Antioxidant Balance and Regulation in Tomato Genotypes of Different Color

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ABSTRACT. Antioxidants, antioxidant capacity, and the expression of isoprenoid metabolism–related genes and two pigmentation–related transcription factors were studied in four native and four hybrid tomato (Solanum lycopersicum) genotypes with different-colored fruit. Red fruit genotypes were associated with greater lycopene, β-carotene, lipophilic antioxidant capacity, and greater chromoplast-specific lycopene β-cyclase (CYC-B) transcript levels. Orange fruit genotypes had greater concentrations of tocopherols and greater transcript levels of homogentisate phytyl transferase (VTE-2), 1-deoxy-D-xylulose phosphate synthase (DXS), and 4-hydroxyphenylpyruvate dioxygenase (HPPD). The yellow fruit genotype was greater in total polyphenol and hydrophilic antioxidant capacity with greater expression of geranylgeranyl reductase (GGDR), phytol kinase (VTE-5), phytoene synthase (PST) 2, lycopene β-cyclase (LCY-B), SINAC1, and SINAC4. Greater levels of individual antioxidants were associated with specific coloration of tomato fruit. Moreover, the negative correlations between the expression of PSY1 and VTE-5, and between lycopene and chlorophyll, suggest a balance between carotenoids, tocopherols, and chlorophylls. The results of this study support either the direct commercialization of tomatoes with different color fruit or use of their genotypes in breeding programs to increase antioxidant levels among existing cultivars.

Tomato fruit are an excellent source of antioxidants and contribute significantly to human health because of their anti-inflammatory, antiallergenic, and antithrombotic properties. Carotenoids and tocopherols are among the major lipophilic antioxidants present in tomatoes, whereas hydrophilic antioxidants include vitamin C and polyphenols (Viuda-Martos et al., 2014). Polyphenols are powerful antioxidants that have been reported to interfere with the initiation, promotion, and progression of cancer and can be divided into different groups according to their structure. The main tomato polyphenols are hydroxycinnamic acids, flavanones, flavonols, and anthocyanins (Marti et al., 2016). Tomato fruit has been widely used as a model for the study of fleshy fruit ripening because of its unique characteristics, such as the presence of well-characterized ripening mutants, reduced plant size, a well-characterized genome, and a short life cycle (Liu et al., 2015).

The red pigmentation of the ripe fruit is one of the most recognizable features of the commercial tomato. The change from green to red occurs due to the degradation of chlorophyll and the accumulation of carotenoids. The carotenoids that accumulate in commercial tomato fruit are lycopene (~90%), β-carotene (5% to 10%) and lutein (1% to 5%), with only trace concentrations of other compounds (Stahl and Sies, 2003).

In recent years, the biosynthetic pathways of carotenoid, tocopherol, and chlorophyll have been studied because of their importance in understanding the regulatory cross-talk that contributes to the nutritional quality of tomato fruit (Almeida et al., 2014; Quadrana et al., 2013). Carotenoids, tocopherols,
and chlorophylls are isoprenoid-derived compounds that are synthesized through the 2-C-methyl-D-erythritol-4-phosphate pathway with geranylgeranyl pyrophosphate as a common precursor (Almeida et al., 2014). Chlorophyll metabolism is closely linked to carotenoid and tocopherol biosynthesis, which are the major lipid-soluble antioxidants (Lira et al., 2016). In the metabolic pathway of carotenoid biosynthesis, the enzyme PSY catalyzes the condensation of 2-geranylgeranyl pyrophosphate to form lycopene (Liu et al., 2015). The cyclization of lycopene for the formation of the luteins in chromoplasts, and PSY2 is associated with chloroplasts.

Carotenoids and an immediate precursor of lycopene (Liu et al., 2015). The first step of the tocopherol pathway is catalyzed by HPPD involving the reduction of 4-hydroxyphenylpyruvate to homogentisate, which is further decarboxylated and then condensed with a prenyl donor by the prenyltransferase, VTE-2. The prenyl donor for tocopherol biosynthesis, phytol diphosphate, also can be synthesized from phytol recycling after chlorophyll degradation by VTE-5 (Quadrana et al., 2013). Therefore, it is considered that tomato fruit of different colors are an interesting model for the study of the isoprenoid–metabolic cross-talk of tocopherol, chlorophyll, and carotenoid pathways. NAC (NAM/ATAF1/2/CUC2) genes constitute one of the largest families of plant-specific transcription factors and influence a diverse set of developmental processes. Although NAC genes are involved in stress responses, SINAC-1 (S. lycopersicum NAC-1) and SINAC-4 (S. lycopersicum NAC-4) have been associated with fruit development and carotenoid accumulation (Zhu et al., 2014).

Mexico is a center of diversification and domestication of tomato and thus offers a wide range of native genotypes with fruit of different colors, shapes, and sizes. These genotypes could be integrated into breeding programs aiming to increase the nutraceutical properties of commercial cultivars and recover antioxidant compounds that have been lost through the selection process (Rodriguez et al., 2013). The aim of this study was to evaluate the variation in carotenoid and tocopherol content among selected hybrid and native tomato lines. In addition, the expression of isoprenoid metabolism–related genes and two pigmentation-related transcription factors were determined, as well as assays for antioxidant capacity of the lipophilic and hydrophilic fractions, total polyphenols, flavonoids, anthocyanins, vitamin C, and chlorophylls.

Materials and Methods

Plant material and collection

Eight tomato genotypes with different-colored fruit were used for this study (Fig. 1). Four native tomato lines, including 15014 [red (Rn)], 15006-A [orange (On)], 15011 [yellow (Yn)], and 15440 [purple (Pn)] were obtained from Colegio de Postgraduados, Mexico. The lines were collected from the following locations: O_N, San Gabriel Chilac, Puebla, Mexico (lat. 18.3261°N, long. 97.3485°W, altitude 1232 m); R_N and Y_N, Xicotepec, Puebla (lat. 20.187°N, long. 97.966°W, altitude 1142 m); and P_N, Tehuacán, Puebla (lat. 18.429°N, long. 97.466°W, altitude 1890 m). They were subject to individual selection (pedigree method for three generations) so that they would be homozygous lines. Four tomato hybrid experimental lines, H1329 (light red; LR_H), H1326 (light orange; LO_H), and HN (chartreuse; CH_H), were obtained from Universidad Autónoma de Chapingo. All genotypes used in this study were part of the Mexican Network of Plant Genetic Resources (Red Mexicana de Recursos Fitogenéticos).

The studied lines, native and hybrid, were planted in a completely randomized block design with three replications, and each experimental unit comprised 10 plants. Native genotypes and hybrid experimental lines were cultivated simultaneously at 22 to 30 °C, with a relative humidity of 75% in two greenhouses 2.4 km apart under the same environmental light conditions. The first greenhouse was located at lat. 19.4633°N, long. 98.9075°W, altitude 2295 m and the second was at lat. 19.4865°N, long. 98.9011°W, altitude 2295 m. Plants were grown in volcanic sand substrate and a Steiner nutrient solution (Steiner, 1961) leading to plants with a single stem. Fruit were selected when ripe on the basis of a color chart developed specifically for each genotype according to their maximum color development (Namitha et al., 2011). The fruit were harvested free of mechanical damage, physical defects, pests, and blemishes and transported immediately after harvest to the Autonomous Metropolitan University-Iztapalapa. A total of 81 pieces of fruit (three biological replicates of 27 fruit) of each genotype were washed, and the seeds and placenta were removed. Hue was measured in three pieces of fruit of each replicate in three different sections of the fruit surface with a colorimeter.
Extraction of the lipophilic and hydrophilic fractions

Lipophilic and hydrophilic antioxidants were sequentially extracted with 75% (v/v) aqueous methanol and dichloromethane (1:1 v/v) and then separated by 50 mM Tris-HCl buffer, pH 7.5. One gram of liquid nitrogen–powdered fruit tissue was used for each extraction. Samples were centrifuged in the dark at 4 °C for 5 min at 2599 g. Each phase (lipophilic and hydrophilic) was used to assess antioxidant composition and capacity. Data were recorded on a fresh weight basis.

In vitro antioxidant capacity

Antioxidant capacity was assayed in both hydrophilic and lipophilic extracts with three techniques: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, the 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, and the cupric ion reducing antioxidant capacity (CUPRAC) method. The ABTS assay was performed according to Re et al. (1999) with minor modifications. An ABTS solution was prepared according to Re et al. (1999). This was diluted in 96% (v/v) ethanol for lipophilic extracts. Absorbance was measured at 734 nm after 10 min of reaction.

The DPPH assay was performed using the method described by Brand-Williams et al. (1995). Absorbance was measured at 515 nm after 15 min of reaction.

The CUPRAC assay was performed according to Özyürek et al. (2011). In it, 1 mL of CuCl2, 1 mL of NH4Ac buffer, 1 mL of neocuproine, and 1.1 mL of sample were diluted in water (hydrophilic phase) or 96% ethanol (lipophilic phase) for a total volume of 4.1 mL. Absorbance was measured at 450 nm.

All the results were acquired by interpolating absorbance on a calibration curve obtained with Trolox and recorded on a fresh weight as micromoles Trolox equivalents (TE) per gram fresh weight.

Hydrophilic antioxidant assays

**Vitamin C (ascorbic acid).** Five grams of powder of each sample was homogenized in the dark with a hand blender (2614; Oster, Boca Raton, FL), filtered through gauze, and centrifuged for 5 min at 2599 g, at 4 °C. The amount of ascorbic acid was determined by isocratic elution with high-performance liquid chromatography (HPLC), as described by Nour et al. (2010). Chromatographic separation was performed with an HPLC system (1260 Series; Agilent Technologies, Santa Clara, CA), using 50 mM potassium dihydrogen orthophosphate buffer, pH 2.8, as a mobile phase (flow rate of 0.7 mL·min⁻¹), a 250 × 4.6-mm, 5-μm analytical column (HyperSil Gold aQ; Thermo Fisher Scientific, Waltham, MA), and diode array detection at λ = 254 nm. The calibration curve was produced using a commercial standard (Sigma-Aldrich, St. Louis, MO).

**Total phenolics.** Hydrophilic extracts were used to determine total polyphenols. The hydrophilic extract was incubated with Folin-Ciocalteau reagent 1:10 v/v and 7.5% w/v sodium carbonate for 1 h. The total polyphenol content was determined by spectrophotometry at 765 nm. The results were acquired by interpolating absorbance on a calibration curve obtained with gallic acid. Results were expressed on a fresh weight basis as micrograms of gallic acid equivalents.

**Total flavonoids.** Hydrophilic extracts were used to determine total flavonoid content using the method of Chang et al. (2002). The standard curve was obtained with quercetin, and the absorbance was measured at 415 nm. The results were expressed on a fresh weight basis as micrograms of quercetin equivalents.

**Total anthocyanins.** Hydrophilic extracts were used for the determination of anthocyanin content by the differential pH method reported by Wrolstad (1993). The absorbance was measured at 520 and 700 nm at pH values of 1.0 and 4.5. The results were expressed as micrograms on a fresh weight basis.

**Lipophilic antioxidant assays**

**Carotenoids.** Carotenoids were extracted from 5 g of liquid nitrogen–powdered fruit using a mixture of hexane/acetone/ethanol 50:25:25 [v/v/v (López-Vidal et al., 2016)]. The filtrate was injected into an HPLC (1200 Series; Agilent Technologies) equipped with a 250 × 4.6-mm, 5-μm column (XTerra C18; Waters Corp., Milford, MA). A multiple wavelength detector (λ = 459 nm) was used. The mobile phase consisted of acetonitrile/methanol/dichloromethane (43:43:14 v/v/v), and the flow was 1 mL·min⁻¹. Commercial standards (Sigma-Aldrich) were used for the identification and quantification of lycopene, β-carotene, and lutein. The results were expressed as micrograms on a fresh weight basis.

**Vitamin E (tocopherols).** The lipophilic extract was used to measure tocopherols according to Méne-Saffrané and Della-Penna (2010). The filtrate was injected into an HPLC (1200 Series, Agilent Technologies) equipped with a 250 × 4-mm, 5-μm column (LiChrospher 100 Diol; Merck, Darmstadt, Germany) at 25 °C. A fluorescence detector was used with an excitation and emission wavelength of 296 and 340 nm, respectively. The mobile phase consisted of hexane/methyl tert-butyl ether (90:10 v/v), and the flow rate was 1 mL·min⁻¹. For the identification and quantification of α-, β-, γ-, and δ-tocopherols, calibration curves were constructed using commercial-grade HPLC standards (Millipore, Burlington, MA). Results were expressed as micrograms on a fresh weight basis.

**Chlorophyll assay**

The extraction and assay of chlorophylls A and B used the spectrophotometric method described by Costache et al. (2012). One gram of liquid nitrogen–powdered fruit was mixed with a 90% v/v methanol aqueous solution. The measurements were carried out at wavelengths of 666 and 653 nm.

**Expression of the genes encoding vitamin E and carotenoid biosynthetic enzymes**

A total of 11 genes of the pathway of carotenoids, tocopherols, and chlorophylls were selected for this study according to their relevance as regulators. RNA isolation was performed according to the technique reported by Chang et al. (1993). Six grams of fruit was crushed to a powder in liquid nitrogen. The purity and concentration of the RNA were determined by spectrophotometry (absorbance 260/280 nm), and the integrity was verified by 1% w/v agarose gel electrophoresis stained with ethidium bromide. Nine regulatory genes of the pathway of carotenoids, tocopherols, and specific oligonucleotides for genes VTE-2, VTE-5, DXS, and HPPD were used as reported by Almeida et al. (2014) (Table 1), whereas the rest of the oligonucleotides were designed using the software Primer3Plus (Untergasser et al., 2007).
The identity of amplification products was verified before performing reverse transcription quantitative polymerase chain reaction (RT-qPCR). The amplification efficiency was found to be between 95% and 100% for all the studied genes. Two micrometers of RNA were treated with 1 U-µL⁻¹ DNase I, RNase free (Thermo Fisher Scientific). The transcripts of interest were amplified by RT-qPCR using the Express SYBR GreenER qPCR Supermix kit (Thermo Fisher Scientific). One microliter of RNA treated with DNase was added per tube in a final volume of 10.5 µL. The 18S RNA fragment was used as a reference gene because it showed minimal variability in its expression between genotypes. The following temperature program was used: reverse transcription at 37 °C for 10 min, initial denaturation at 95 °C for 3 min, 94 °C for 30 s, annealing temperature of 58 °C (PSY1, PSY2, CYC-B, and LCY-B), or 60 °C (GGDR, VTE-2, VTE-5, and HPPD) for 20 s, and an extension temperature of 72 °C for 30 s. Thirty cycles were required for all genes, except for LCY-B, which required 32 cycles. Relative mRNA expression was calculated by the 2⁻ΔΔCt method (Villa-Hernández et al., 2013). A value of 1 was arbitrarily assigned to every replicate of the mRNA expression of the RN genotype, and the relative expression of the other genotypes was calculated using this genotype as reference. Three biological replicates were used and every replicate was loaded two times to minimize the error.

**Statistical analysis**

Analyses were carried out using analysis of variance with the Tukey test (significance level of 0.05) using Prism (version 7.0; GraphPad Software, San Diego, CA). Principal component analysis (PCA) was performed using Statgraphics Plus for Windows (version 4.0; StatPoint, Warrenton, VA).

**Results and Discussion**

**In vitro antioxidant capacity**

In all genotypes, a greater antioxidant capacity was found in the hydrophilic extract compared with the lipidic extract (Table 2). Differences in antioxidant capacity among genotypes showed the same trend across all three determination methods; however, differences in the lipophilic phase were less discriminating when using the DPPH and CUPRAC methods, probably because the detection wavelengths used for these techniques (515 and 450 nm, respectively) were close to the wavelength at

| Table 1. Forward and reverse sequences of oligonucleotides for the 11 genes studied and reference gene (18S) (Almeida et al., 2014). |
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| **Gene** | **Forward sequence** | **Reverse sequence** |
| Phytoene synthase 1 (PSY1) | 5'-ACCTGATGATGCCAACAGTCGTTATCAAAATGC-3' | 5'-CCATCCGAGACGTCGTCAATG-3' |
| Phytoene synthase 2 (PSY2) | 5'-TGCGTATGGCACTTGTGAAAGT-3' | 5'-CGATCCCAAAGGCGTCAATG-3' |
| Lycopene β-cyclase (LCY-B) | 5'-GCCGCGTATGCTCAATG-3' | 5'-CCATCCGAGACGTCGTCAATG-3' |
| Chromoplast-specific lycopene β-cyclase (CYC-B) | 5'-AGTTCAATTCGATGCTCAATG-3' | 5'-CCCAAGAGGCGTCAATG-3' |
| Geranylgeranyl reductase (GGDR) | 5'-CCAGGCGGTGGAAGAAGTTC-3' | 5'-GGCGGTGGAAGAAGTTC-3' |
| VTE-2 | 5'-CCATCCGAGACGTCGTCAATG-3' | 5'-CCCAAGAGGCGTCAATG-3' |
| VTE-5 | 5'-CGATCCCAAAGGCGTCAATG-3' | 5'-CCCAAGAGGCGTCAATG-3' |
| 1-Deoxy-D-xylulose phosphate synthase (DXS) | 5'-GCACTGTTGTGTTTACG-3' | 5'-GGGATAGTTCTACGTTG-3' |
| p-Hydroxyphenylpyruvate dioxygenase (HPPD) | 5'-CACAGCCGTTGAAGAATTG-3' | 5'-CCAGGCGGTGGAAGAAGTTC-3' |
| SINAC-1 | 5'-GCACTTGGATGTTGATTTGACG-3' | 5'-GGGATAGTTCTACGTTG-3' |
| SINAC-4 | 5'-CCATGCTTCGATGCTCAATG-3' | 5'-CCCAAGAGGCGTCAATG-3' |
| 18S | 5'-AAACCGGTTATGCTCAATG-3' | 5'-CCCAAGAGGCGTCAATG-3' |

| Table 2. Antioxidant capacity of tomato genotypes by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, cupric ion reducing antioxidant capacity (CUPRAC), and t2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) methods. Trolox equivalents (TE) values represent the means from three biological replicates of 27 fruit each. |
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| Genotype | DPPH Lipophilic fraction [µmol/g] | Hydrophilic fraction [µmol/g] | CUPRAC Lipophilic fraction [µmol/g] | Hydrophilic fraction [µmol/g] | ABTS Lipophilic fraction [µmol/g] | Hydrophilic fraction [µmol/g] |
| RN | 0.012 ± 0.005 a | 0.041 ± 0.002 c | 6.20 ± 0.19 a | 8.61 ± 0.38 b | 2.05 ± 0.05 a | 7.48 ± 0.85 c |
| ON | 0.011 ± 0.005 b | 0.046 ± 0.006 b | 6.42 ± 0.08 b | 8.89 ± 0.30 b | 0.85 ± 0.01 d | 8.02 ± 0.11 b |
| YN | 0.008 ± 0.0002 d | 0.054 ± 0.0017 a | 3.51 ± 0.10 d | 12.9 ± 0.36 a | 0.50 ± 0.06 e | 11.08 ± 0.22 a |
| PN | 0.009 ± 0.0002 c | 0.028 ± 0.0005 d | 3.78 ± 0.25 c | 5.61 ± 0.34 d | 0.51 ± 0.02 e | 3.72 ± 0.10 d |
| LRH | 0.012 ± 0.0011 a | 0.037 ± 0.0005 c | 4.44 ± 0.22 b | 9.60 ± 0.86 b | 1.21 ± 0.03 c | 7.30 ± 0.12 c |
| LOH | 0.011 ± 0.0002 b | 0.032 ± 0.0006 d | 4.36 ± 0.02 b | 7.28 ± 0.66 c | 0.84 ± 0.02 d | 7.15 ± 0.04 c |
| CH | 0.005 ± 0.00004 e | 0.012 ± 0.0006 c | 3.02 ± 0.35 d | 3.29 ± 0.43 e | 0.40 ± 0.01 e | 1.86 ± 0.04 e |
| BH | 0.010 ± 0.0003 b | 0.040 ± 0.0003 e | 4.56 ± 0.25 b | 6.44 ± 0.92 c | 1.76 ± 0.03 b | 7.55 ± 0.10 c |

aRN = red native; ON = orange native; YN = yellow native; PN = purple native; LRH = light red hybrid; LOH = light orange hybrid; CH = chartreuse hybrid; BH = black hybrid.

bValues followed by the same letter in each column are not significantly different among each other according to the Tukey multiple comparison test (α = 0.05).
which carotenoids were detected (400–500 nm). This suggested that the most appropriate method to measure antioxidant capacity in tomatoes was ABTS followed by CUPRAC and then DPPH.

The YN genotype had the greatest antioxidant capacity in the hydrophilic fractions from the three determination methods (0.054, 12.9, and 11.08 μmol·g⁻¹ TE for DPPH, CUPRAC, and ABTS, respectively). For the lipophilic fractions, greater antioxidant capacities were observed among the red genotypes. The RN genotype had the greatest lipophilic antioxidant capacity with values of 0.0126 (DPPH), 5.20 (CUPRAC), and 2.05 (ABTS) μmol·g⁻¹ TE.

**Hydrophilic antioxidants**

**Vitamin C.** Ascorbic acid levels were greatest in the RN and ON genotypes [665 and 472 μg·g⁻¹, respectively (Table 3)] and the lowest was in the genotype YN at 3.4 (± 0.1) μg·g⁻¹. Hernández-Suárez et al. (2008) found ascorbic acid levels in Dorothy, Boludo, and Dunkan cultivars of Tenerife-grown tomatoes of 138 to 157 μg·g⁻¹. In addition, according to Gould (1992), cultivars could be considered a significant source of ascorbic acid with contents higher than 200 μg·g⁻¹; on this basis, five of the eight genotypes analyzed in this work [C11, LRH, ON, PN, and RN] would be considered a significant source of ascorbic acid because they had values >200 μg·g⁻¹. At physiological pH, vitamin C exists as monooanion form called ascorbate, which has proven roles in stress tolerance in tomato and fulfills numerous roles, mainly through its antioxidant potential (Baldet et al., 2013).

**Total Polyphenols.** The YN genotype had the greatest polyphenol content when compared with the rest of the genotypes [64.1 μg·g⁻¹ gallic acid equivalents vs. 10.2–49.8 μg·g⁻¹ gallic acid equivalents (Table 3)], as well as the greatest total antioxidant capacity and antioxidant capacity of the hydrophilic fractions. We proposed that this genotype did not need to develop high levels of vitamin C as a defense response against abiotic stress because of its high polyphenol content. Our results also indicate that polyphenols have a greater overall contribution to antioxidant capacity when compared with carotenoids and other antioxidants. Barros et al. (2012) determined the polyphenol content in red and yellow tomato cultivars from Portugal and found greater levels in the yellow genotype (54.23 μg·g⁻¹ gallic acid equivalents). Therefore, it was suggested that there was a relationship between the content of phenolic compounds and yellow coloration. It was also proposed that polyphenol contents had been reduced as a result of plant domestication, because wild genotypes contained greater contents of these compounds (Di Paola Naranjo et al., 2016). The total polyphenol content also might be correlated with fruit size, because the bigger genotypes of this study (C11 and PX) had the lowest levels. A possible explanation for this could be that smaller fruit genotypes have a larger surface area in relation to their volume and thus require greater protection against environmental changes, particularly in native genotypes. Furthermore, phenolic compounds in tomato fruit are concentrated in the skin (Bovy et al., 2002). The negative correlation between polyphenols and fruit size has been reported previously and it also suggests that development of cultivars of commercial fruit size with high polyphenol contents may be difficult (Hanson et al., 2004).

**Flavonoids.** Flavonoids are a group of polyphenolic compounds with important anti-inflammatory and antiallergenic effects. The main flavonoids in tomato are quercetin, kaempferol, and myricetin. In addition to carotenoids, flavonoids, which accumulate mainly in the epidermis, play an important role in the color of tomatoes (Karlova et al., 2014). We found the greatest total flavonoid content in genotype ON, followed by B11 and RN (Table 3). Among flavonoids, anthocyanins are purple pigments whose accumulation increases resistance to pathogens in tomato fruit (Zhang et al., 2013). Here, anthocyanins were found in the B11, PX, and RN genotypes [3.15, 1.22, and 0.26 μg·g⁻¹, respectively (Table 3)]. Our results suggest that native genotypes could be used to improve the levels of flavonoids in commercial cultivars.

**Lipophilic antioxidants**

**Carotenoids.** Fruit color is associated with flavor, health, and nutritional content because carotenoids are precursors of various aroma volatiles and play an important role in photo-protection and inhibiting lipid peroxidation. The strong antioxidant capacity, and the multiple benefits to human health, explains why we seek to select tomato genotypes with greater

Table 3. Total polyphenols, total flavonoids, total anthocyanins, and chlorophyll a and b in selected tomato genotypes. Values represent the means from three biological replicates of 27 fruit each. All values are expressed in a fresh weight basis.

| Genotype  | Polyphenols (gallic acid equivalents) | Flavonoids (quercetin equivalents) | Anthocyanins | Chlorophyll a | Chlorophyll b | Total chlorophyll | Vitamin C |
|-----------|--------------------------------------|------------------------------------|--------------|---------------|---------------|-----------------|-----------|
| RN        | 46.3 ± 30 b² | 0.16 ± 0.09 c | 0.26 ± 0.17 c | nd | nd | nd | 665 ± 3.4 a |
| ON        | 49.8 ± 6.6 b | 1.08 ± 0.08 a | nd | 14 ± 0.3 d | 8.9 ± 1 b | 22 ± 0.7 c | 472 ± 0.9 b |
| YN        | 64.1 ± 1.9 a | 0.02 ± 0.01 e | nd | nd | nd | nd | 0.34 ± 0.1 g |
| PN        | 21.0 ± 5.6 e | 0.06 ± 0.01 e | 1.22 ± 0.3 b | 227 ± 0.6 a | 69 ± 1.4 a | 296 ± 1.7 a | 222 ± 1 d |
| LRH       | 41.6 ± 2.1 c | nd | nd | nd | nd | nd | 339 ± 0.02 c |
| LOH       | 34.3 ± 9.8 d | 0.13 ± 0.01 d | nd | nd | nd | nd | 163 ± 2.4 e |
| C11       | 10.2 ± 3.1 f | nd | nd | 155 ± 0.02 b | 64 ± 0.06 a | 219 ± 0.15 b | 339 ± 7.1 c |
| B11       | 39.7 ± 0.41 c | 0.52 ± 0.14 b | 3.15 ± 1.0 a | 28 ± 0.02 c | 2.9 ± 0.1 b | 31 ± 0.07 c | 7.92 ± 12.4 f |

²RN = red native; ON = orange native; YN = yellow native; PN = purple native; LRH = light red hybrid; LOH = light orange hybrid; C11 = chartreuse hybrid; B11 = black hybrid.

²Values followed by the same letter in each column are not significantly different among each other according to the Tukey multiple comparison test (α = 0.05).

nd = not detected.
contents of carotenoids and other antioxidants (Klee and Giovannoni, 2011; Namitha et al., 2011).

Lycopene is among the main functional compounds in tomato fruit because of its strong antioxidant role (Stahl and Sies, 2003). Here, significant differences were found in the levels of lycopene (Table 4), which ranged from 3.59 (± 0.007) to 44.57 (± 0.99) μg·g⁻¹. The greatest levels were found in the RN genotype (44.57 μg·g⁻¹) followed by LRH and BH genotypes; the YN and CH genotypes had the lowest levels. The values found in this study were consistent with those reported by Lewinsohn et al. (2005), who found levels of 0.04 to 63.6 μg·g⁻¹ of lycopene. Lycopene can decrease the formation of erythema.

Table 4. Tocopherol and carotenoid contents of tomato genotypes. Values represent the means from three biological replicates of 27 fruit each. All values are expressed in a fresh weight basis.

| Genotype  | α-Tocopherol | β-Tocopherol | δ-Tocopherol | γ-Tocopherol | Tocopherol | α-Carotene | Lutein |
|-----------|--------------|--------------|--------------|--------------|------------|------------|--------|
|           | Mean ± SE (μg·g⁻¹) | | | | | | |
| RN        | 2.02 ± 0.14 a | 0.25 ± 0.02 d | 0.02 ± 0.018 c | 0.61 ± 0.102 b | 44.6 ± 0.49 a | 3.01 ± 0.09 a | 0.86 ± 0.012 b |
| O N       | 5.51 ± 0.04 b | 0.41 ± 0.04 c | 0.01 ± 0.003 c | 0.51 ± 0.004 b | 11.0 ± 0.11 d | 1.41 ± 0.03 b | 0.47 ± 0.106 b |
| Y N       | 1.57 ± 0.03 d | nd           | nd           | nd           | 3.93 ± 0.003 e | 0.36 ± 0.05 c | 1.12 ± 0.013 a |
| P N       | 1.23 ± 0.02 e | 0.22 ± 0.01 d | 0.01 ± 0.008 c | 0.10 ± 0.005 c | 11.5 ± 0.13 d | 0.21 ± 0.13 c | 0.40 ± 0.011 d |
| LR H      | 1.76 ± 0.06 d | 0.57 ± 0.00 b | 0.13 ± 0.03 a | 2.09 ± 0.026 a | 26.7 ± 0.09 b | 2.82 ± 0.02 a | 0.54 ± 0.017 c |
| LO H      | 7.90 ± 0.1 a  | 0.76 ± 0.05 a | 0.16 ± 0.051 a | 2.23 ± 0.238 a | 10.1 ± 0.02 d | 1.37 ± 0.26 b | 0.41 ± 0.002 d |
| C H       | 0.50 ± 0.02 f | 0.41 ± 0.01 c | 0.05 ± 0.017 b | nd           | 3.59 ± 0.01 e | 0.14 ± 0.01 c | 0.55 ± 0.008 c |
| B H       | 1.15 ± 0.05 e | 0.15 ± 0.02 e | 0.15 ± 0.002 e | 0.33 ± 0.008 b | 22.1 ± 0.14 c | 1.60 ± 0.04 b | 0.63 ± 0.013 bc |

R N = red native; O N = orange native; Y N = yellow native; P N = purple native; LR H = light red hybrid; LO H = light orange hybrid; C H = chartreuse hybrid; B H = black hybrid.

Values followed by the same letter in each column are not significantly different among each other according to the Tukey’s multiple comparison test (α = 0.05).

nd = not detected.

Fig. 2. Heat map representation of log2-fold change in RNA expression of carotenoid and tocopherol-related genes and NAC transcription factors in tomato compared with the native red (RN) genotype. Tocopherol, chlorophyll, and carotenoid biosynthetic pathways adapted from Almeida et al. (2016) and Lira et al. (2016) are indicated in orange, green, and red, respectively. The genes (GGDR = geranylgeranyl reductase; HPPD = 4-hydroxyphenylpyruvate dioxygenase; PSY-1 = phytoene synthase 1; PSY-2 = phytoene synthase 2; CYC-B = chromoplast-specific lycopene β-cyclase; LCY-B = lycopene β-cyclase; DXS = 1-deoxy-D-xylulose phosphate synthase; VTE-5 = phytol kinase; VTE-2 = homogentisate phytyl transferase; MEP pathway = non-mevalonate pathway; PP/PDP = phytyl diphosphate; GGDP = geranylgeranyl diphosphate; MBPQ = 2-methyl-6-phytyl-1,4-benzoquinol; and DMBQ = 2,3-dimethyl-6-geranylgeranylbenzoquinol) are indicated by the color of the genotype that showed the greatest transcript levels (red, orange, or yellow): O N = orange native; Y N = yellow native; P N = purple native; LR H = light red hybrid; LO H = light orange hybrid; C H = chartreuse hybrid; B H = black hybrid.
of the skin and inhibit prostate, breast, colorectal, lung, and pancreatic cancer. Greater intake of lycopene is recommended for patients with cancer and cardiovascular disease [35–75 mg daily (Stahl and Sies, 2003)].

β-carotene is a red–orange pigment and a precursor of vitamin A considered an essential compound for vision. It also prevents photo oxidative damage and sunburn (Almeida et al., 2014; Lewinsohn et al., 2005; Stahl and Sies, 2003). In this study, β-carotene levels were 80% to 90% lower than lycopene levels, ranging from 0.14 to 3.0 µg g⁻¹. The greatest β-carotene levels were found in the RN and LRH genotypes (Table 4).

Lutein was high in the YN genotype [1.12 ± 0.01 µg g⁻¹ (Table 4)]. Lutein is a yellow pigment synthesized in both chloroplasts and chromoplasts and promotes eye and skin health (Almeida et al., 2014). The values seen here were similar to those of Perry et al. (2009), who found a content of 0.32 µg g⁻¹ in round tomatoes.

Tocopherols. α-tocopherol, the most abundant form in tomatoes, was greatest in the orange genotypes (7.89 and 5.5 µg g⁻¹ in ON and LOH genotypes, respectively). This was close to values reported for Naomi red cherry tomato (7.4 µg g⁻¹), a genotype that is rich in α-tocopherol (Zanfini et al., 2010). In our study, β, δ, and γ tocopherol content was greatest in LRH and LOH genotypes (Table 4). These results support the use of tomato genotypes of different color either directly as food sources or in breeding programs to rescue functional compounds such as carotenoids, tocopherols, anthocyanins and vitamin C.

Chlorophylls

Some mature tomato genotypes may contain chlorophyll because it does not completely degrade during ripening (Carrillo-López and Yahia, 2014). We found the greatest levels of chlorophyll a in the BH genotype, followed by PN [0.276 and 0.227 mg g⁻¹, respectively (Table 3)]. Chlorophyll b was greater in the PN and CH genotypes (0.069 and 0.064 mg g⁻¹, respectively). In terms of total chlorophyll content, significant differences were found between genotypes, with the content greatest in BH (0.305 mg g⁻¹) followed by PN (0.296 mg g⁻¹), CH (0.219 mg g⁻¹), and finally ON genotype (0.022 mg g⁻¹).

Costache et al. (2012) reported an average of 0.25 mg g⁻¹ of total chlorophylls in cherry genotypes. The high levels of chlorophylls, carotenoids (mainly lycopene), and anthocyanins suggested that the black and purple color of these genotypes was the result of a combination of chlorophylls with these pigments.

Expression of enzyme-encoding genes of the carotenoid and tocopherol biosynthetic pathways

Chlorophylls, carotenoids, and tocopherols are isoprenoid-derived compounds that share a common precursor, geranylgeranyl diphosphate (Quadrana et al., 2013). The chlorophyll and tocopherol prenyl side chains derive from phytol diphosphate (PPD), which in turn is generated by GGDR. PPD for tocopherol biosynthesis also can originate from phytol kinase (VTE5) phosphorylation of phytol to phytol-phosphate. Recent evidence indicates that PPD for tocopherol biosynthesis is mostly supplied by chlorophyll degradation (Almeida et al., 2016; Lira et al., 2016). In this work, the transcript levels of PSY1 were greater in RN, LRH, and BH genotypes, whereas the lowest levels were found in the YN and CH genotypes (Fig. 2; Table 5). According to Lois et al. (2000), the increase in expression of PSY1 during fruit ripening is correlated with lycopene accumulation and is induced by ethylene. The CYC-B transcript levels were greatest in genotypes with greater lycopene and β-carotene levels. Hwang et al. (2016) found a greater CYC-B expression in orange genotypes with greater β-carotene content, suggesting a relationship between CYC-B expression and the levels of this carotenoid. A similar trend was found here with the genotypes with greater levels of β-carotene (RN, LRH, ON, LOH, and BH) also having significantly greater CYC-B transcript levels.

Table 5. Relative mRNA expression values of carotenoid and tocopherol-related genes and NAC transcription factors in tomato genotypes compared with the RN genotype.

| Gene              | Genotype | Mean ± SE | RN   | NO  | YN   | PN   | LRH  | LOH  | CH   | BH   |
|-------------------|----------|-----------|------|-----|------|------|------|------|------|------|
| GGDR              |          |           | 1.00±0.00 b | 1.640±0.5 b | 28.314±1.7 a | 0.397±0.03 b | 0.837±0.24 b | 0.740±0.13 b | 15.127±0.94 a | 1.624±0.12 b |
| HPPD              |          |           | 1.00±0.00 d | 1.922±0.22 b | 0.882±0.12 d | 1.296±0.03 c | 2.818±0.37 a | 1.358±0.09 c | 1.485±0.24 c |
| PSY1              |          |           | 1.00±0.00 a | 0.833±0.18 a | 0.176±0.09 c | 0.532±0.33 b | 0.787±0.10 a | 0.626±0.10 a | 0.124±0.06 a |
| PSY2              |          |           | 1.00±0.00 c | 1.223±0.19 c | 5.105±0.45 a | 0.968±0.15 b | 1.405±0.38 c | 0.826±0.13 d | 8.710±0.52 a |
| CYC-B             |          |           | 1.00±0.00 a | 0.784±0.10 a | 0.611±0.02 b | 0.193±0.15 c | 0.792±0.15 a | 0.980±0.11 a | 1.040±0.03 a |
| LCY-B             |          |           | 1.00±0.00 a | 1.338±0.35 c | 10.568±0.84 a | 0.660±0.17 d | 1.329±0.17 c | 0.538±0.11 d | 0.370±0.20 b |
| DXS               |          |           | 1.00±0.00 c | 5.072±1.50 a | 2.067±0.54 b | 0.923±0.31 c | 2.105±0.82 b | 0.506±0.12 a | 1.680±0.53 b |
| VTE-5             |          |           | 1.00±0.00 a | 0.495±0.16 c | 1.778±0.56 a | 0.734±0.16 b | 0.256±0.03 d | 0.314±0.02 d | 1.456±0.37 a |
| VTE-2             |          |           | 1.00±0.00 c | 4.512±0.38 a | 2.089±0.99 b | 2.474±0.63 b | 2.664±0.3 b  | 5.064±0.41 a | 0.964±0.39 c |
| SINAC-1           |          |           | 1.00±0.00 a | 2.881±0.67 b | 6.833±0.22 a | 3.056±1.02 b | 0.291±0.01 d | 0.894±0.08 c | 1.469±0.25 c |
| SINAC-4           |          |           | 1.00±0.00 b | 1.396±0.18 b | 1.884±0.13 a | 0.760±0.24 a | 0.422±0.04 d | 0.596±0.06 c | 1.163±0.25 b |

aGGDR = geranylgeranylglycyl reductase; HPPD = 4-hydroxyphenylpyruvate dioxygenase; PSY-1 = phytoene synthase 1; PSY-2 = phytoene synthase 2; CYC-B = chloroplast-specific lycopene β-cyclase; LCY-B = lycopene β-cyclase; DXS = 1-deoxy-D-xylulose phosphate synthase; VTE-5 = phyto kinase; VTE-2 = homogentisate phytyl transferase.

RN = red native; ON = orange native; YN = yellow native; PN = purple native; LRH = light red hybrid; LOH = light orange hybrid; CH = chartreuse hybrid; BH = black hybrid.

Values followed by the same letter in each column are not significantly different among each other according to the Tukey’s multiple comparison test (α = 0.05).

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PSY2, found exclusively in chloroplasts, was five times greater in the YN and CH genotypes than in the RN genotype. The transcript levels of LCY-B were also five times greater in the YN genotype than in the RN genotype, which also had greater lutein contents. These results suggest that the LCY-B enzyme was more involved in the conversion of δ-carotene to α-carotene for lutein synthesis than CYC-B (Namitha et al., 2011).

Regarding the genes of the tocopherol pathway, the transcript levels of GGDR were greatest in the YN and CH genotypes. VTE-5, associated with chlorophyll recycling, had a 1.8-fold greater relative expression in the YN and CH genotypes compared with the RN genotype. In contrast, the transcript levels of HPPD, DKS, and VTE-2 were greater in ON and LOH genotypes, which also had the greatest tocopherol contents. Almeida et al. (2014) report that the expression of VTE-2 increases with tocopherol synthesis.

NAC transcription factors

SINAC1 is the most-studied NAC transcription factor in tomato. Ma et al. (2014) reported that overexpression of SINAC1 resulted in reduced carotenoids by altering carotenoid pathway flux and decreasing ethylene synthesis, leading to yellow and orange mature fruit. In the present study, we found a greater expression of SINAC1 in both native and hybrid yellow fruit with lower lycopene and β-carotene levels, followed by those with orange, dark coloration, and finally red fruit (Table 5). Our results confirmed that SINAC1 expression was highly associated with carotenoid content and color of the fruit.

By comparison, SINAC4 has been reported to play an important role in carotenoid accumulation during tomato fruit ripening, acting as a positive regulator by modulating the hormone ethylene and therefore carotenoid pigmentation. Zhu et al. (2014) reported that fruit from RNAi transgenic tomato plants displayed orange color in both the pericarp and placenta, which implied decreased accumulation of lycopene and elevated β-carotene. A reduced expression of the gene PSY1 was reported, with the chromoplast and chloroplast lycopene β-cyclases (CYC-B and LCY-B) being upregulated in SINAC4 RNAi fruit as compared with controls. In the present study, a greater expression of SINAC4 was found in yellow fruit, suggesting that SINAC4 might be associated with yellow carotenoid lutein production, which is regulated mainly by LCY-B (Namitha et al., 2011). Moreover, a greater expression in native genotypes compared with hybrid genotypes suggests that although SINAC4 has been associated with coloration, it also has been reported that it responds to several stress factors such as dehydration (Zhu et al., 2014). Native genotypes show greater biotic and abiotic stress responses, which allow them to grow in soils with limited moisture availability and tolerate high temperatures.

PCA and Pearson correlation coefficient analysis

Tomato fruit of different colors are an interesting model to understand the isoprenoid–metabolic cross-talk of the tocopherol, chlorophyll, and carotenoid pathways. In this work, we found that greater levels of specific antioxidants were associated with particular colorations of tomato fruit. PCA was carried out to study the correlation between all studied parameters (Fig. 3A). Red genotypes (RN and LRH) were associated with lycopene, β-carotene, lipophilic antioxidant capacity, total polyphenols, vitamin C, and greater relative transcript expression of PSY1 and CYC-B (both chromoplastic) but not with tocopherols, which indicated that in these genotypes, carotenoids made a greater contribution to lipophilic antioxidant capacity (Table 6). Orange genotypes (ON and LOH) correlated with tocopherols, flavonoids, VTE-2, HPPD, and DKS expression. A positive correlation between HPPD and VTE-2, and negative correlations between PSY1 and VTE-5 (–0.8192) and between lycopene and chlorophyll (–0.6209) suggested a balance between chlorophylls, tocopherols, and carotenoids. These correlations were consistent in light of the chloroplast-to-chromoplast transition during fruit ripening that results in massive chlorophyll degradation and lycopene accumulation. The results of this study suggested compensation between antioxidant levels, which in turn was associated with a

Table 6. Statistically significant Pearson product moment correlations (P values below 0.05). These correlation coefficients range between –1 and +1 and measure the strength of the linear relationship between the variables.

| Parameter | Correlation coefficient |
|-----------|------------------------|
| Total polyphenols vs. lutein | 0.94 |
| Total polyphenols vs. hydrophilic TEAC | 0.96 |
| Lycopene vs. β-carotene | 0.89 |
| Lycopene vs. lipophilic TEAC | 0.89 |
| β-carotene vs. lipophilic TEAC | 0.89 |
| Total tocopherols vs. HPPD | 0.91 |
| Total tocopherols vs. VTE-2 | 0.85 |
| GGDR vs. VTE-5 | 0.87 |
| GGDR vs. LCY-B | 0.96 |
| GGDR vs. PSY-2 | 0.99 |
| LCY-B vs. PSY-2 | 0.97 |
| HPPD vs. VTE-2 | 0.92 |

TEAC = Trolox-equivalent antioxidant capacity; HPPD = 4-hydroxyphenylpyruvate dioxygenase; VTE-2 = homogentisate phytol transferase; GGDR = geranylgeranyl reductase; VTE-5 = phytol kinase; LCY-B = lycopene β-cyclase; PSY-2 = phytoene synthase 2.
particular color of the fruit. Although the B_{11} genotype was associated with anthocyanins, the Y_{N} genotype was associated with lutein, total polyphenols, hydrophilic antioxidant capacity, and PSY2, LCY-B, VTE-5, and GGDR expression. The red genotypes analyzed here were a source of both lycopene, associated with greater PSY1 expression, and β-carotene, whose content was associated with greater CYC-B expression, whereas the Y_{N} genotype presented the greatest total polyphenol content, resulting in a greater in vitro antioxidant capacity of the hydrophilic fraction. Native genotypes of different color are a significant source of functional compounds that have been lost in commercial red genotypes through selection processes. These genotypes could be used either directly as food or in breeding programs to recover greater levels of functional compounds, such as carotenoids, tocopherols, anthocyanins, and vitamin C.

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