin) via β-carotene hydroxylase (β-CH) (Blas et al., 2010). However, in cultivars with red pulp, the conversion of lycopene in cyclic carotenoids

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is suppressed or even inhibited, leading to lycopene accumulation (Yan, Gao, Shen, & Zhou, 2011).

Although several studies have compared the expression of genes related to carotenoid biosynthesis in carotenoid-rich fruits (Yan et al., 2011; Ronen, Carmel-Goren, Zamir, & Hirschberg, 2000) focusing on the correlation between gene expression and the synthesis and antioxidant capacity of carotenoids during the ripening of cv. 'Golden' and cv. 'Sunrise Solo' papayas are still elusive.

In this context, the aim of this study was to evaluate the expression of genes involved in carotenoid synthesis in two distinct papaya cultivars ('Golden' and 'Sunrise Solo') during ripening and to correlate gene expression with carotenoid levels and peroxyl scavenging capacity.

2. Materials and methods

2.1. Material

Methyl-*tert* butyl ether (MTBE) and methanol were acquired from Merck (Darmstadt, Germany), and other analytical-grade reagents were purchased from Labsynth (Diadema, Brazil). Samples and solvents were filtered through Millipore membranes (Billerica, MA, USA) (0.45 μm) before high performance liquid chromatography (HPLC) analysis. All-*trans*-β-carotene (99.5%), all-*trans*-β-cryptoxanthin (99.0%), all-*trans*-lutein (98.0%), and all-*trans*-lycopene (99.0%) standards were acquired from Sigma-Aldrich (Darmstadt, Germany). Azobisisobutyronitrile (AIBN) was obtained from Mig Quimica (São Paulo, Brazil), and the fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$, MW = 504.43 g/mol) was acquired from Invitrogen™ (Carlsbad, CA).

2.2. Samples

Papaya fruits from cv. 'Golden' and cv. 'Sunrise Solo' were acquired directly from the farmer located in Jaguaré city (latitude 18°54′10″S and longitude 40°04′16″W), Espírito Santo State, Brazil. All the fruits were in their initial stage of ripening (at approximately 150 days after anthesis) and did not have any prior hot nor ethylene treatment. Nine specimens of each cultivar were carefully selected (no injuries) and allowed to ripen at 20 °C and 90% humidity. Three ripening stages (green, intermediate, and ripe) were distinguished according to the peel color as described elsewhere (Tatagiba & Oliveira, 2000; Fonseca et al., 2007; De Oliveira & Vitória, 2011). The specimens analyzed (Figs. 2SM and 3SM — Supplementary Material) on the first day consisted of papayas in their first stage of ripening (green), including fruits with 0 to 25% yellow skin (Fig. 2SM — G1 and S1). In the second stage (intermediate), the third day of the experiment, the fruits showed 25 to 75% yellow skin (Fig. 2SM — G2 and S2). In the third stage, analyzed on the fifth day of the study, the fruits had 75 to 100% yellow skin (ripe) (Fig. 2SM — G3 and S3). Each sample was obtained by manual peeling and removing the seeds. The pulps were homogenized N_2 using pestle and mortar, the samples were stored at −80 °C.

2.3. Carotenoid analysis

Carotenoids were exhaustively extracted with acetone from 15 g of papaya pulp from both cultivars at each of the 3 ripening stages (green, intermediate, and ripe) and transferred into light petroleum/diethyl ether (2:1), saponified with 10% methanolic KOH overnight at room temperature, washed to remove all traces of alkali, concentrated in a rotary evaporator (T < 38 °C), and stored under nitrogen atmosphere in a freezer (De Rosso & Mercadante, 2007a; Barreto et al., 2011). The analysis was carried out using a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps (model LC-20AD), an on-line degasser, and a Rheodyne injection valve (Rheodyne LCC, Rohnert Park, CA) with a 20 μL loop. The equipment included, connected in series, a photodiode array detector (PDA) (Shimadzu, model SPD-M20A) and a mass spectrometer with an ion-trap analyzer and atmospheric pressure chemical ionization source (APCI) from Bruker Daltonics, model Esquire 4000 (Bremen, Germany). Carotenoid separation was carried out on a C_{30} YMC column (5 μm, 250 × 4.6 mm i.d.) (Waters, Wilmington, MA) using as mobile phase a linear gradient of methanol/MTBE (methyl *tert*-butyl ether) from 95:5 to 70:30 in 30 min, to 50:50 in 20 min, and maintaining this proportion for 35 min. The flow rate was 0.9 mL/min, and the column temperature was set at 28 °C. The experimental conditions for identification and quantification by HPLC–DAD–APCI–MS^2 were the same as previously described (De Rosso & Mercadante, 2007a; Silva, Rodrigues, Mercadante, & De Rosso, 2014).

Carotenoid identification was based on the following parameters: elution order on reverse phase (C_{30}); UV–visible (absorption wavelength ($\lambda_{máx}$), spectral fine structure (%III/II), and peak *cis* intensity); co-chromatography with authentic standards; and comparison of the mass spectrum with available data (Enzell & Back, 1995; Britton, Liaaen-Jensen, & Pfander, 2004; Crupi, Preedy, & Antonacci, 2012). For quantification, calibration curves were constructed for all-*trans*-β-cryptoxanthin, all-*trans*-lutein, all-*trans*-lycopene and all-*trans*-β-carotene, with a minimum of 7 concentration levels (5 to 60 μg/mL). The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the parameters of each standard curve: LOD = 3.3 × SD / S and LOQ = 10 × SD / S, where SD is the standard deviation of the response and S is the slope of the curve. For the four analytical curves of carotenoids, $R^2 = 0.99$, the limit of detection was 0.1 μg/mL, and the limit of quantification was 0.5 μg/mL. The carotenoids present in low concentrations in the samples or whose standards were not available, such as all-*trans*-neoxanthin, all-*trans*-violaxanthin, and *cis*-lutein, were quantified using the curve of all-*trans*-lutein; 5,6-epoxy-β-cryptoxanthin was quantified using the curve of all-*trans*-β-cryptoxanthin; and 13-*cis*-lycopene, prolycopene, and phytofluene were quantified using the all-*trans*-lycopene curve. The carotenoid concentration was expressed in μg/g of fresh matter. All analyses were performed in triplicate.

2.4. Quantitative analysis of gene expression by real-time PCR (qPCR)

Gene expression analyses were accomplished according to Fabi et al. (2012). The procedures in 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments — MIQE' were strictly followed according to Bustin et al. (2009). Total RNA was extracted from papaya pulp according to Fabi, Cordenunsi, Seymour, Lajolo, and Do Nascimento (2009) with modifications. One hundred milligrams of pulp was extracted using the ConcertTM Plant RNA Reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, the extracted RNA was treated with DNAse, and integrity was determined by agarose gel electrophoresis. RNA concentration was spectrophotometrically determined using a Synergy H1 spectrophotometer (Biotek Instruments Inc., Vermont, USA). cDNA synthesis was achieved using the Revert Aid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). Primers were designed as previously described by Bustin et al. (2009) and are depicted in Supplementary Material (Table 1SM). The accession numbers from the GenBank nucleotide database used to analyze the gene sequences under study were as follows: DQ666828 for the phytoene synthase gene; FJ599643.1 for the lycopene β-cyclase gene; DQ666830.2 for the phytoene desaturase gene; FJ812088.1 for the ζ-carotene desaturase gene, and HQ998850 for the β-carotene hydroxylase gene. Amplicons were confirmed by cloning and sequencing. The control gene used in the experiments was the elongation factor gene (*TEF*), present on chromosome LG9 contig 1059 (ABIM01001059), analyzed by the following primers: papaya_tef_f: 5′-GTTAAGAACGTTGCCGTGAAG 3′ and papaya_tef_r: 5′-ATGTGAAGTTGGCTGCTTCCT-3′. The cycle threshold (CT) comparison method described by Livak and Schmittgen (2001) was used to evaluate