Anthocyanin enrichment of tomato (*Solanum lycopersicum* L.) fruit by metabolic engineering

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Transgenic tomato plants accumulating high amounts (70–100 fold) of anthocyanin in the fruit were developed by the fruit specific expression of two transcription factors, *Delila* and *Rosea*1 isolated from *Antirrhinum majus*. The transgenic tomato plants were identical to the control plants, except for the accumulation of high levels of anthocyanin pigments throughout the fruit during maturity, thus giving the fruit a purplish colour. The total carotenoids, including lycopene levels were unaffected in the anthocyanin-rich fruits, while its antioxidant capacity was elevated. The gene expression analysis confirmed the elevated expression of the downstream genes of the anthocyanin pathway due to the expression of the transcription factors and the expression levels coincided with the fruit ripening stages, highest expression occurring during the breaker stage. Anthocyanin-rich tomato fruit is important in view of the protective function of these compounds on consumption against a number of lifestyle-related diseases.

Keywords: Anthocyanin, carotenoids, metabolic engineering, transgenic tomato.

DIETARY anthocyanins are potent antioxidants, and modifiers of cell signalling pathways and their consumption is known to confer protection against cardiovascular diseases (CVDs), cancers, diabetes, arthritis, etc.[1,2]. They belong to flavonoids, a large group of polyphenolic compounds abundant in some fruits and vegetables, such as pomegranate, Indian black berry or jamun (*Syzygium cumini* L.), red/blue grapes, purple corn, purple cabbage, etc. The World Health Organization (WHO) estimates that 80% CVDs and one-third of cancers can be avoided by healthier diet (WHO Fact Sheet, Global Strategy on Diet, Physical Activity and Health). Anthocyanins are one such group of nutraceuticals known to play a protective function against chronic diseases. Increased consumer awareness of the protective functions of health foods and higher incidence of CVDs and cancers in the population has increased the demand for health foods, including the anthocyanin-rich fruits and vegetables.

However, anthocyanins are not present in the edible parts of some important crop plants, such as tomato, which is one of the most consumed vegetables worldwide. Naturally tomato fruits accumulate only traces of anthocyanins mainly in the peel[3] and small amounts of other flavonoids, such as naringenin chalcone and flavonols in the fruit[4,5].

Tomato is an ideal candidate for anthocyanin enrichment due to its widespread regular dietary intake in fresh, cooked and processed forms. Higher agronomic productivity, year-round availability and amenability for genetic engineering, coupled with consumption pattern make tomato a suitable crop for anthocyanin enrichment[6]. Attempts have been made to breed tomato fruits with enhanced anthocyanin content by classical plant breeding[7–10]. The genetic characterization of these plants revealed that the alleles, Aubergine (*Abg*), Anthocyanin fruit (*Aft*) and atroviolacea (*atv*) exhibited varying levels of anthocyanin accumulation in the epidermis only but not throughout the fruit; also the fruit size was negatively correlated with anthocyanin content. Tomato fruits with significantly higher levels of carotenoids and flavonoids were obtained by the fruit-specific RNA interference-mediated suppression of an endogenous photomorphogenesis-related regulatory gene DE-ETIOLATED 1 (*DET1*), demonstrating that the regulatory genes could be effectively employed in metabolic engineering to enhance the levels of a wide range of phytonutrients[11]. Manipulation of transcription factors is an ideal strategy to modify the expression of multiple target genes. In tomato, overexpression of Anthocyanin 1 (*Ant1*), a transcription factor regulating anthocyanin production has led to the accumulation of anthocyanins in fruit skin and a layer immediately below it[12]. Similarly, two transcription factors Leaf Color (*LC*) and Colorless1 (*C1*) from maize were introduced into tomato to induce anthocyanin accumulation in fruits[13], however no anthocyanin accumulation could be observed,

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whereas the accumulation of other flavonoids increased marginally. The variable effect of transcription factors in anthocyanin accumulation may be related to the target genes in different crops. The anthocyanin biosynthesis is regulated by R2R3-MYB, MYC of bHLH (basic helix–loop–helix) family of transcription factors and *WD*-40 like proteins whose regulatory effects vary among species[14,15]. In *A. majus* flowers, the two transcription factors, a bHLH-type *Del* and a MYB-related *Ros*1 interact to induce anthocyanin biosynthesis[16,17]. Thus by careful selection and fruit-specific expression of the transcription factors, it was possible to enhance the anthocyanin content of the tomato fruits.

The fruit-specific ectopic expression of the two transcription factors, Rosea1 (*Ros*1) and Delila (*Del*) from *A. majus* involved in upregulation of the genes of the anthocyanin pathway in tomato resulted in anthocyanin accumulation throughout the fruit of cultivar Micro-Tom[18]. However, Micro-Tom is not commercially cultivated and was originally developed for ornamental purposes. It harbours two major recessive mutations: dwarf (*d*) and miniature (*mnt*)[19] and is a preferred model plant for molecular research of fruit development.

In this experiment we set out to enrich the anthocyanin content of the fruits of a commercially cultivated tomato cultivar, Arka Vikas by fruit-specific expression of two transcription factors *Ros*1 and *Del* by *Agrobacterium*-mediated transformation. The transformed tomato plants had accumulated high levels of anthocyanin throughout the fruit on maturity and were phenotypically similar to the wild type plants, except for the dark chocolate to purple colour of the fruits.

Materials and methods

Gene cloning and vector construction

Del and *Ros*1 genes were obtained from the total RNA isolated from the field-grown floral petals of *A. majus*. Total RNA was isolated from 100 mg of floral petals using TRI® reagent (Sigma-Aldrich, USA). RNA was quantified and diluted to a concentration of 1 µg/µl with nuclease-free water. DNAase I (MBI Fermentas Life Sciences, USA) treatment was performed to remove traces of DNA in the RNA preparation. cDNA was synthesized using Revert-Aid First Strand cDNA synthesis kit (MBI Fermentas Life Sciences) in a reaction volume of 20 µl comprising 1.2 µg DNAase I treated RNA, 1 µl oligo-dT primer, 1 µl dNTP mix and 1 µl reverse transcriptase. Reverse transcription was performed according to the manufacture's protocol. Further, the cDNA was diluted five times and used for PCR amplification. PCR amplification of *Del* and *Ros*1 was carried out by employing gene-specific primers (Table 1) using cDNA. *E8* promoter was isolated from the tomato genomic DNA by PCR using specific primers. A promoter fragment of 2175 bp was cloned in pTZ57R/T and sequenced. The PCR reactions have been performed using the high-fidelity *Taq* DNA polymerase with 3′–5′ exonuclease proof-reading activity (Fermentas Life Sciences, USA).

Construction of Del and Ros1 expression cassette

Del and *Ros*1 were used for the construct preparation, individually under the control of the *E8* promoter and *Nos* terminator (*Nos-T*). The *E8* promoter cloned in pTZ57R/T was PCR-amplified using specific primers containing *Kpn*I and *Xho*I restriction sites in the forward and reverse primers respectively. The PCR product was digested with the above restriction endonucleases (RE) and cloned in pBluescript II KS+ vector. *Del* gene cloned in pTZ57R/T was PCR-amplified using specific primers containing RE sites, *Xho*I and *Pst*I for forward and reverse primers respectively. The amplified product was cloned in pBluescript II KS+ between *Xho*I and *Pst*I sites, in front of the *E8* promoter. *Nos-T* was amplified from pBI121 vector by PCR amplification using specific primers with RE sites, forward primer with *Pst*I site and reverse primer with *Bam*HI site, and was inserted between the same sites of the pBluescript containing *E8* promoter and *Del* gene. Initially, the assembling of fragments was done using pBluescript II KS+. Similarly, the *Ros*1 cassette contains *E8* promoter (2172 bp) :: *Ros*1 (663 bp) :: *Nos-T* (249 bp) inserted between the *Bam*HI–*Aat*II, *Aat*II–*Bcu*I and *Bcu*I–*Not*I restriction sites respectively. The *Del* cassette consists of *E8* promoter (2172 bp) :: *Del* (1967 bp) :: *Nos-T* (249 bp) inserted between the *Kpn*I and *Bam*HI restriction sites. The *Del* cassette was inserted between *Kpn*I and *Bam*HI sites of pBluescript II KS+ containing *Ros*1 cassette. The combined cassette (7510 bp) was inserted into a binary vector, pGreen II 0029 between *Kpn*I and *Not*I and designated as pGAntho (Figure 1). It was mobilized into *A. tumefaciens* strain EHA105 containing pSOUP plasmid by electroporation.

Tomato transformation and regeneration

Transformation of tomato with pGAntho construct was carried out by *Agrobacterium*-mediated gene transformation. Cotyledons and hypocotyls obtained from 10–12-day-old seedlings were used for transformation as described by Manamohan *et al.*[20]. The acclimatized plants were transferred to pots and grown in a biosafety net house to observe the anthocyanin accumulation in the fruits; the fruits were harvested in vine ripe stage for biochemical analysis.

Sampling for analysis

Sampling for biochemical analysis was performed using pGAntho transgenic tomato, vector control transgenic

Table 1. List of primers used in this study

Sl. No.	Primer	Primer sequence 5′–3′ Forward	Reverse	Amplicon length
1	*E8* P	gtttca**GGTACC**GAATTCATTTTTGACATC	gtttca**CTCGAG**AATCTCAATATGAGGATG	2172
2	Delila	gtttca**CTCGAG**AGAGGATTCAAGAATGGC	cttagt**CTGCAG**ATAGTGAGCATAAATCAA	1967
3	Nos-T	gtttca**ACTAGT**GATCGTTCAAACATTTGG	gtttca**GCGGCCGC**GATCTAGATGTAACATAGATG	249
4	*E8* P	gtttca**GGATCC**GAATTCATTTTTGACATC	gtttcag**ACGTCA**ATCTCAATATGAGGATG	2172
5	Rosea	gtttcag**ACGTCA**TGGAAAAGAATTGTCGT	gtttca**ACTAGT**TTAATTTCCAATTTGTTG	663
6	Nos-T	gtttca**ACTAGT**GATCGTTCAAACATTTGG	gtttca**GCGGCCGC**GATCTAGTAACATAGATG	249
7	Actin	TGGTATTGTGTTGGACTCTGG	AATCACGACCAGCAAGATCC	107
8	Del	AAGGCTTCTGATACGGACAAG	TCTTACGGCTTCCATCACTTC	165
9	Rosea	AGAGTATGGTGAAGGGAAATGG	CCGACCTCTTTTGATATTTGGC	118
10	CHI	TGCGGTTGGTAATTGTTTTCTC	TCCTCATTCTTTCCACCTGTAAG	109
11	F3H	TTACCCAAAGTGTCCAGAGC	AACGGGCTGAACAGTGATC	148

Bold letters represents the RE sites. Lowercase letters before the RE sites represent the anchor sequences which are helpful for efficient restriction digestion. Primers 1–3 were used for constructing *Del* cassette and transgenic screening; primers 4–6 were used for *Ros*1 construct preparation as well as transgenic screening, whereas, primers 7–11 were used for expression analysis.

Figure 1. Schematic diagram of the pGAntho gene construct in pGreen II.

tomato containing empty plant transformation vector pGreen II, wild type (WT) control tomato and commercial control tomato fruits obtained from the market.

Biochemical analysis of tomato fruits

Estimation of anthocyanin: Total anthocyanin content in the tomato fruit was determined by pH-differential spectrophotometry[21,22]. Briefly, 2 g of tomato fruit with pulp and skin was ground into a fine paste using 5 ml of distilled water as solvent. The extract was centrifuged at 9000 rpm for 10 min and filtered through Whatman No. 1 filter paper. The above extraction steps were repeated thrice and the final volume was made to 25 ml. Samples were diluted in a ratio of 1 : 2 with aqueous buffers of pH 1.0 (0.025M KCl) and pH 4.5 (0.4 M sodium acetate) separately and absorbance of triplicates was monitored at 510 and 700 nm for samples in both the buffers within 30 min. The total monomeric anthocyanins were calculated using the following formula and expressed as cyanidin-3-glucoside equivalent.

$$\text{Amount of anthocyanin (mg/g)} = A/26900 \times 449.2 \times 3 \times 25/\text{Amount of sample (mg)} \times 100.$$

Estimation of antioxidants: Total antioxidant activity of the anthocyanin-enriched tomato fruits was estimated by ferric reducing antioxidant power (FRAP) assay[23]. Briefly, the FRAP working solution was prepared by adding 0.3 M sodium acetate buffer, 10 mM TPTZ (2,4,6 tripyridyl-2-triazine) solution in 40 mM HCl and 20 mM ferric chloride ($FeCl_3$) in the ratio of 25 : 2.5 : 2.5 respectively. Two grams of tomato fruit was homogenized and made into a paste using 80% ethanol (10 ml) as solvent. The extract was centrifuged at 9000 rpm for 10 min and the supernatant was filtered using cotton wool. The above steps were repeated thrice and finally the volume of the extract was made up to 50 ml. The samples were left at 4°C overnight to settle down. The extract was mixed with 6 ml of FRAP reagent and incubated at 37°C for 60 min. Absorbance of coloured product was read at 593 nm, and the solution containing all reagents except the fruit extract was used as blank. Amount of antioxidants present in the sample was calculated using the following formula and expressed in terms of % AEAC (ascorbic acid equivalent capacity).

$$\text{Amount of antioxidants (\%)} = \text{concentration of sample } (\mu g) \times FV/V \times 100/\text{weight of sample (g)},$$

where FV is the final volume (ml) and V is the volume taken for estimation (ml).

Estimation of carotenoids: Total carotenoids were estimated using acetone extract of tomato fruits according to Thimmaiah[24]. Two grams of sample was homogenized in a mortar and pestle to make a fine paste. Then a known volume of acetone was added and allowed to settle for 2 min; then filtered using Whatman No. 1 filter paper.

This step was repeated until the paste turned yellow to colourless, then the filtrate volume was made up to 50 ml with acetone. The filtrate was mixed and incubated overnight at 4°C. Absorbance of filtrate was read at 450 nm after it warmed to room temperature and carotenoids were estimated using the formula: $C = D \times V \times f \times 10/2500$, where C is the amount of carotenoids (mg/100 g), D the absorbance at 450 nm, V the volume of original extract (ml), f the dilution factor and 2500 the average extinction coefficient of the pigments.

Estimation of lycopene: Lycopene was estimated as described by Thimmaiah[24]. Two grams of tomato fruit was extracted with acetone and the volume was made up to 50 ml. In a separating funnel 20 ml of petroleum ether was added and mixed with the extract, and to this 2 ml of 5% sodium sulphate (Na_2SO_4) was added and incubated at room temperature for 15 min. The colourless extract was collected in a beaker and orange coloured extract was taken back into a separating funnel and the above extraction was repeated until the extract became colourless. To this, 10 g of anhydrous sodium sulphate was added and incubated in dark for 60 min. Then the extract was filtered and volume made up to 50 ml using petroleum ether. Absorbance was read at 503 nm and the amount of lycopene was calculated using the formula:

$$\text{Lycopene (mg/100 g)} = (3.1206 \times \text{absorbance} \times \text{dilution}/\text{weight of sample (g)} \times 1000) \times 100.$$

Gene expression analysis by quantitative real-time PCR

For total RNA extraction, three stages (green, breaker and red-ripe) of tomato fruits were selected. RNA was extracted from 100 mg of tissue using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich,USA). DNA contamination in RNA sample was removed by treating with RNAase-free DNAase I and purified by phenol–chloroform (25 : 25) extraction. Quality and quantity of RNA were analysed by resolving in agarose gel (1.2%) and by estimating the ratio at A_{260}/A_{280} using Nanodrop1000™ respectively. Reverse transcription (RT) was performed in a total reaction volume of 20 µl comprising 1 µg total RNA, 0.3 µl (60 units) reverse transcriptase, 1 µl oligo-dT primer and 1 µl dNTP mix (10 mM) according to the manufacturer's instructions (Fermentas Lifescience INC, USA). RT negative control comprising all components except template was included. Further, 1 : 10 diluted cDNA was used for PCR amplification employing gene specific primers (Table 1).

The target gene expression was quantified by quantitative real-time (Q-RT PCR) (Light Cycler 480II Roche, Switzerland) using DyNAmo™ HS SYBR® Green qPCR Kit (Finzymes, Finland). β-actin was used as reference gene for normalization. Q-RT PCR was performed in 20 µl reaction volume comprising 10 µl SYBR green master mix, 0.5 µl each of forward and reverse primers (5 µM each), 5 µl of 1 : 10 diluted cDNA template and 4 µl molecular biology-grade water and the following cycling parameters were used: 95°C for 15 min followed by 40 cycles of 94°C for 10 sec, 59°C for 30 sec and 72°C for 30 sec. Melt-curve analysis of amplicons was performed to assess the specificity of amplification. The relative expression levels of target genes were calculated using $2^{-\Delta\Delta C}$T method[25].

Transgene segregation analysis in T_1 plants

Line no 8, a primary transformant, was selected for segregation analysis of the progeny. Of the 66 T_1 plants, 51 plants were positive for the transgene while 15 tested negative. Visual screening was carried for the purple and red fruit colours.

Chromaticity analysis

To determine the colour, hue and chroma of the fruits of anthocyanin-enriched tomato and WT plants, a hand-held portable colorimeter (Konica Minolta CR-10) was used. The $L^*a^*b^*$ system (L^* refers to lightness of colour, a^* to the proportion of red (positive values) and green colour (negative values), while b^* defines the proportion of yellow (positive values) and blue colour (negative values).

Data analysis

Statistical analysis of biochemical data was carried out using one-way ANOVA with three replicates using GraphPad Prism v.5 (GraphPad Software, Inc., USA) at $P < 0.05$. Q-RT PCR data were statistically analysed using one-way ANOVA with quantification cycle (C_q) values of three replicates utilizing GraphPad Prism v.5 (GraphPad Software, Inc., USA) at $P < 0.05$. Segregation was analysed statistically using X^2 test. Chromaticity data of fruits was statistically analysed using one-way ANOVA with three replicates of eight T_1 lines.

Results

Plant transformation and screening for the transgene

Forty-six primary tomato transformants were generated using *Agrobacterium*-mediated transformation using pGAntho binary vector. PCR screening using *Del*, *Ros*1 gene-specific primers confirmed the presence of the transgene in the primary transformants and two lines were selected for further analysis.

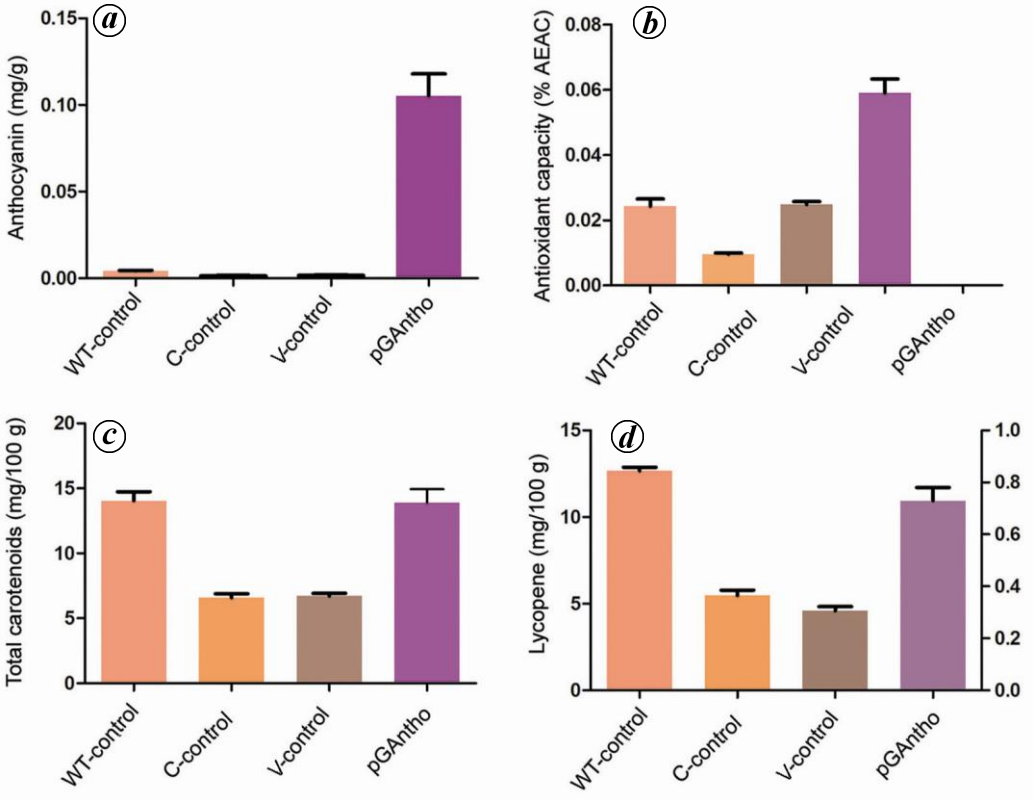

Figure 2. Biochemical analysis of tomato fruit. ***a***, Anthocyanin content; ***b***, Antioxidant capacity; ***c***, Total carotenoid content; ***d***, Lycopene content. Error bars represent standard error mean of triplicates. WT, Wild type; C, Commercial; V, vector and pGAntho, Transgenic tomato.

Biochemical analysis

Estimation of anthocyanins: Anthocyanin estimation revealed significantly ($P > 0.05$) higher accumulation of anthocyanins in the transgenic fruits compared to the WT, vector control and commercial control fruits. The average anthocyanin content of the transgenic fruit was 0.1 mg/g fresh weight, which was 70–100 fold higher than that of the control fruits (Figure 2 *a*).

Estimation of antioxidants: The antioxidant capacity was significantly higher ($P > 0.05$) in the transgenic fruits (0.0506% AEAC) than in the controls and was 6.2 times higher than that of the commercial control fruits and nearly double than that of the WT control fruits (Figure 2 *b*).

Estimation of carotenoids: The total carotenoid content of the transgenic fruits (13.89 mg/100 g) was on par with that of the WT control, while it was nearly two fold higher than that of commercial control fruits (Figure 2 *c*).

Estimation of lycopene: The lycopene content of the transgenic fruits and WT control fruits did not differ significantly and was 10.93 mg and 12.65 mg per 100 g fruit respectively, whereas commercial control fruits had only half as much lycopene as the anthocyanin-rich fruits Figure 2 *d*).

Gene expression analysis

Del and *Ros*1 expression was highly dependent on fruit developmental stages. The expression levels were lower in green stage. In breaker-stage fruits, the expression of *Del* and *Ros*1 showed 10.15 and 24.43 folds upregulation respectively. However, in red-ripe fruit, the expression levels were drastically reduced (Figure 3 *a*). Further, the expression of target genes of *Del* and *Ros*1, viz. chalcone isomerase (*CHI*) and flavanone-3-hydroxylase (*F3H*) were analysed across the development stages of the fruits. The expression levels of *CHI* and *F3H* were 5.69 and 6.63 fold higher respectively, in breaker stage compared to green and red-ripe stages. In addition, the expression levels of *CHI* and *F3H* were several fold higher in the transgenic fruits compared to the WT fruits (Figure 3 *b*).

Segregation analysis of the transgenic progeny

Segregation analysis by progeny testing of the transgenic line no. 8 indicated transgene segregation in a Mendelian fashion. Visual screening of fruit colour confirmed its

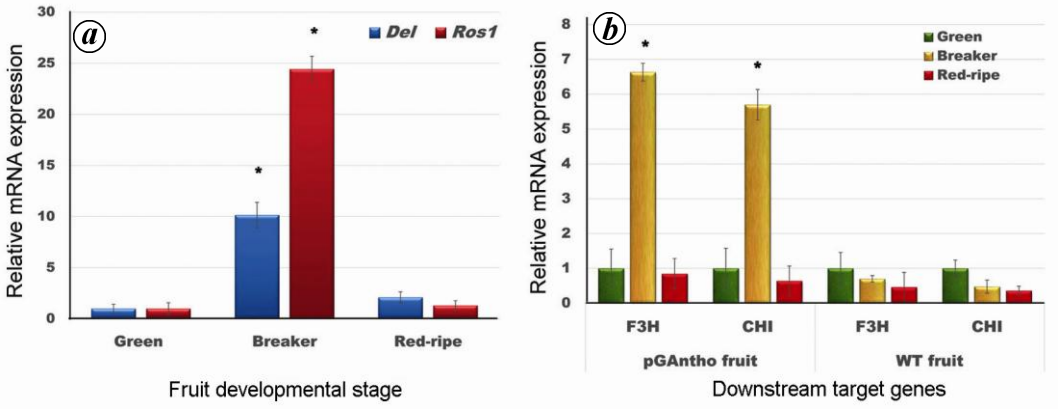

Figure 3. Expression analysis of *Del* and *Ros*1 and their targets: ***a***, Relative mRNA levels of *Del* and *Ros*1 at three developmental stages of fruit. ***b***, Relative mRNA levels of *F3H* and *CHI* induced by *Del* and *Ros*1 at three developmental stages of fruit.

segregation with nearly 75% of the plants showing purple colouration and 25% showing red colouration.

Chromaticity analysis

The *a*+ and *b*+ values obtained for the control fruit were higher indicating the intense red colour of the fruit, whereas the anthocyanin-enriched fruits displayed lower values for both *a*+ and *b*+ indicating purple colour. It was also observed that *L*+ value for red tomato displayed higher proportion of lightness of 32.87, whereas lower values were observed for lightness in all the transgenic tomato fruits (Table 2).

Discussion

Tomato plants contain flavonoids, including anthocyanin and small quantities of intermediary compounds of the flavonoid pathway such as naringenin chalcone and some flavonols such as kaempferol and quercetin glycosides mainly in the vegetative tissues[4,13]. In tomato fruit, flavonoids accumulate mainly in the peel, which is only about 5% of total fruit weight[6,9]. Various approaches, such as classical plant breeding[8] and transgenics[3], have been employed to accumulate flavonoids in tomato fruits, but the flavanoid accumulation was mostly restricted to the pericarp of the fruit. Thus on the basis of whole fruit fresh weight, flavonoid accumulation was low. Efforts to introgress genes derived from wild species of tomato were made[8]. However the pigment accumulation was limited to the peel and a thin layer beneath it (http://hort.oregonstate.edu/purple_tomato.faq). Careful selection of the complementing transcription factors regulating the genes of the anthocyanin pathway is crucial for anthocyanin enrichment in tomato. In this regard, the transgenic tomato (cv. Micro-Tom) plants were developed which accumulated more than hundred-fold anthocyanin compared to the WT tomato fruits[14]. But, Micro-Tom is not used for commercial cultivation, hence, the commercially cultivated cultivar tomato Arka Vikas was selected for enriching the anthocyanin content in the fruit. It is also an open-pollinated tomato variety with good combining ability for use in breeding programmes.

We have developed transgenic tomato plants producing anthocyanin-rich fruits by the ectopic expression of two transcription factors, *Del* and *Ros*1 from *A. majus*. *Del* is a MYC-type bHLH transcription factor that affects the pigmentation of corolla tube and is known to be required for the activation of late anthocyanin biosynthesis genes, including flavanoid 3′-hydroxylase (*F3′H*), dihydroflavonol 4-reductase (*DFR*), anthocyanin synthase (*AS*) and UDP-glucose 3-o-flavanoid transferase (*UGFT*) in *A. majus*[16,26]. *Ros*1 is a R2R3 MYB transcription factor that determines the pattern and intensity of floral anthocyanin pigmentation by modulating the expression of a number of structural genes of the anthocyanin biosynthesis such as *F3′H*, *F3H*, flavonols synthase (*FLS*), *DFR* and *UFGT* which are highly dependent on *Ros*1 for induction, whereas anthocyanidin synthase (*ANS*) and anthocyanin transporter (*AT*) were less dependent on *Ros*1 for induction in *A. majus*[16]. The Delila protein can itself bind to the G-box in the promoter region of the target genes independently[27] and also by forming a complex with the MYB factor such as *Ros*1 and *WD*-40 factors[28]. Genetic studies reveal that anthocyanin biosynthesis is regulated at the transcriptional level by a ternary complex of proteins, MYB-bHLH-WD40 proteins[6,29]. The *E8*:*Del*:*Ros*1 transgenic tomato plants developed were investigated for the expression levels of *Del* and *Ros*1 transcription factors and their target genes, viz. *CHI* and *F3H* at three different developmental stages of tomato fruit. A dramatic increase of 10- and 25-fold in the transcript levels was observed for *Del* and *Ros*1 genes respectively, in breaker stage compared to the green and red-ripe fruits of transgenic tomato. This increased expression might be

Table 2. Chromaticity analysis of WT control and pGAntho transgenic tomato fruits

	Wild-type control	pGAntho transgenic T_1 tomato lines							
		8–33	8–23	8–16	8–17	8–19	8–17	8–13	8–61
a+	21.33 ± 0.4	8.9 ± 0.5	8.6 ± 1.4	9.9 ± 0.7	12.1 ± 1.2	10.63 ± 0.2	14 ± 0.6	8.8 ± 0.2	7.57 ± 0.5
b+	16.17 ± 0.3	5.33 ± 0.3	6.53 ± 0.5	6 ± 0.3	8.27 ± 1.0	8.33 ± 0.29	10.4 ± 0.7	5.46 ± 0.4	5.66 ± 0.4
L+	32.87 ± 0	25.97 ± 0.6	27.4 ± 0.3	26.83 ± 0.1	27.53 ± 0.4	28.03 ± 0.8	29.37 ± 0.4	26.27 ± 0.1	26.43 ± 0.4

Values are represented for mean ± SEM.

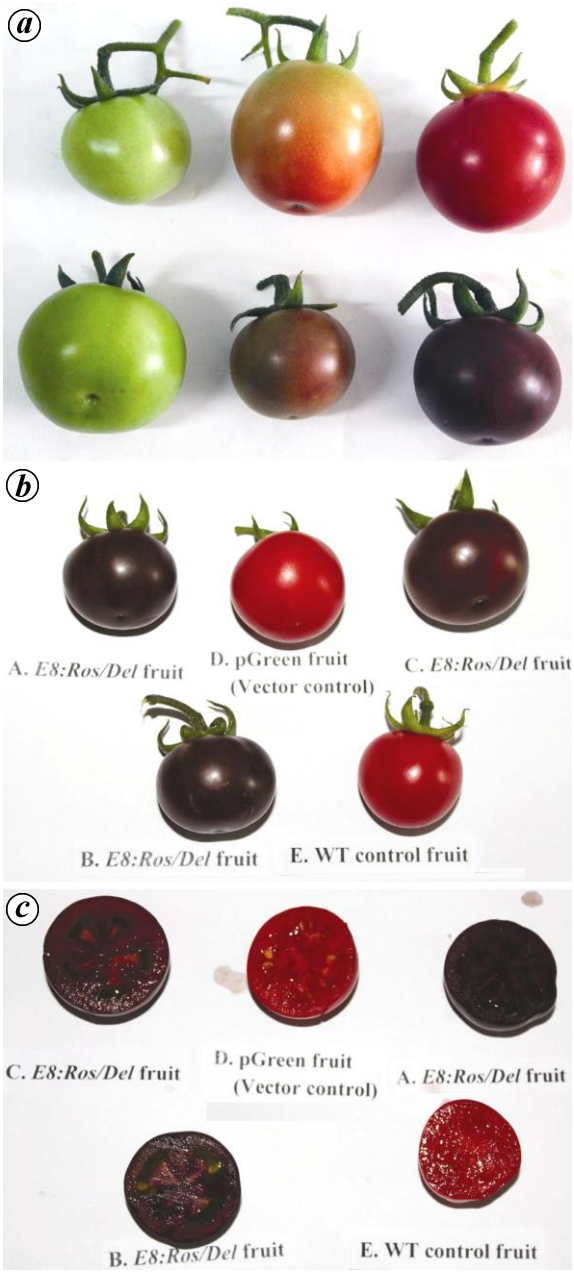

Figure 4. Tomato fruit colour in pGAntho (*E8:Ros/Del*) transgenic, WT-control and vector control. ***a***, Fruit colour during developmental stages of the fruit, from left green, breaker and red-ripe of WT-control (top) and pGAntho (bottom). ***b***, Comparison of ripened fruits of pGAntho, WT-control and Vector control. ***c***, Transverse section of the ripened fruits of pGAntho, WT-control and Vector control.

responsible for the biosynthesis and accumulation of high levels of anthocyanins throughout the tomato fruit. In order to understand whether the increased expression of *Del* and *Ros*1 leads to the enhanced expression of the genes of the anthocyanin biosynthetic pathway, we analysed the expression of two target genes of *Del* and *Ros*1, viz. *CHI*, a key enzyme responsible for the isomerization of chalcone to naringenin and *F3H*, a key enzyme in the diverging point of the flavonoid biosynthetic pathway, leading to the synthesis of anthocyanins and flavanols. In pGAntho transgenic fruits, *CHI* and *F3H* expression was 10.5- and 12-fold higher respectively, than in the WT fruits (Figure 3 *b*). We also observed a developmental stage-dependent regulation, where the expression levels of *CHI* and *F3H* were 5.69- and 6.63-fold higher respectively, in breaker stage compared to green and red-ripe stages (Figure 3 *b*).

It was reported that the main bottleneck in anthocyanin accumulation in tomato fruits was insufficient expression of *CHI*, thus, in the transgenic fruits significantly higher expression of *CHI* compared to WT would have resulted in accumulation of high levels of anthocyanin. The strong accumulation of anthocyanin in transgenic tomato fruits may also be contributed by the composition and interaction of the MYC factors and MYB factors leading to the overexpression of genes of the anthocyanin biosynthetic pathway. The regulatory pattern of MYC and MYB transcription factors varies in monocots and dicots, unlike the *LC* of maize, in *Antirrhinum*, the anthocyanin pathway genes are regulated by three genes, *Delila*, *Eluta* and *Rosea* which activate different parts of the anthocyanin pathway[26]. However, the ectopic expression of *Delila* in tomato and tobacco has led to a strong pigmentation of floral and vegetative parts respectively, which in part may be explained by strong induction of *DFR* expression[30].

On similar lines, a transposon insertion mutation in a R2R3 MYB factor, purple (*Pr*) in cauliflower causes intense purple curd instead of white; the mutation in the promoter region overexpresses the *Pr* gene, which interacts with bHLH factors to activate the structural genes of anthocyanin biosynthesis. The *Pr* gene may be the functional equivalent of *Ros*1 in *Brassica*. Harbinger DNA transposon insertion in the upstream regulatory region of promoter of the *Pr-D* allele was responsible for the

upregulation of the *Pr* gene in inducing phenotypic change in the plant[31].

The highest anthocyanin content of the transgenic fruits (0.13 mg/g fresh weight) was 70–100 fold higher than that of the control fruits. Anthocyanins are also known to be potent antioxidants. The antioxidant capacity of the transgenic tomato fruits as measured by AEAC showed that it was 2.5–6 times higher compared to control fruits. This may be due to the increased anthocyanin content in the transgenic fruits. The anthocyanin and carotenoid biosynthetic pathways are known to operate independently in plants. Similarly, in this experiment we have observed that in anthocyanin-rich tomato fruits the total carotenoid and lycopene levels were similar to those of the WT fruits. The genetic analysis of the anthocyanin accumulating lines has shown that the carotenoid levels in them has remained unaltered, suggesting that anthocyanin accumulation does not affect the carotenoid biosynthesis[9].

The segregation of the transgene in the T_1 progeny exhibited a single copy integration and stable Mendelian inheritance pattern as the transgene segregated in a ratio 3 : 1 of transgenic to null. The chromaticity analysis of the control and transgenic tomato fruits has clearly shown the colour differences among these fruits; the control fruits tending towards intense red, whereas the pGAntho transgenic fruits show a tendency towards intense blue to purple colour (Table 2 and Figure 4).

The protective effects of flavanoid-rich tomatoes in cancers and CVDs have been demonstrated in transgenic mice with *Trp*53$^{-/-}$ mutation, where significantly prolonged survival was observed compared to the mice fed on WT tomato fruit or standard laboratory diet[18], whereas the markers of cardiac health were significantly improved in transgenic mice expressing C-reactive human protein and fed with high flavonoid tomato peel[32]. It was observed that an 8-week feeding of the rat with maize-derived, anthocyanin-rich diet provided protection to the rat heart against ischemia-reperfusion injury[33]. The cardio-protection was associated with increased myocardial glutathione levels, suggesting that dietary anthocyanins might modulate cardiac antioxidant defences.

In conclusion, we have developed cultivated tomato cv. Arka Vikas producing fruits rich in anthocyanins, while retaining natural high carotenoid levels of tomato inherited in a stable Mendelian fashion. The transgenic approach has achieved high levels of anthocyanin content throughout the fruit. The fruit is of value for its health benefits as it is rich in anthocyanins along with naturally occurring carotenoids. In addition to the direct consumption, these fruits can also be utilized to extract biological food colourants and anthocyanin compounds used as a nutraceuticals.

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