Up-regulation of the vacuolar invertase TAI gene may contribute to the accumulation of carotenoids in tomato fruits

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Abstract: Generally, fleshy fruit ripening is accompanied by its sweetening and color change caused by dramatic molecular, biochemical and physiological conversions. Species of the tomato clade differ in color of ripe fruits. Carotenoids accumulate in the fruit chromoplasts of red-fruited species, and the fruits of primitive green-fruited tomato species remain green due to the numerous blocks of carotenogenesis. In this study, the sugar and carotenoid content, as well as the expression patterns of the four genes encoding main fruit-specific sucrose cleavage enzymes TAI, SUS1, NI2 and LIN5, were characterized in immature green, mature green and ripe fruits of *S. lycopersicum* cultivars and the wild green-fruited tomato species *S. habrochaites* and *S. peruvianum*. An analysis of the sugar and carotenoid content confirmed that carotenoids and hexoses accumulate in the ripe fruits of the red-fruited *S. lycopersicum* cultivars, while green-fruited tomatoes produce fruits enriched for sucrose and containing only trace amounts of carotenoids. The *SUS1*, *LIN5* and *NI2* expression showed no correlation with fruit color and hexose content. In fruits of green-fruited species, all analyzed genes were expressed in a similar way. *TAI* expression was extremely specific for cultivars, being maximal in ripe fruit, characterized by carotenoid and hexose accumulation in fruits. The findings suggest that vacuolar invertase TAI, rather than apoplastic invertase LIN5 or cytoplasmic sucrose synthase SUS1 and invertase NI2, may specify sugar composition in ripe fruits.

Key words: sucrose hydrolysis genes; red- and green-fruited tomato species; carotenoid biosynthesis.

1. Introduction

Solanum section Lycopersicon includes the cultivated tomato *S. lycopersicum* and its 12 wild relatives that differ significantly in fruit physiology, biochemistry and morphology (Peralta et al., 2008). This makes tomato species an attractive model for studying the mechanism of fleshy fruit development from initiation to full maturity.

Ripening, converting mature fruit from inedible to edible form, is an irreversible process associated with perceptible organoleptic and visible alterations in texture, flavor, aroma, color, and size, due to dramatic molecular, metabolic, biochemical and physiological changes driven by internal and external signaling (Seymour et al., 2013). Ripening determines crop yield, shelf life, and fruit quality traits, including dry matter amount, content, and nutritional value (Li, Van Eck, 2007). The recently evolved so-called red-fruited tomato species (*S. lycopersicum*, *S. pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*) have a yellow to red color of ripe fruits due to carotenoid synthesis (mainly beta-carotene and lycopene) and chlorophyll degradation (Llorente et al., 2016). Ripe fruits, formed by more ancient green-fruited tomato species, do not accumulate carotenoids and, as a result, stay green (Kilambi et al., 2017). The basic mechanisms of ripening are similar in both sets of tomato species and accompanied by high ethylene biosynthesis, fruit softening, and sugar content increment. However, they differ in sugar composition in ripening fruits: red-fruited species accumulate glucose and fructose, and green-fruited tomatoes contain sucrose (Beckles et al., 2012). The exact molecular mechanisms of mutual regulation of sugar metabolism and fruit pigmentation have not yet been fully understood. There is some evidence that sugars can act as signaling molecules to activate carotenoid biosynthesis and, as a result, the fruit acquires a different color. For example, changing the sugar concentration in mandarin (*Citrus unshiu*), watermelon (*Citrullus lanatus*) and tomato fruits alters their pigmentation (Iglesias et al., 2001; Telef et al., 2006; Zhang et al., 2017). Thus, a connection can be proposed between the sucrose-hexose ratio and the synthesis of carotenoids. In carbohydrate metabolism, glucose is the central molecule, sucrose is the most common form of translocated sugar, and sucrose-hexose interconversion is one of the main reactions (Winter, Huber, 2000). In plants, four sucrose cleavage enzymes are known: cytoplasmic sucrose synthase (SUS), and three invertases, acid vacuolar (TAI), acid apoplastic (LIN) and neutral cytoplasmic (NI) (Winter, Huber, 2000).

In this study, new data were obtained on the possible relationships between carbohydrate and carotenoid metabolism by analyzing fruit-specific expression patterns of *SUS1*, *TAI*, *LIN5* and *NI2* and fruit biochemical composition (sugars, carotenoids) during ripening in green- and red-fruited tomato species.

2. Materials and methods

Accessions of cultivated (*S. lycopersicum* cv. Silvestre Recordo, Red Cherry and Fioletovii) and wild (*S. habrochaites* and *S. peruvianum*) tomato species (*Solanum* section Lycopersicon) were provided by the N. I. Vavilov Institute of Plant Genetic Resources (St.-Petersburg, Russia) and grown in a greenhouse with a 16-h/8-h (28 °C/23 °C) light-dark cycle (light intensity, 300–400 μmol m^{-2} s^{-1}).

Total RNA was extracted from immature green (IF), mature green (MF) and fully ripe (RF) fruits using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and analyzed by gel electrophoresis and fluorimetry (Qubit® Fluorometer, Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA) and an oligo-dT primer and quantified by fluorimetry. Gene-specific primers separated by at least one big intron were designed to amplify fragments of coding sequences: SUSrtF (5′-GATTTCGAGCCTTTCACTGC-3′)/SUSrtR (5′-AGGTATTCCTCTGCCTTCC-3′) (for *SUS1* gene), TAIrtF (5′-GAGGCTCCGGGAGTTGGTAA-3′)/TAIrtR (5′-CC AAATCTTGACGGAGGCAG-3′) (for *TAI*), LIN5rtF (5′-TGTCAAGAATGTTCATAGAACTC-3′)/LIN5rtR (5′-TGAATGAGCCCAAATAATATTGC-3′) (for *LIN5*) and NI2rtF (5′-ACTCTTGCTGCTAATGATCCTAA-3′)/NI2rtR (5′-ACTTCTTCATATTTGTTATCATCGAG-3′) (for *NI2*). Quantitative real-time PCR (qPCR) was performed in two biological and three technical replicates using 2.5 ng of cDNA, developed primers, and SYBR Green and ROX RT-PCR mixture (Syntol, Moscow, Russia) at the following conditions: denaturation at 95 °C for 5 min, and 40 cycles at 95 °C for 15 s and 60 °C for 40 s. The expression levels were normalized to that of the tomato reference genes *Expressed* and *Actin 2/7* (Expósito-Rodríguez et al., 2008; Bemer et al., 2012). Statistical analysis was performed using Graph Pad Prism version 7.02 (San Diego, CA, USA; https://www.graphpad.com/scientifc-software/prism/).

Biochemical analysis was performed in two biological replicates. To assess sucrose, glucose and fructose content, 1 g of fruit material was ground in liquid nitrogen, diluted with 10 ml of 80 % ethanol, centrifuged at 10,000 rpm for 15 min, and analyzed by HPLC (Varian ProStar, VARIAN INC., Palo Alto, CA, USA) using an HPLC Refractive Index Detector 102M (Stayer, Moscow, Russia) and an Agilent Carbohydrate Analysis Column (150 mm × 4.6 mm, 5 μM); isocratic elution was performed with 75 % v/w acetonitrile as a mobile phase; the flow rate was maintained at 1.5 ml/min and the temperature, at 30 °C. Total carotenoids content was measured by Nagata and Yamashita equation (Nagata, Yamashita, 1992).

3. Results and discussion

Three *S. lycopersicum* cultivars and two wild tomato species, *S. habrochaites* and *S. peruvianum*, considered in this study represent two diverse groups varying in fruit pigmentation and sugar composition (Table 1). The same fruit samples of examined accessions were taken at three time points, including growth (IF), mature (MF) and ripe (RF) stages, both for biochemical and qPCR analyses.

An analysis of the sugar and carotenoid contents confirmed that carotenoids and hexoses accumulate in ripe fruits of the red-fruited *S. lycopersicum* cultivars, while green-fruited tomatoes produce fruits enriched for sucrose and containing trace amounts of carotenoids (see Table 1). The obtained data are fully consistent with the previously observed carbohydrate biochemistry of fruits in red- and green-fruited tomatoes (Peralta et al., 2008; Beckles et al., 2012) and suggest a negative correlation between the accumulation of monosaccharides and the presence of carotenoids.

To find out the possible factors influencing the difference in carbohydrate composition and, likely, in the implementation of the carotenoid biosynthesis in tomato fruits, *SUS1*, *TAI*, *LIN5* and *NI2* expression patterns were determined and compared in ripening fruits of red-fruited cultivars Silvestre Recordo, Red Cherry and Fioletovii and the green-fruited species *S. habrochaites* and *S. peruvianum* (Figure 1). These genes were selected for analysis among their paralogs because their products were shown to have fruit-specific activity (Winter, Huber, 2000; Baxter et al., 2005).

The transcription levels of the analyzed genes varied considerably relative to each other (see Figure 1). The *SUS1*, *LIN5* and *NI2* transcripts were found in fruits of both red- and green-fruited tomatoes, which indicates the absence of any correlation of their expression with fruit color.

In fruits of green-fruited species, all analyzed genes were expressed in a similar way. The *LIN5* transcription level was the highest in mature green fruit and absent in ripe fruit. *SUS1* transcription was maximal in immature green fruits and decreased during the transition from stage IF to stage RF.

Our observations suggest that primitive green-fruited species may have retained an ancestral expression pattern for each analyzed sucrose cleavage gene, as was also demonstrated for carotenoid metabolism genes (Kilambi et al., 2017).

In contrast, the *SUS1* and *LIN5* transcription varied between cultivars of the red-fruited species without correlation with the sugar and carotenoid content in ripe fruit.

Thus, *S. lycopersicum* cultivars, which, due to intensive tomato breeding, differ significantly in fruit color, sweetness and other related traits, may have diversified expression of sucrose catabolism genes with a unique pattern for each cultivar.

Unlike these genes, *TAI* expression was extremely specific to cultivars, being maximal in ripe fruit, characterized by carotenoid and hexose accumulation in fruits. Our observation is consistent with previous data on *TAI* expression in ripe fruits of other red-fruited tomato species (Slugina et al., 2017).

TAI activity in the vacuole may lead to accumulation of symplastic glucose in the cell, which may stimulate chromoplast formation, as shown for watermelon (Zhang et al., 2017).

Differences in fruit color in tomato species and cultivars may indicate the uniqueness of carotenoid biosynthesis regulation in each genotype.

Fruits of red-fruited species accumulate carotenoids at high levels in carotenoid sequestering substructures inside chromoplasts (Li, Van Eck, 2007). Fruit carotenogenesis coincides with the chloroplast into chromoplast transformation (Kilambi et al., 2017). In green-fruited species, fruits do not have orange/red color due to the numerous blocks of fruit-specific chromoplast formation and carotenoid synthesis (Kilambi et al., 2017).

4. Conclusions

The findings suggest that vacuolar invertase TAI, rather than apoplastic invertase LIN5 or cytoplasmic proteins, sucrose synthase SUS1 and invertase NI2, may be a key enzyme in determining sugar composition in ripe fruit. The role of cytoplasmic and apoplastic sucrose hydrolysis probably consists in maintaining the basic sucrose-hexose balance in the cells and in supplying dividing and expanding cells with energy

Table 1
Sugar and carotenoid content in immature green (IF), mature green (MF) and ripe (RF) fruits of *S. lycopersicum* cultivars and two wild tomato species

Accession	Fruit stage	Glucose, mg/g fresh weight	Fructose, mg/g fresh weight	Sucrose, mg/g fresh weight	B-carotene, mg/g fresh weight
S. habrochaites	IF	0.313	0.373	0.174	
	MF	0.3865	0.493	0.4375	
	RF	0.2285	0.339	1.5845	< 0.001
S. peruvianum	IF	0.551	0.749	0.246	
	MF	0.628	0.842	0.3125	
	RF	0.024	0.399	1.328	< 0.001
S. lycopersicum cv. Silvestre Recordo	IF	0.837	0.812	0.181	
	MF	0.9885	1.2075	0.149	
	RF	1.3205	1.702	0.099	0.098
S. lycopersicum cv. Red Cherry	IF	0.916	0.952	0.172	
	MF	0.818	0.917	0.201	
	RF	0.893	1.203	0.055	0.08
S. lycopersicum cv. Fioletovii	IF	0.938	0.998	0.096	
	MF	0.987	1.160	0.184	
	RF	1.069	1.324	0.044	0.091

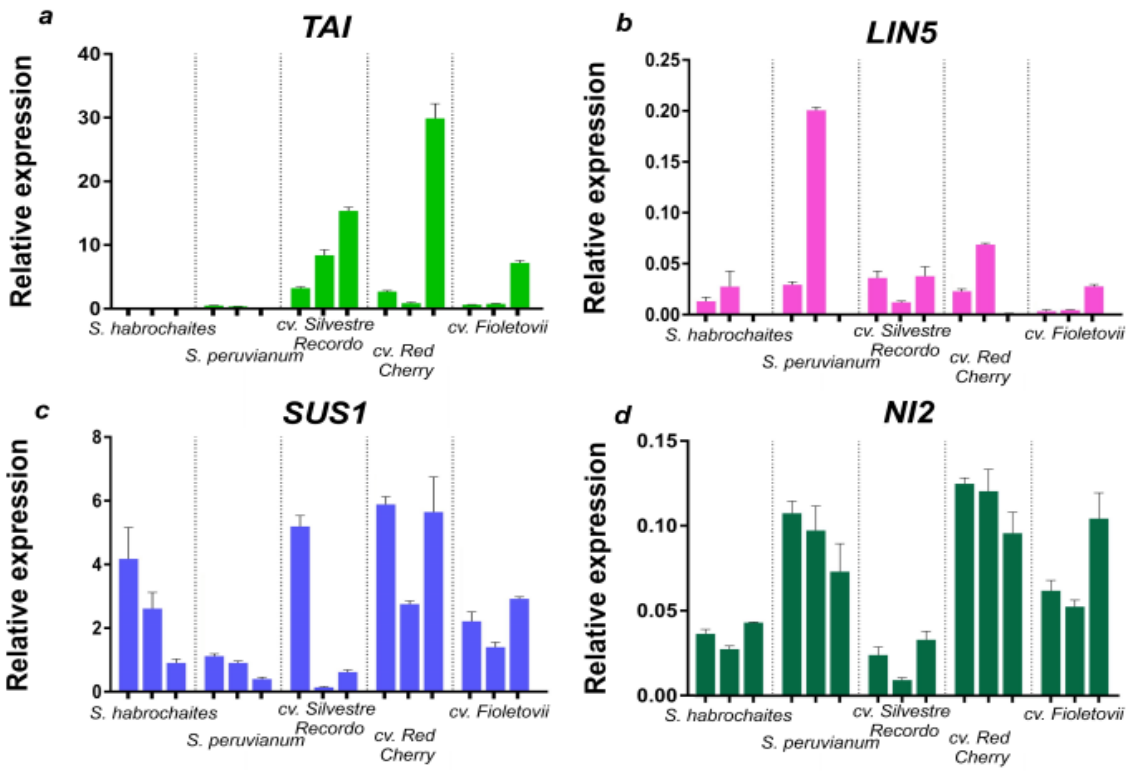

Figure 1. Expression patterns of *TAI* (*a*), *LIN5* (*b*), *SUS1* (*c*) and *NI2* (*d*) in immature green, mature green and ripe fruits of *S. lycopersicum* cultivars and two wild tomato species.

and carbon to synthesize the necessary compounds during fruit growth up to the stage of maturation.

Given the available evidence of a positive correlation between the sugar content and carotenoids in fruits, studying the genetic networks of fruit-specific carbohydrate metabolism may contribute to new alternative approaches to the manipulation of carotenoid levels in food crops.

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