RESEARCH ARTICLE

Expression profile of genes coding for carotenoid biosynthetic pathway during ripening and their association with accumulation of lycopene in tomato fruits

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Abstract

Fruit ripening process is associated with change in carotenoid profile and accumulation of lycopene in tomato (*Solanum lycopersicum* L.). In this study, we quantified the β-carotene and lycopene content at green, breaker and red-ripe stages of fruit ripening in eight tomato genotypes by using high-performance liquid chromatography. Among the genotypes, lycopene content was found highest in Pusa Rohini and lowest in VRT-32-1. To gain further insight into the regulation of lycopene biosynthesis and accumulation during fruit ripening, expression analysis of nine carotenoid pathway-related genes was carried out in the fruits of high lycopene genotype—Pusa Rohini. We found that expression of phytoene synthase and β-carotene hydroxylase-1 was four and thirty-fold higher, respectively, at breaker stage as compared to red-ripe stage of fruit ripening. Changes in the expression level of these genes were associated with a 40% increase in lycopene content at red-ripe stage as compared with breaker stage. Thus, the results from our study suggest the role of specific carotenoid pathway-related genes in accumulation of high lycopene during the fruit ripening processes.

[Smita S., Rajwanshi R., Lenka S. K., Katiyar A., Chinnusamy V. and Bansal K. C. 2013 Expression profile of genes coding for carotenoid biosynthetic pathway during ripening and their association with accumulation of lycopene in tomato fruits. *J. Genet.* **92**, 363–368]

Introduction

Pulp colour changes from green via breaker to red colour in the tomato fruits. These colour changes are caused by the accumulation of specific pigments such as chlorophylls, carotenoids, flavonoids or betalains (Tanaka *et al.* 2008). β-carotene, lycopene, lutein, violaxanthin and zeaxanthin are the important carotenoids present in most of the cultivated tomato and also provide dietary nutrition to humans (Bramley 2002). Therefore, tomato is preferred as an important model plant for studying carotenoid biosynthesis pathway during fruit ripening (Wiebke and Ralph 2009). The pathway is highly active in tomato fruits during ripening, leading to the accumulation of specific carotenoids. At the green stage of fruit ripening, lutein accumulates in high amount; lutein is helpful to humans in protecting the retina against damaging irradiation and age-related macular degeneration (Handelman *et al.* 1988; Seddon *et al.* 1994; Moeller *et al.* 2006). At the red-ripe stage of fruit ripening, lycopene accumulates in a high level due to the differential expression of genes for enzymes involved in conversion of lycopene to other carotenoids (Hirschberg 2001). Lycopene provides defense against development of type 2 diabetes mellitus, and helps to maintain prostate health (Stacewicz-Sapuntzakis and Bowen 2005). It is an important intermediate with strong antioxidant properties and is involved in the biosynthesis of many other carotenoids such as β-carotene which is responsible for colouration in fruits and flowers.

Several studies have described carotenoid biosynthesis regulation at molecular level in plants and its stimulation by light (Cunningham and Gantt 1998; Hirschberg 2001; Fraser and Bramley 2004; Liu *et al.* 2004). Different types of regulatory mechanisms at transcriptional and

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Keywords. carotenoid biosynthesis; carotenoid pathway genes; tomato; lycopene; Pusa Rohini; ripening.

posttranscriptional levels are involved in specific carotenoid accumulation (Sauret-Güeto *et al.* 2006). The 1-deoxy-D-xylulose-5-phosphate synthase (DXS) enzyme of methylerythritol phosphate (MEP) pathway that supplies isoprene precursors is a rate limiting enzyme in carotenoid biosynthesis during tomato fruit ripening (Lois *et al.* 2000; Rodriguez-Concepcion *et al.* 2001; Rodríguez-Concepción *et al.* 2003). The carotenoid biosynthesis begins with the formation of phytoene from geranylgeranyl-diphosphate (GGPP). One of the important gene with highest activity during fruit ripening is phytoene synthase (PSY) in tomato (Fraser *et al.* 2007). Recently, Lee *et al.* (2012) analysed the correlation between carotenoid content and gene expression profiles at breaker and red-ripe stage of fruit ripening in tomato (Lee *et al.* 2012). Likewise, several efforts have been made to engineering carotenoid metabolism. These studies have provided insight into the regulatory mechanisms of carotenoid accumulation and development of high lycopene tomato varieties (Giuliano *et al.* 2000, 2008; Römer and Fraser 2005; Fraser *et al.* 2007).

In the present study, lycopene and β-carotene content of eight different tomato genotypes were quantified at three different stages (green, breaker and red-ripe) of fruit ripening. Expression levels of selected genes coding for carotenoid biosynthesis were also investigated at the same developmental stages to establish gene-to-metabolite links in high lycopene tomato variety, Pusa Rohini.

Materials and methods

Plant materials

Tomato of eight genotypes namely Pusa Rohini, Pusa Sadabahar, H-24, Pusa Gaurav, Pusa Ruby, Pusa 120, VRT-32-1 and Pusa Uphaar were grown in plastic pots at National Phytotron Facility, Indian Agricultural Research Institute, New Delhi, India. Fruits were harvested at three ripening stages viz. mature green, breaker and red-ripe that were immediately frozen in liquid nitrogen and stored at −80°C to perform further experiments.

Extraction, separation and quantification of carotenoids by HPLC

Fruit tissues without seeds (2.5 g) were weighed; flesh frozen and homogenized using liquid nitrogen and placed in a 250 mL amber colour conical flask for carotenoid extraction. Samples threshold values in the were extracted with 25 mL of hexane:acetone:ethanol (2:1:1) on an incubator shaker at 140 rpm for 10 min at 25°C. Further, to separate the orange hexane layer from the bottom aqueous layer, sample was shaken for 5 min. The organic phase containing lycopene was pooled with the first extractant and injected in high-performance liquid chromatography (HPLC, Waters^{TM}) for profiling of carotenoids. Known amount of pure standards were used as reference and detected at 471 nm (figure 1, a&b in electronic supplementary material at http://www.ias.ac.in/jgenet/).

Pigment separation was performed by YMC S-5 μm (250 × 4.6 mm) carotenoid column (Wurbs *et al.* 2007), at a flow rate of 2 mL/min in the mobile phase of methyl tertiary-butyl ether:methanol (7:3). The injection volume was 20 μL. Lycopene eluted at approximately 15 min. A standard of lycopene and β-carotene (90–95% pure from Sigma Chemicals, St. Louis, USA) was run with each set of samples on the HPLC. The concentration of standards used is specified in table 1 in electronic supplementary material which ranged between 0.12 and 400 μg. Data analysis was carried out by using the software Empower ver. 2 (Waters, Milford, USA) and the chromatographic peaks were identified by comparison of retention times with standards of lycopene and β-carotene. In our study, five of the biological replicates were used for each genotype at each stage.

Transcript profiling of carotenoid related genes by qRT-PCR

Good quality RNA was isolated from pericarp of fruits of *Solanum lycopersicum* L cv. Pusa Rohini (high lycopene) and VRT-32-1 (low lycopene), using trizol method (Ambion, Austin, USA) and extracted RNA was reverse transcribed by Ambion RETRO script kit. RT-PCR was carried out for nine selected carotenoid biosynthesis pathway-related genes. The qRT-PCR reactions (20 μL) containing 10 ng of cDNA were carried out in an Eppendorf Realplex-4 Mastercyclerep gradients machine (Hamburg, Germany). Tomato *SIE1α* gene was used as the endogenous control. Auto baseline and threshold values in the realplex software (ver. 1.5) were used. The qRT-PCR reactions were carried out at 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s each by the method described previously by Katiyar *et al.* (2012). The threshold cycles (CT) of each test target were averaged for triplicate reactions and the values were normalized according to the CT of the control products. Gene expression data were normalized by subtracting the mean of reference gene (*SIE1α* gene) CT value from individual CT values of corresponding target genes (ΔCT). The relative fold change in breaker and red-ripe stages as compared with green stage of fruit was calculated by using expression values of green stage as a calibrator for the respective genotype. The fold change value was calculated by using $2^{-\Delta\Delta CT}$ method. ΔΔCT was calculated by subtracting ΔCT of a gene in reference sample from ΔCT of that gene in sample of interest. Average of three replicates was used for calculating the expression levels which were further transformed on $\log_2$ scale. The primers used for the amplification for all the genes and a control primer are given in table 2 in electronic supplementary material.

Results and discussion

The carotenoid (β-catotene and lycopene) content in eight genotypes of tomato fruits at three different stages of fruit

Table 1. Genotypic differences in lycopene and β-carotene content of tomato during different stages of fruit ripening.

Tomato varieties	Green fruit (μg/g fresh wt.)		Breaker fruit (μg/g fresh wt.)		Red fruit (μg/g fresh wt.)	
	Lycopene	β-carotene	Lycopene	β-carotene	Lycopene	β-carotene
Pusa Rohini	ND	ND	1.0	ND	23.0	0.55
Pusa Gaurav	ND	ND	3.5	0.40	17.0	1.00
H-24	ND	0.15	1.5	0.10	15.0	0.40
Pusa Sadabahar	ND	0.25	1.5	0.55	12.0	0.90
Pusa Ruby	ND	ND	3.0	0.10	12.0	0.35
Pusa 120	ND	0.2	1.3	0.40	11.0	0.40
Pusa Uphar	ND	ND	ND	0.35	10.0	0.85
VRT-32-1	ND	0.25	ND	0.1	1	0.1

ND, non detectible.

ripening were measured using HPLC method. Lycopene and β-carotene levels of different genotypes of tomato were calculated and shown in table 1. Maximum lycopene was observed in Pusa Rohini and minimum in VRT-32-1, at red-fruit stage of fruit ripening (figure 1). In case of β-carotene, all the genotypes have less than 1 μg/g fresh weight of tissue, and maximum β-carotene was detected in Pusa Gaurav. Lycopene concentration increased from green fruit stage to the red fruit in all eight tomato genotypes examined here (table 1). Similarly, the β-carotene level also increased from green fruit to red fruit in majority of the tomato genotypes viz. Pusa Ruby, Pusa Uphaar, Pusa Sadabahar, Pusa Gaurav and Pusa 120, except VRT-32-1 where a decreasing trend was observed. HPLC analyses revealed that Pusa Rohini has the highest and VRT-32-1 has lowest amount of lycopene at red stage of fruit maturity (figure 2, a&b in electronic supplementary material). We observed highest lycopene content at red-fruit stage in Pusa Rohini, which surprisingly showed lowest lycopene content at breaker stage as compared to other genotypes. Lycopene at retention time (Rt) 12.016 for breaker and 11.814 for red stage was major which comprises around 100 and 86.83% at this detection nanometer in Pusa Rohini genotype. Therefore, this genotype was chosen for further analysis at transcriptional level to know the correlation between expression patterns of carotenoid-related genes and carotenoid accumulation at different stages of fruit ripening.

Expression profiling was performed for nine genes potentially involved in the carotenoid biosynthesis pathway in the high lycopene accumulating tomato genotype, i.e. Pusa

Figure 1. Phenotype representation of tomato genotype (a) VRT-32-1 and (b) Pusa Rohini at red-ripe fruit stage.

Rohini to establish gene-to-metabolite links during fruit ripening. In the present study, transcriptional regulation of nine genes namely *DXR* (1-deoxyxylulose 5-phosphate reductoisomerase), *IPI* (IPP isomerase), *PSY* (phytoene synthase), *PDS* (phytoene desaturase), *ZDS* (ζ-carotene desaturase), *CRTLb* (lycopene β-cyclase), *CRTRb1* (β-carotene hydroxylase-1), *VDE* (violaxanthin de-epoxidase) and *ZEP* (zeaxanthin epoxidase) involved in carotenoid biosynthesis pathway was analysed using qRT-PCR (figure 2). Expression levels of *IPI*, *PSY*, *CRTLb* and *VDE* showed sharp increase in their transcript abundance from breaker-to-red stage as compared to green fruit stage in Pusa Rohini. *CRTLb* and *VDE* were induced 36.27 and 35.79 folds at red-ripe stage, respectively (figure 3). The *IPI* was highly expressed

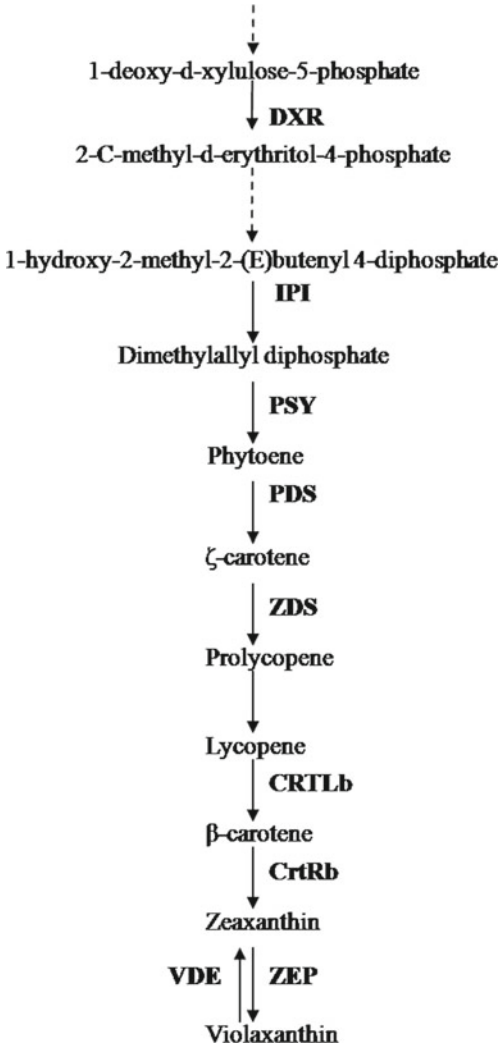

Figure 2. Schematic presentation of the carotenoid biosynthetic pathway in plants. *DXR* 1-deoxyxylulose-5-phosphate reductoisomerase, *IPI*, IPP isomerase; *GGDS*, GGPP synthase; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, z-carotene desaturase; *CRTLb (LCYB)*, lycopene cyclase; *CHYB (CrtRb)*, carotenoid e-ring hydroxylase; *ZEP*, zeaxanthin epoxidase; *VDE*, violaxanthin de-epoxidase (same abbreviation used throughout the manuscript).

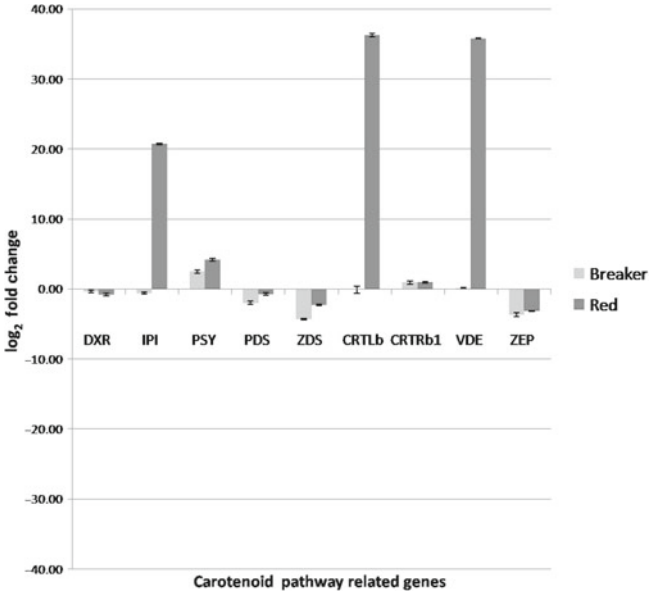

Figure 3. The qRT-PCR expression profile of different carotenoid biosynthesis pathway genes. Relative expression values of carotenoid pathway-related genes in Pusa Rohini at breaker and red stage were calculated using the expression at green stage as base level (used as calibrator).

at breaker stage. The expression of *PSY* was highest among all carotenoid biosynthesis pathway-related genes at breaker stage. *ZDS* was highly downregulated at breaker stage as compared to red-ripe stage.

The sudden increase in lycopene content was observed at red stage as compared to breaker stage. This increase in lycopene was associated with the upregulation of *PSY*, *CrtLb* and *CRTRb1* genes at breaker stage. We presume that high *PSY* expression at breaker stage might lead to high accumulation of carotenoids, which latter converted into lycopene at red stage. Several other studies also showed a high correlation between accumulation of lycopene and expression levels of *PSY1*, *CRTLb*/*LCY-B* and *CYC-b* (chromoplast-specific lycopene cyclase) transcripts in tomato fruits (Giuliano *et al.* 1993; Corona *et al.* 1996; Ronen *et al.* 2000). DXS activity is the rate limiting step in MEP pathway that supplies isoprene precursors (Carretero-Paulet *et al.* 2002), consequently, limiting the carotenoid biosynthesis pathway in tomato (Lois *et al.* 2000; Rodriguez-Concepcion *et al.* 2001; Rodríguez-Concepción *et al.* 2003). Instead of the synchronized upregulation of *PSY* and *DXS*, the expression of *DXS* genes was found to be downregulated. *ZEP* and *VDE* were upregulated at breaker stage, but down regulated at red-ripe stage of fruit development. The correlation between a decrease in *ZEP* expression and increase in lycopene content observed at red-ripe stage in our study is consistent with previous reports, where increase in level of carotenoid was achieved by silencing *ZEP* (Römer *et al.* 2002), *LCY-e* and *CRTRb* genes (Van *et al.* 2007). The *ZDS* expression gradually increased from green to breaker and slightly reduced at

red-ripe stage. Conversion of phytoene to lycopene is catalysed by both PDS and ZDS by desaturation reactions via ζ-carotene. In our study, we found upregulation for *ZDS* at breaker stage as compared to red-ripe stage. We observed a decreasing trend of β-carotene accumulation from green to the red ripe stage in VRT-32-1 genotypes (table 1). We presume the induction of the carotenoid biosynthesis pathway genes might be responsible for higher lycopene content in the elite tomato cultivars.

These results suggest that transcriptional regulation of carotenoid biosynthesis pathway genes is a key mechanism of lycopene accumulation. The chemical nature and biological activity of enzymes involved in carotenoid biosynthetic pathways in plants are well known (Cunningham and Gantt 1998; Hirschberg 2001; Wiebke and Ralph 2009; Smita *et al.* 2013). However, the genotypic differences in gene regulation and its correlation with pigment content are still lesser known in tomato. In conclusion, understanding the molecular basis of genotypic differences in carotenoid biogenesis and accumulation will help to develop designer tomato fruits with desired carotenoid content. Characterization of the promoters of the candidate genes identified in this study and identification of transcription factors will help development of genotypes having higher lycopene content and antioxidant property.

Acknowledgement

The authors thank Department of Biotechnology, Government of India for funding the research work.

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Received 15 March 2013, in revised form 5 May 2013; accepted 15 May 2013
Published on the Web: 23 October 2013