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PAPER

Expression of carotenoid biosynthetic pathway genes and changes in carotenoids during ripening in tomato (*Lycopersicon esculentum*)

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To study the expression pattern of carotenoid biosynthetic pathway genes, changes in their expression at different stages of maturity in tomato fruit (cv. Arka Ahuti) were investigated. The genes regulating carotenoid production were quantified by a dot blot method using a DIG (dioxigenin) labelling and detection kit. The results revealed that there was an increase in the levels of upstream genes of the carotenoid biosynthetic pathway such as 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), 4-hydroxy-3-methyl-but-2-enyl diphosphate reductase (Lyt B), phytoene synthase (PSY), phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS) by 2–4 fold at the breaker stage as compared to leaf. The lycopene and β -carotene content was analyzed by HPLC at different stages of maturity. The lycopene (15.33 ± 0.24 mg per 100 g) and β -carotene (10.37 ± 0.46 mg per 100 g) content were found to be highest at 5 days post-breaker and 10 days post-breaker stage, respectively. The lycopene accumulation pattern also coincided with the color values at different stages of maturity. These studies may provide insight into devising gene-based strategies for enhancing carotenoid accumulation in tomato fruits.

1. Introduction

Carotenoids are one of the largest group of isoprenoid compounds biosynthesized by all photosynthetic bacteria, cyanobacteria, algae and higher plants. In plant systems, carotenoids play a major role in light harvesting and photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation and serve as precursors for the biosynthesis of the phytohormone abscisic acid (ABA).^{1,2} In recent years there has been considerable interest in the dietary carotenoids due to their provitamin A activity.^{3,4} β -Carotene is the most potent dietary precursor of vitamin A, the deficiency of which leads to xerophthalmia, blindness and premature death.⁵ Vitamin A deficiency has been reported as the most common dietary problem affecting children aged 1–4 years. Other known attributes of carotenoids include their high antioxidant potential and ability to prevent onset of certain cancers.⁶

Plants preferably synthesize carotenoids *via* the recently identified 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway (Fig. 1) rather than the mevalonic acid pathway.^{7,8} Whilst both pathways produce isopentenyl pyrophosphate (IPP), the DOXP pathway is plastidic in nature and leads to the formation of carotenoids, phytol, plastoquinone-9, and diterpenes.⁹ IPP leads to the formation of geranylgeranyl pyrophosphate (GGPP) which is the precursor for the formation of carotenoids. The

most important step in the carotenoid biosynthesis pathway is the head-to-head condensation of two GGPP molecules to form a colourless compound phytoene.^{10,11} This two-step reaction is catalyzed by the enzyme phytoene synthase (PSY). The tomato contains two isoforms of the PSY gene, Psy-1 and Psy-2. The Psy-1 encodes the fruit-ripening-specific isoform, whilst Psy-2 predominates in green tissues, including mature green fruit and has no role in carotenogenesis in ripening fruit.¹² The phytoene undergoes a series of four desaturation reactions to form phytofluene, ζ -carotene, neurosporene and finally lycopene. The four sequential desaturations are catalyzed by two related enzymes in plants; phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS). A carotenoid isomerase (CRTISO) activity is additionally required to transform the poly *cis* lycopene (pro-lycopene) to the all *trans*-isomer. Cyclization of lycopene marks the branching point in the plant carotenoid pathway. Lycopene β -cyclase (LCYB) catalyzes lycopene to produce β -carotene in a two-step reaction that creates one β -ionone ring at each end of the lycopene molecule. In the other branch, δ -carotene is produced by the addition of one ϵ -ring to lycopene in the presence of lycopene ϵ -cyclase (LCYE).^{13,14} Xanthophylls are formed by the oxygenation of carotenes, typically by the addition of hydroxyl, epoxy or keto groups.

The ripening of tomato (*Lycopersicon esculentum*) fruit is a highly regulated process, during which co-ordinated genetic and biochemical events take place leading to changes in fruit texture, aroma, color and flavour.¹⁵ One of the most important noticeable changes during ripening is the increase in the carotenoid content. This is evident in the form of a change in

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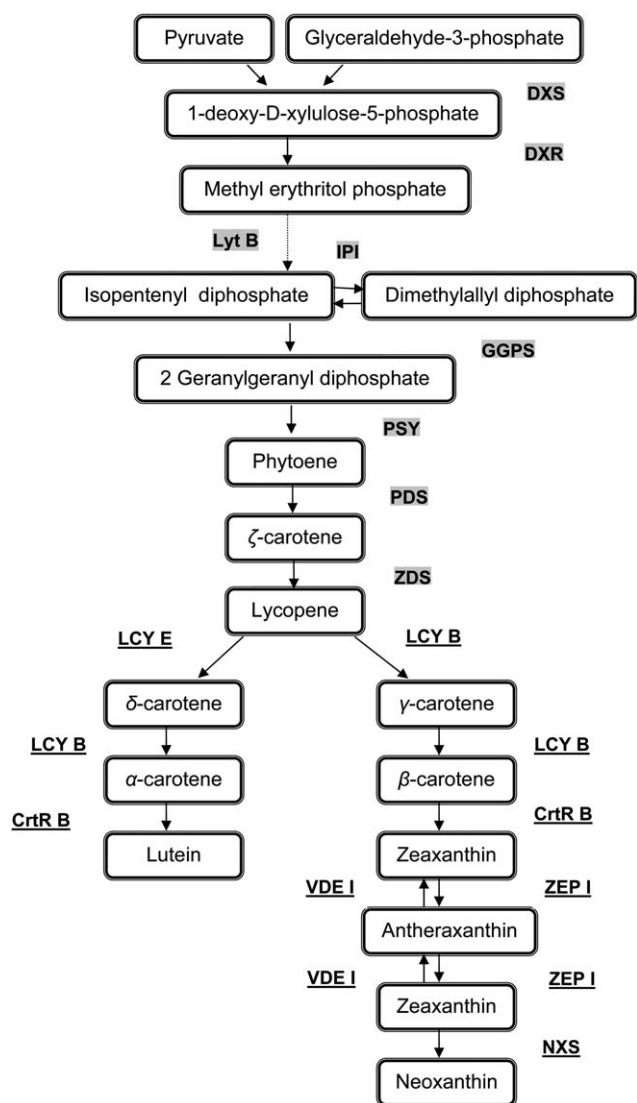


Fig. 1 Carotenoid biosynthetic pathway in higher plants showing upstream (shaded) and downstream (underline) genes used in our study.

pigmentation due to massive accumulation of lycopene and the disappearance of chlorophyll.¹⁶ The genes of lycopene biosynthesis are strongly induced during fruit development,¹⁷ while the genes encoding cyclization enzymes are downregulated.¹⁸ Therefore, the present study was undertaken to study the gene expression pattern of the complete carotenoid biosynthetic pathway in the Indian cultivar Arka Ahuti and the accumulation pattern of β -carotene and lycopene at various stages of maturity were characterized.

2. Materials and methods

2.1 Plant material

Tomato seeds cv. Arka Ahuti was obtained from the Indian Institute of Horticultural Research, Hessarghatta, Bangalore. The tomato plants were grown in the Agri-Horticulture Department of CFTRI. Fruits were harvested at different stages of maturity [green (GR), breaker (BR), 3 days post-breaker (BR 3), 5 days post-breaker (BR 5), 7 days post-breaker (BR 7),

10 days post-breaker (BR 10) and 14 days post-breaker (BR 14)] and the tissues were frozen using liquid nitrogen and stored at -80°C (SOLOW, USA) until further use.

2.2 Transcript analysis of carotenoid biosynthetic genes

Extraction of phagemid DNA from *E. coli* clones and PCR.

From the glycerol stock of *E. coli* clones harbouring different carotenoid biosynthetic genes shown in Table 1 (kindly provided by Dr Avtar K. Handa, Purdue University, USA), a loopful of culture was inoculated into LB broth with $50\text{ }\mu\text{g mL}^{-1}$ ampicillin and incubated overnight in a shaker incubator (Alpha Scientific, Bangalore, India) at 37°C . Phagemid DNA was extracted from these cultures as described by Sambrook *et al.*¹⁹ The DNA thus obtained was quantified spectrophotometrically and the inserts were confirmed using T3 (5'- ATTAACCTCAC-TAAAGGGA-3') and T7 (5'- TAATACGACTAACTA-TAGGGAGA -3') primers. The PCR was carried out in a thermal cycler (Master Cycler, Eppendorf, Germany) using Taq DNA polymerase (Bangalore Genei, Bangalore) and the PCR program used was initial denaturation at 94°C for 2 min followed by 30 cycles with 94°C for 30 s, 50°C for 30 s, 72°C for 1 min and final extension of 10 min at 72°C . The amplified PCR products were electrophoresed on a 1% agarose gel.

Dot blot preparation, hybridization and analysis. The phagemid DNA (20 ng) was manually spotted onto a positively charged nylon membrane (Roche Diagnostics, Germany), wetted by keeping over a pre-soaked (2x SSC) 3M Whatman filter paper, UV cross linked in a UV crosslinker at 12000 J s^{-1} (UVP, UK) for 2 min, briefly rinsed in sterile Milli-Q water and air-dried. The membrane was stored at $2-8^{\circ}\text{C}$ until the hybridization experiments were carried out.

For the transcript analysis of different carotenoid biosynthetic pathway genes, RNA extraction was carried out using TRI Reagent (Sigma Aldrich, Bangalore, India) from tomato leaf and fruit tissues (pulp with skin) at different stages of maturity following manufacturer's protocol. Briefly, the tissues ($\approx 50-100\text{ mg}$) were finely ground in liquid nitrogen and homogenized in 1 mL of TRI reagent. The homogenate was stored at room temperature (RT) for 5 min for complete dissolution of the nucleoprotein complexes. Chloroform (200 μL) was added and shaken vigorously for 15 s; the samples were incubated at RT for 15 min and centrifuged at 10 000 rpm for 15 min at 4°C . The upper aqueous layer was transferred to a fresh Eppendorf tube and RNA was precipitated by adding 500 μL of isopropanol, and then incubated at RT for 10 min. The samples were centrifuged at 10 000 rpm for 10 min at 4°C . The RNA pellet obtained was washed with 75% ethanol by vortexing and centrifuged at 10 000 rpm for 10 min at 4°C . The ethanol was removed and the pellet was briefly air-dried for 3–5 min and solubilized in 50 μL of DEPC (diethyl pyro carbonate) water by heating at $55-60^{\circ}\text{C}$ for 10 min. The RNA was analyzed by electrophoresis in a 1% agarose-formaldehyde gel and quantified spectrophotometrically. The concentration of RNA ($\mu\text{g mL}^{-1}$) was calculated by multiplying A_{260} by the dilution factor and extinction co-efficient ($1\text{ }A_{260} = 40\text{ }\mu\text{g RNA per mL}$).

$$A_{260} \times \text{dilution factor} \times 40 = \mu\text{g RNA per mL}$$

Table 1 Different carotenoid clones used in the study

Clone no.	EST ID	NCBI GI	Genes
Upstream genes			
cTOF13G5	EST472325	5059159	1-Deoxy-D-xylulose-5-phosphate synthase (DXS)
cTOA23B1	EST544380	15679907	1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)
cLEG44J6	EST441030	10943130	4-Hydroxy-3-methyl-but-2-enyl diphosphate reductase (Lyt B)
cTOB1O5	EST296226	6528386	Isopentenyl pyrophosphate isomerase (IPI)
cLEG9L24	EST399227	643093	Geranylgeranyl pyrophosphate synthase (GGPS)
cLEG31P23	EST411956	10804314	Phytoene synthase (PSY)
cLES9I16	EST260738	19286	Phytoene desaturase (PDS)
cTOA27N22	EST545748	6470254	ζ-Carotene desaturase (ZDS)
Downstream genes			
cLES3N4	EST258963	3005982	Lycopene ε cyclase (LCY E)
cLEC71N1	EST533455	1006672	Lycopene β cyclase (LCY B)
cTOD10L9	EST355779	5870597	β-Carotene hydroxylase (CrtR-B)
cTOD9L3	EST355566	1772984	Zeaxanthin epoxidase (ZEP I)
cTOF1J5	EST468977	24636984	Violaxanthin de-epoxidase (VDE I)
cTOF17I10	EST473193	8249884	Neoxanthin synthase (NXS)
cLEG71H1	EST589157	2769641	9- <i>cis</i> -Epoxycarotenoid dioxygenase (VP-14)

The cDNA probe was prepared from the extracted RNA from tissues at different stages of maturity. The reaction mixture consisted of 10 µg of RNA, 1 µL oligo dT (10 mM) primer and DEPC water to make up the volume to 20 µL. The contents were incubated at 70 °C for 10 min and cooled in ice immediately. To this, a mixture containing 5× assay buffer for reverse transcriptase, DTT (0.1M), 10 mM dNTP mix (containing DIG labelled UTP) and M-MuLV (Moloney murine leukaemia virus) reverse transcriptase (20 U) was added and incubated at 37 °C for 1 h in a water bath (Julabo, Bangalore). The reaction was stopped by heating at 70 °C for 15 min followed by cooling on ice.

Hybridization and detection was done using a DIG DNA labelling and detection kit (Roche Diagnostics, Germany). Briefly, the membrane (7 × 7 cm) was hybridized for 30 min at 62 °C using 5 mL of pre-heated DIG Easy hybridization solution. The DIG labelled cDNA probe was denatured by heating in boiling water for 5 min and rapidly cooled on ice. The denatured probe was mixed with 3.5 mL of DIG Easy hybridization solution and hybridized with the membrane at 62 °C overnight with gentle shaking. Stringency washes and detection were carried out following the manufacturer's instructions. The blots were scanned using an HP ScanJet 8250 scanner and stored in JPEG format at 200 dpi resolution. The image was quantified by Adobe-Photoshop software.²⁰

2.3 Extraction of carotenoids and HPLC analysis

Tomato fruit (pulp with skin) tissues (≈ 250 mg) at different stages of maturity were ground in liquid nitrogen. Carotenoids were extracted using 1 mL of hexane : acetone : methanol (2 : 1 : 1 v : v : v) along with Sudan I as internal standard.²¹ Samples were vortexed thoroughly and centrifuged at 10 000 rpm for 5 min at 4 °C. The supernatant was collected in a fresh Eppendorf tube, dried in N₂ gas and redissolved in 200 µL of the

mobile phase. All the extractions were carried out in dim light conditions.

The extracts were subjected to HPLC analysis in an Agilent 1100 liquid chromatograph instrument using ODS-P column (Inertsil 4.6 × 250 mm, 5 µm). The mobile phase consisted of methanol : tetrahydrofuran (95 : 5 v : v) with a flow rate of 1 mL min⁻¹. Detection was done at 450 nm using a UV detector and the column temperature was maintained at 30 °C. The amount of sample injected was 20 µL and the analysis was carried out thrice for each sample.

2.4 Colour measurement

The colour of tomato fruits at different stages of maturity were measured using Hunter Lab Labscan XE colour measuring system (Hunter Associates Laboratory Inc, Raton, Virginia, USA). The readings were recorded in terms of *L*, *a* and *b* values. The *L* (lightness value) ranges from 0 (black) to 100 (white). The *a* (negative value for green to positive value for red) and *b* (negative value for blue to positive value for yellow) axes have no specific numerical limits.

2.5 Statistical analysis

Analysis of variance was done with a completely randomized experiment with three replications and means were compared using Duncan's multiple range test (DMRT).²²

3. Results and discussion

3.1 Transcript analysis of various carotenoid biosynthetic genes

The expression pattern of the upstream genes (genes responsible for lycopene accumulation) such as DXS (1-deoxy-D-xylulose-5-phosphate synthase), DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase), Lyt B (4-hydroxy-3-methyl-but-2-enyl diphosphate reductase), IPI (isopentenyl pyrophosphate isomerase), GGPS (geranylgeranyl pyrophosphate synthase), PSY,

PDS and ZDS as well as downstream genes (genes responsible for converting lycopene to xanthophylls) such as LCY E, LCY B, Crt RB (β -carotene hydroxylase), ZEP I (zeaxanthin epoxidase), VDE I (violaxanthin de-epoxidase), NXS (neoxanthin synthase) and VP 14 (9-*cis*-epoxycarotenoid dioxygenase) of the carotenoid biosynthetic pathway was studied using cDNA probe synthesized from different maturity stage RNA. At the breaker stage, relative expressions of almost all upstream genes of carotenoid biosynthetic pathway increased significantly. At the 'breaker' stage of ripening, the color changes from green to pink because of the accumulation of lycopene, resulting from increased synthesis of carotenoid enzymes. The upstream genes of isoprenoid pathway such as DXS, GGPS and IPI showed a 1–2 fold increase, whereas, DXR, LYT B, PSY, PDS and ZDS genes increased 2–3 fold (Fig. 2). Similarly, the expression levels of LCY B and LCY E showed an increase of 1–2 fold and xanthophyll biosynthetic genes Crt RB, ZEP I, NXS and VP 14 increased by 2–3 fold (Fig. 3). Higher levels of gene expression of the central isoprenoid pathway during early fruit development in tomatoes has been reported.^{23,24} The mRNA levels for the enzymes PSY and PDS that produce lycopene increase 10–20 fold at the breaker stage of ripening, but the mRNAs of both lycopene cyclases, LCY B and LCY E disappear.^{17,18,25} Studies have revealed that the increase in PSY and PDS expression during the breaker stage is due to transcriptional regulation.²⁵

The highest accumulation of lycopene was observed at the 5 day post-breaker stage. Even though, the levels of upstream genes such as DXS, IPI, GGPS and PDS were high, increased levels of downstream genes LCY B, LCY E and NXS were also observed, which may be responsible for degradation of lycopene in subsequent stages. Similarly, at the BR 10 stage, highest levels of β -carotene accumulation coincided with the higher expression of xanthophyll biosynthetic genes such as Crt RB, ZEP I, NXS along with LCY E. This may be the probable reason for the decrease in the lycopene content leading to its conversion to other carotenoids. The increasing level of expression of carotenogenic genes DXS, PSY, PDS, ZDS, CRTISO, LCY B, LCY E and CHY B (β -carotene hydroxylase) has been observed during petal development of chrysanthemum also.²⁶

We observed a differential expression of carotenoid biosynthetic pathway genes in tomato fruits during ripening. Transcriptional upregulation of carotenoid biosynthesis during maturity have been shown in tomato,^{27,28} tobacco,²⁹ daffodil,³⁰ marigold³¹ and *Gentia lutea*.³² Regulation at the enzymatic level

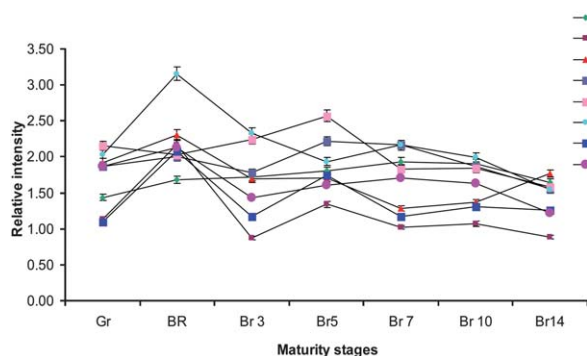


Fig. 2 Gene expression pattern of upstream genes of carotenogenic pathway in tomato tissues at different stages of maturity.

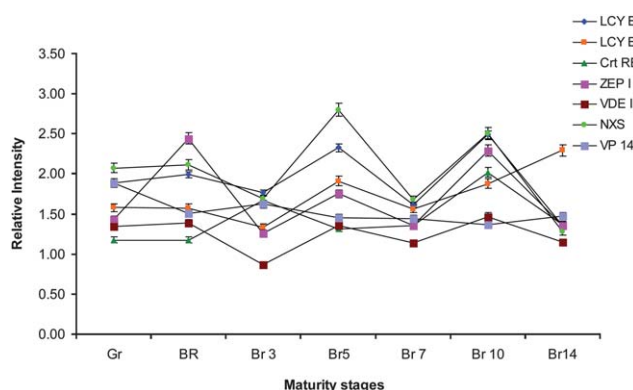


Fig. 3 Gene expression pattern of downstream genes of carotenogenic pathway in tomato tissues at different stages of maturity.

is predicted to account for the higher concentration of lycopene in fruits of the tomato mutants *old-gold*¹³ and *old-gold-crimson*.³³ The hypothesis that differential gene expression is the major reason for the accumulation of lycopene is further corroborated by the accumulation of δ -carotene in the fruits of the delta mutant, which results from increased transcription of CrtL-e (LCY E),²⁸ and by the synthesis of β -carotene in fruits of the beta mutant, which is caused by the upregulation of a second lycopene β -cyclase gene, Cyc-b.¹³ A similar upregulation of carotenoid gene expression during fruit development has been found in bell pepper,^{34–37} melon³⁸ and *Satsuma mandarin* (*Citrus unshiu* Marc).³⁹

In our study also, increased transcription levels of GGPS, PDS and ZDS may be responsible for lycopene accumulation at the early stages of ripening (up to BR 5) and increased expression levels of downstream enzymes (Fig. 3) in later stages may lead to the formation of β - β -, β - ϵ -carotenoids and xanthophylls to a lesser extent. The lycopene accumulation in autumn olive fruit also coincided with upregulation of upstream genes GGPS, PSY, PDS and ZDS and downregulation of LCY B and BCH (β -carotene hydroxylase) genes.⁴⁰

3.2. Lycopene and β -carotene content

Based on the HPLC chromatograms, the lycopene content showed a gradual increase from the green stage up to the 5 day post-breaker stage and the β -carotene up to the 10 day post-breaker stage (Table 2). The lycopene content (15.33 ± 0.24 mg per 100 g) was found to be highest at the BR 5 stage and highest β -carotene content (10.37 ± 0.46 mg per 100 g) was observed at the BR 10 stage. A decrease in the lycopene content was observed in the fully ripe stage.

The lycopene accumulation in tomatoes which accounted for 90–95% of the total carotenoids was found to vary from 9.1–12.4 mg per 100 g in different cultivars under varying growth conditions.⁴¹ Brandt *et al.*⁴² studied the lycopene content in tomato Daniella F1 variety at three different stages of ripening and found the highest lycopene content to be 10.3 ± 1.6 mg per 100 g in green house grown tomatoes and 6.8 ± 0.8 mg per 100 g in open field grown tomatoes at the fully ripe stage. Similarly, in Pitanga (*Eugenia uniflora* L.) cultivar, the lycopene content was found to be 3.35 mg per 100 g in partially ripe and 7.11 mg per 100 g in ripe fruits.⁴³

Table 2 Lycopene and β -carotene content at different stages of maturity in tomato

Tomato tissues	Lycopene (mg per 100 g)	β -Carotene (mg per 100 g)
Green (GR)	1.42 $\pm$ 0.14 ^c	0.46 $\pm$ 0.13 ^c
Breaker (BR)	2.76 $\pm$ 0.82 ^c	3.47 $\pm$ 0.93 ^d
3 days post-breaker (BR 3)	12.70 $\pm$ 0.87 ^b	4.12 $\pm$ 0.98 ^d
5 days post-breaker (BR 5)	15.33 $\pm$ 0.24 ^a	5.90 $\pm$ 0.84 ^c
7 days post-breaker (BR 7)	11.97 $\pm$ 0.74 ^{bc}	7.57 $\pm$ 0.66 ^b
10 days post-breaker (BR 10)	11.42 $\pm$ 0.75 ^c	10.37 $\pm$ 0.46 ^a
14 days post-breaker (BR 14)	9.58 $\pm$ 0.80 ^d	8.12 $\pm$ 0.96 ^b

Values are mean $\pm$ SD of 3 replications. Values in each column followed by same letter are not significantly different ($p \leq 0.05$).

In tomatoes the content of β -carotene has been shown to increase from the green (0.33 mg per 100 g) stage to the fully ripe stage (3.68 mg per 100 g at 24 days post-breaker).¹⁶ In our study also, we have found that content of β -carotene increased from 0.46 $\pm$ 0.13 mg per 100 g at the green stage to 10.37 $\pm$ 0.46 mg per 100 g at 10 days post-breaker. Studies on the carotenoid composition of other fruit also showed that β -carotene concentration increased as the fruit ripens. The concentration of β -carotene was found to be 0.54 and 1.2 mg per 100 g in partially ripe and 1.24 and 3.81 mg per 100 g in fully ripe fruits of two varieties of acerola.⁴⁴

The accumulation of carotenoids is dependent on the regulation of different carotenogenic enzymes. Apart from this, sequestration of carotenoids in non-photosynthetic tissues has been well established in the accumulation of carotenoids.^{45,46} It has also been reported by Cunningham *et al.*,⁴⁷ that the proportions of cyclic carotenoids may be controlled by the substrate specificity of β - and ϵ -cyclases.

3.3. Colour measurement

The L , a and b values of tomato fruit at different stages of maturity are shown in Fig. 4. The negative ' a ' value indicates the intensity of green colour and positive value indicates red colour. The green stage of the tomato fruit showed a more negative value due to the green colour. The breaker stage showed a less negative value due to a color change from green to pink. The remaining stages showed positive values for ' a ' with the highest being at the 5 day post-breaker stage, which is indicative of the highest lycopene accumulation at this stage (Table 2). Thus, the ' a ' value

showed a typical sigmoid curve where there is no noticeable change when fruits are still green (green to breaker stage) and increases as the colour changes from light red to fully red stage. These results are similar to the one observed by Lopez Camelo and Gomez.⁴⁸ The positive ' b ' value indicates a yellow color and it remained almost constant throughout ripening. The fact that the change in ' b ' value is negligible has been shown by other studies also.^{48,49} The ' L ' value is a measure of the lightness of the tomato fruit, which decreases during ripening indicating darkening of fruits (Fig. 4).

4. Conclusion

The present study shows the differential expression pattern of the complete carotenoid biosynthetic pathway genes for tomato cv. Arka Ahuti. The expression pattern coincided with the accumulation of the major carotenoid pigment, lycopene. The complete gene expression analysis of the present study may provide insight into devising strategies for enhancing carotenoid accumulation in tomato fruits.

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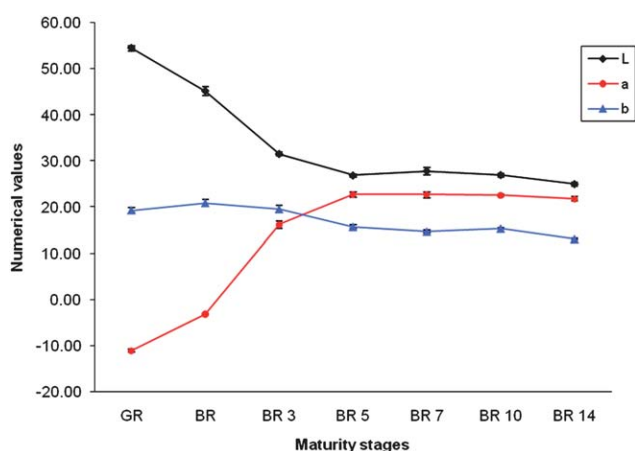


Fig. 4 Color values of tomato at different stages of maturity.

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