Phenotypic and Molecular Characterization of Anthocyanin Biosynthesis and Accumulation in Fruit Tissues of a Selected Tomato Mutant

Khaldoun O. Al Sane

***Abstract*—**Anthocyanins are present in a wide range of plant tissues, principally flowers and fruit, but also storage organs, roots, tubers and stems. Anthocyanins are able to play important physiological roles including protection of tissues from photo-inhibition caused by high levels of visible light, and from oxidative damage, and controlling the transcription of genes required for growth and development. In tomato (Solanum lycopersicum L.), anthocyanins are normally synthesized only in vegetative tissues. Surprisingly, Aft mutant (accession LA1996) displays anthocyanin accumulation in the fruit. In the present work, I have characterized a mutant in which elevated levels of anthocyanins were shown in its fruit tissues exposed to sunlight. The molecular regulation of anthocyanin biosynthetic pathway in this mutant and in its genetic background (Ailsa Craig, AC) has been, also, described.

***Keywords*—** Anthocyanin, biosynthetic pathway, molecular regulation, Solanum lycopersicum L., tomato mutant

I. Introduction

FLAVONOIDS represent a large class of plant phenolic compounds, of which anthocyanins are the most wide-spread pigments due to their unique structures and thus a wide range of chemicals are derived along their biosynthetic pathway [21]. Anthocyanins are present in a wide range of plant tissues, principally flowers and fruit, but also storage organs, roots, tubers and stems [11]. Anthocyanins and flavonoids are usually found in the vacuoles of almost every cell type in the epidermal, ground, and vascular tissues of all vegetative organs [11], [27]. Anthocyanins- Greek anthos (flower) and kyanos (dark blue)- are colored pigments that give flowers their characteristic red, purple, and blue colors [11], [19]. However, the possible physiological roles of anthocyanins in vegetative tissues have perplexed scientists for well over a century [11]. They added, several different factors can affect the final color of the fruit or flower.

Although the selective pressure that has driven the occurrence of anthocyanins and other flavonoids in such disparate vegetative structures remains far from obvious, plant physiologists have made significant progress over the past decade in elaborating the consequences of cellular anthocyanins on plant function [1], [9], [11].

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Moreover, there are several limitations to the current supply of anthocyanin pigments. The plant sources are very limited. As a result, food manufacturers have a narrow color range from which to choose. In addition, the supply could be affected by long cultivation times, seasonal and climatic variations, pest and disease attack and the increasing cost of intensive agriculture coupled with the decreasing availability of low-cost arable land [24]. All of these factors affect quality and quantity of supply. As the demand for more prepared and processed food increases and the expectation of high health products rises, food manufactures are requiring a wider range of colorants and antioxidant additives. One alternative source for the production of anthocyanins is by using plant cell cultures [24], [20].

New knowledge for genetic engineering enhanced agronomic and nutritional traits in plants. It seems likely that anthocyanin regulation, biosynthesis and accumulation will continue to serve as an important experimental model for understanding cellular metabolism for some time to come [12], [13], [19]. An excellent and convenient model for such an approach is tomato since it is among the most important, commonly consumed vegetables in human diets worldwide [4], [28], [29]. Many enzymes required for the production of different flavonoid classes have been characterized [14], [30]. In tomato (Solanum lycopersicum L.), the anthocyanins biosynthetic pathway had been described [5], [14], [26]. Further, the principal genes involved in the anthocyanin biosynthetic pathway in tomato have been recently identified [8], [14], [32]; Even though, their expression and exact involvement in genetic regulation of anthocyanin production is still not fully described.

In tomato fruit, only small amounts of flavonoid biosynthetic intermediates are accumulated, whereas anthocyanins are usually not synthesized [22], [27]. Nevertheless, several tomato mutant alleles result in altered levels of anthocyanin accumulation in fruits and/or vegetative tissues. They have been isolated and catalogued as monogenetic stocks by the Tomato Genetic Resource Center, (TGRC, University of California, Davis, http://tgrc.ucdavis.edu). There is, now available, a considerable number of tomato accessions that display phenotypes characterized by anthocyanin intensification (Table 1), partial or complete absence, or any other form of anthocyanin alteration in fruits and vegetative tissues as well. For instance, Aft (accession LA1996) and Abg (accession

LA3668) mutants display anthocyanin accumulation in the fruit (reviewed by [10], Table 1) compared to the wild type (Ailsa Craig, AC). Therefore, I have chosen this mutant for the study. Furthermore, other tomato mutants, which are unable to synthesize anthocyanins, have been isolated after submission of wild type seeds to chemical mutagenic treatment [3].

Since anthocyanin mutations can be utilized as genetic markers, our research interest was focused on studying the gene(s) encoding the different steps along the anthocyanin biosynthetic pathway in different tomato genotypes. In the present work, Anthocyanin fruit (Aft) tomato mutant, accession LA1996, has been described and characterized. Besides, the work was performed to understand and monitor the molecular regulation of anthocyanin biosynthetic pathway in the mutant experimented and characterized. Reference [16] reported the presence and quantity of anthocyanins in Aft tomato fruits, and concluded that the simple inheritance of Aft makes the utilization of this gene in existing tomato germplasm feasible. In the current study, I went one step ahead in studying a part of the molecular regulation of anthocyanin biosynthesis in this mutant.

II. Materials and Methods

A. Plant material and growth conditions

Tomato (Solanum lycopersicum L.) seeds of Anthocyanin fruit (Aft) mutant (accession LA1996) and its genetic background, AC (Ailsa Craig) were used in this study. The phenotype of Aft mutant is characterized by elevated levels of anthocyanins in its fruits (the Tomato Genetic Resources Center (TGRC), University of California, Davis, http://tgrc.ucdavis.edu; reviewed by [10], Table 1).

Growth media was prepared in which a hydroponic solution was used in 1:200 dilution applying salt-free-water. The detailed composition of the stock solutions applied is shown in table 2. After growth and setting fruits, sampling was performed taking fruit tissues exposed to sunlight or grown under shade in Aft plants. While a uniform coloration was observed in the wild type (AC); therefore tissues were obtained accordingly. Four different fruit tissues were analyzed; skin, pericarp, placenta, and seeds. Tissues were collected during each developmental stage: mature green, turning red (breaker), and red ripe stages, immediately frozen using liquid N2 and stored at -80 °C for later analyses .

B. RNA extraction and purification

Seedlings were collected, immediately frozen using liquid N2 and stored at -80 °C. The frozen material was later ground to a fine powder with liquid N2 using a mortar and pestle and undergone RNA extraction protocol using the aurintricarboxylic acid method as previously described [23]. RNA was extracted from seedlings grown on Murashige and Skoog solution (control). RNA was, then, subjected to agarose gel electrophoreses on a 1% agarose gel to assess and ensure good quality of the extracted RNA. Further, its quantity was spectrophotometrically determined scoring the readings at 260 and 280 nm.

C. DNase treatment and retrotranscription

To eliminate any possible DNA contamination of our RNA samples, DNase treatment was performed using the TURBO DNA-free kit (Ambion, Austin, TX, USA). RNA (2 µg) from each sample were reverse-transcribed into cDNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA).

D. Real-time reverse transcription polymerase chain reaction (RT-PCR)

The amplification and assessment of fluorescence have been analyzed in PCR reaction plate of 96 wells through an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) and the default ABI Prism 7000 PCR program for PCR conditions.

The expression analysis of the genes: LeDFR (GenBank accession no Z18277) (forward primer:5'-CAAGGCAGAGGGAAGATTCATTTG-3'; reverse primer: 5'-GCACCATCTTAGCCACATCGTA-3' ; TaqMan probe: 5'-ATCCCATCATGCTATCATC-3') and LeANT1 (GenBank accession no DD030645) (forward primer: 5'-AAGTGGATCTCATTTTGAGGCTTCA-3' ; reverse primer: 5'-TCCTTCCGGGAAGTCTACCA-3'; TaqMan probe: 5'-CAACAGATGGTCACTTATTG-3') was performed by real-time reverse transcription polymerase chain reaction (RT-PCR) loading the corresponding primers and following the manufacturer's protocol.

The gene LeEF1A was used as an endogenous control (Lycopersicon esculentum ELONGATION FACTOR 1-ALPHA; GeneBank accession number X14449) (forward primer: 5'-TGCTTGCTTTCACCCTTGGT-3'; reverse primer 5' CGATTTCATCATACCTAGCCTTGGA-3'; and its relative expression was determined. Relative quantification of each single gene expression was performed using the comparative threshold cycle (CT) method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin number 2 (Applied Biosystems).

E. Microscopy and photography

Photos of the fruit tissues of Ailsa Craig and Aft mutant shown in this study were taken using a DS-U2 Nikon digital sight camera (Nikon,Tokyo,Japan) together with a Nikon TMS-F microscope (type 104) connected with the NIS-elements F2.20 imaging software (Laboratory Imaging, Nikon, Tokyo, Japan).

III. Results

A. Phenotypic characterization of peel tissues

The peel tissues of both the mutant Anthocyanin fruit (Aft) and its genetic background (Ailsa Craig) grown in the greenhouse were phenotypically analyzed during different developmental stages; mature green, turning red (breaker) and red ripe stages (Fig. 1). Accumulation of anthocyanins occurred only in fruit tissues of the Aft mutant which were directly exposed to sunlight, whereas a uniform coloration was observed in the wild type (Ailsa Craig) fruits; either exposed to sunlight or grown under shade (Fig. 1).

B. Microscopic characterization of peel tissues

The Anthocyanin fruit (Aft) genotype and Ailsa Craig (the line selected as a wild type) were also microscopically analyzed. Peel tissues of both genotypes were visualized under microscope (Nikon TMS-F microscope (type 104) connected with the NIS-elements F2.20 imaging software, Laboratory Imaging, Nikon, Tokyo, Japan) during different developmental stages; mature green, turning red (breaker) and red ripe stages (Fig. 2). Anthocyanin pigmentation in spot-pattern only in the peel tissues of the mutant fruits could be observed. Those spotted areas were found only in the parts of fruits exposed to sunlight. The same result obtained earlier in the wild type (AC), in which a uniform coloration was observed, was confirmed using the microscopic characterization. The tissues showed the same color either the fruits were exposed to sunlight or grown under shade (Fig. 2).

C. Expression of the genes involved in anthocyanin biosynthesis and accumulation in fruit tissues of Aft tomato mutant s

I have analyzed the expression of the genes (LeDFR and Ant1) hypothesized to be involved in anthocyanin biosynthesis and accumulation in fruit tissues of two tomato genotypes; Anthocyanin fruit (Aft) tomato mutant and in the wild type Ailsa Craig (AC). Four different fruit tissues were analyzed; skin, pericarp, placenta, and seeds. Expression of DFR (Figs. 3a, c, and e) and of Ant1 (Figs. 3b, d, and f) have been detected. Higher expression levels of DFR were reported compared to Ant1 expression in all developmental stages analyzed; mature green, turning red, and red ripe (Figs. 3a-f). These genes were much higher expressed in Aft fruit parts which were exposed to sunlight. Only slight expression levels were detected in the wild type (black bars) and in Aft fruit tissues grown under shade (Figs. 3a-f). Further, Ant1 was not expressed at all during mature green stage (Fig. 3b).

IV. Discussion

Accumulation of anthocyanins in spots in peel tissues of the mutant *Anthocyanin fruit* (*Aft*) fruits was observed (Fig. 1) in all developmental stages; mature green, turning red (breaker) and red ripe. This was confirmed by the microscopic characterization of peel tissues of the mutant fruits compared to its genetic background Ailsa Craig (AC) grown in the greenhouse (Fig. 2). These spotted areas were found only in the parts of fruits exposed to sunlight while a uniform coloration was observed in the wild type (Ailsa Craig) either the fruits were exposed to sunlight or grown under shade (Figs. 1,2). This result was in agreement with a study conducted on apples in which Starking Delicious apples produced higher anthocyanin levels than Fuji and Mutsu under light in the whole fruit skin [2]. In another study, it has been found that in contrast to the control sample that was kept in the dark, natural light increased the total anthocyanin content in cranberry fruit (*Vaccinium macrocarpon* Ait) [3].

Reference [15] concluded that plant pigmentation is affected by a variety of factors. Light, an important plant developmental signal, influences the accumulation of anthocyanins primarily through the activation of the transcription factors that regulate the flavonoid biosynthetic pathway. From their study conducted on maize cells, they found that light induces the fusion of anthocyanin-containing vacuoles, anthocyanic vacuolar inclusion (AVI) described earlier in this study, and the spread of anthocyanins from the inclusions into the vacuolar sap. Their findings suggest a novel mechanism for the action of light on the vacuolar storage of anthocyanin.

Not only the light intensity concern. Other factors affect the accumulation of anthocyanins as well. The light quality, wavelength, light/dark cycles are some examples. The relatively short wavelength band ranging from blue to ultraviolet (UV) was important for the accumulation of anthocyanins in 'Gros Colman' grape berries [18]. In addition, continuous light operation enhanced anthocyanin production more than the light/dark cycle process. These findings are useful for designing and operating photo bioreactors for enhanced anthocyanin production [18]. Further, It has been emphasized that the ultraviolet light is involved in anthocyanin accumulation in 'Gros Colman' grape berries [17]. Therefore, UV permeability of covering materials in greenhouses should be considered.

Expression of the genes (*LeDFR* and *Ant1*) have been analyzed. These genes are involved in anthocyanin biosynthesis and accumulation in fruit tissues of *Anthocyanin fruit* (*Aft*) tomato mutant and in the wild type Ailsa Craig. Four different fruit tissues were analyzed; skin, pericarp, placenta, and seeds (Fig. 3). *DFR* was higher expressed compared to *Ant1* expression levels in all developmental stages analyzed; mature green, turning red, and red ripe (Figs. 3a-f). These biosynthetic genes were much higher expressed in *Aft* fruit tissues which were exposed to sunlight and particularly in skin tissues. Low expression levels were detected in the wild type and in *Aft* fruit tissues grown under shade (Figs. 3a-f). A study was conducted on Arabidopsis [6] and showed strong light induction of the *MYB* genes *PAP1* and *PAP2*. All structural genes (*CHS, DFR, F3H, LDOX*) were consistent with the hypothesis that they have a key role in light induction of anthocyanin biosynthesis. All *bHLH* genes analyzed showed light induction and in the seedlings, their expression preceded that of the late structural genes, suggesting their possible role in light regulation of these structural genes.

Much work has been carried out on the genetic control of anthocyanin biosynthesis [7]. To eliminate the limitations in the expression of anthocyanin biosynthetic pathway, new approaches need to be implemented. Isolation of genes coding for enzymes that is considered the driving force towards anthocyanin production and are under-expressed *in vitro*, returning these key genes to cultures under the control of high-expression promoters to proceed in the pathway could be a successful approach [11], [19].

Moreover, other approaches have been suggested including: developing appropriate vector systems, *in vitro*

transformation of cells [25], generating tomato transformants in which sequences encoding key biosynthetic enzymes or encoding regulatory elements are ectopically expressed [5], [28].

The evaluation of flavonoid gene expression of wild tomato species, as well as mutant lines, can be the first step towards enhancement of flavonoid production. Yet, there are still some important considerations: are the health-protective qualities observed in *in vitro* studies also displayed *in vivo*? Moreover, could *in vivo* anthocyanins production proceed without significant variability compared to the *in vitro* systems?

More investigations need to be carried out, and new insights yielded from different fields like chemistry or biotechnology, and developing industrial-scale extraction and purification processes, will probably increase the application of anthocyanins and anthocyanin-derived pigments in the food industry.

ACKNOWLEDGMENT

The author would like to thank Prof. Pierdomenico Perata (Scuola Superiore Sant' Anna, Italy) for his valuable assistance, support and guidance throughout my work. This research was supported by *the Italian Ministry of University and Research (MiUR), PRIN2006, TomANTHO Project*.

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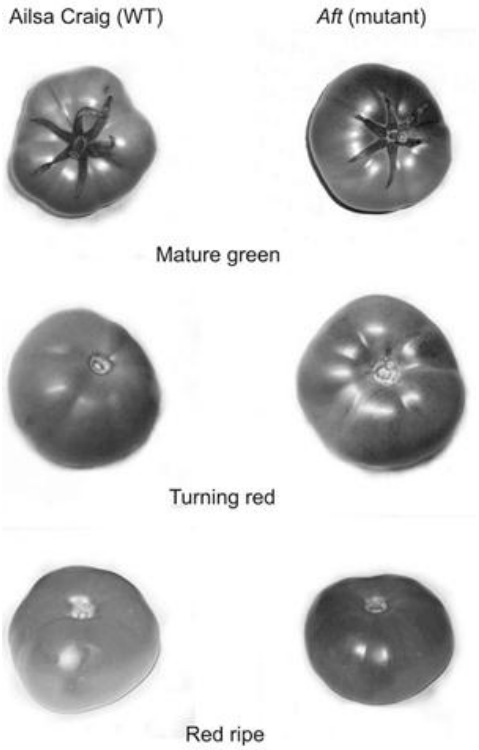

Fig 1 Phenotypic characterization of peel tissues of the mutant Anthocyanin fruit (Aft) and the wild type Ailsa Craig grown in the greenhouse using a hydroponic culture system during different developmental stages; Mature green, Turning red (breaker) and Red ripe. Darker regions in the fruit skin of aft mutant indicate the site of anthocyanin accumulation

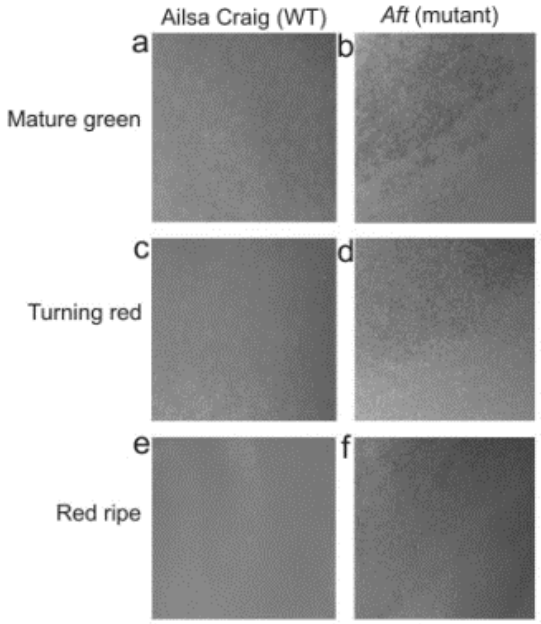

Fig 2 Microscopic characterization of peel tissues of the genotype Anthocyanin fruit (Aft) and its wild type Ailsa Craig (AC) grown in the greenhouse using a hydroponic culture system during different developmental stages; Mature green (a,b), Turning red (c,d) and Red ripe (e,f). Darker regions in the fruit skin of Aft mutant indicate the site of anthocyanin accumulation

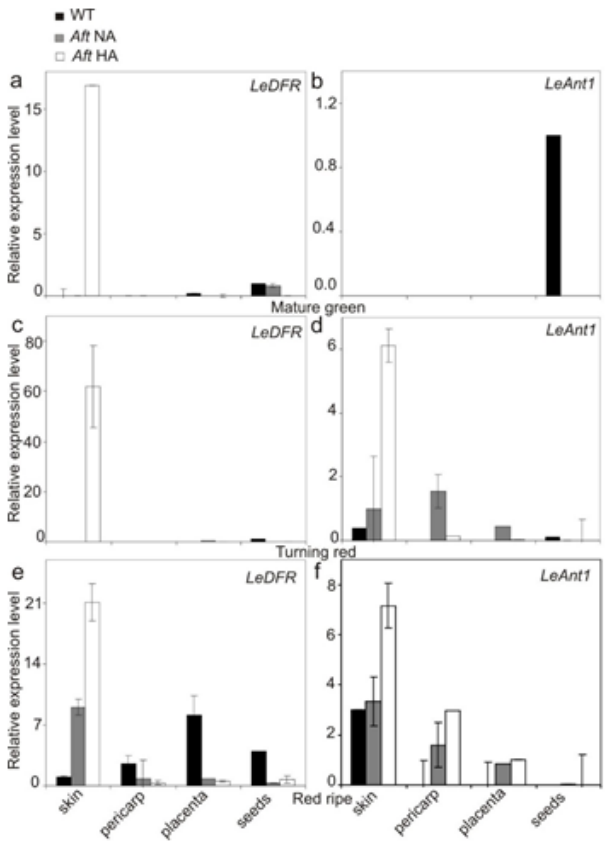

Figure 3. Expression of the genes (LeDFR and Ant1) involved in anthocyanin biosynthesis and accumulation in fruit tissues of two tomato genotypes; Anthocyanin fruit (Aft) tomato mutant and in its wild type Ailsa Craig (AC). Four different fruit tissues were analyzed; skin, pericarp, placenta, and seeds. DFR was higher expressed compared to Ant1 expression levels in all developmental stages analyzed; mature green, turning red, and red ripe (Figs. 3a-f). These biosynthetic genes were much higher expressed in Aft fruit parts which were exposed to sunlight (white bars) and in skin tissues in particular. Low expression levels were detected in the wild type (black bars) and in Aft fruit tissues grown under shade (grey bars) (Figs. 3a-f). Real-time reverse transcription polymerase chain reaction (RT-PCR) was used. Data are means of three replicates ± S.D. The expression of LeEF1A gene was used as the endogenous control to normalize the data.

TABLE I
LIST OF TOMATO MUTANTS DISPLAYING INTENSIFICATION OF ANTHOCYANIN PIGMENTATION *

Gene	Allele	Locus name	Synonyms	Anthocyanin modification	Background	Origin	Accession
Abg		Aubergine		Fruit epidermis purple, particularly on shoulder and where exposed to direct light; also enhanced by wounding	Unknown	SPON	LA3668
Aft		Anthocyanin fruit	*Af*	Anthocyanin in green and ripe fruit; environmentally sensitive, absent when shaded	Unknown	SPON	LA1996
Atv		Atroviolaciu m		Excess anthocyanin on leaves, stems, and fruits	VF-36	SPON	LA0797
Atv		Atroviolaciu m		Excess anthocyanin on leaves, stems, and fruits	Ailsa Craig	SPON	LA3736
Dim		Diminuta		Older leaves gray green with violet veins	Lukullus	RAD	LA0597
dim-2		diminuta-2	*dim2*	Much anthocyanin in hypocotyl, growth zones	Ailsa Craig	RAD	LA3170
Fle		Flexifolia	*fle1*	leaves with strong anthocyanin	Ailsa Craig	RAD	LA3764
Pds		phosphorus deficiency syndrome	*Ph-oid*	Leaves flushed with anthocyanin	Unknown	SPON	LA0813
Per		Perviridis		Leaves darker green, dropping early, anthocyanin strong	Rheinlands Ruhm	RAD	LA0564
Pn		Punctate		Heavy anthocyanin accumulation at base of large trichomes on upper leaf surface	Ailsa Craig	SPON	LA3089
Pn		Punctate		Heavy anthocyanin accumulation at base of large trichomes on upper leaf surface	Unknown	SPON	LA0812
Ppa		purpurea		High anthocyanin content	Lukullus	RAD	LA2054
Vio		Violacea		Heavy anthocyanin on stems and veins	Lukullus	RAD	LA0633
Vio		violacea		Heavy anthocyanin on stems and veins	Ailsa Craig	RAD	LA3734A

* Source: Al Sane et al. 2011; Giovanni et al. 2011

TABLE II
THE COMPOSITION OF THE STOCK SOLUTIONS USED IN THE HYDROPONIC CULTURE SYSTEM IN THE GREENHOUSE IN GROWING AFT MUTANT AND AILSA CRAIG (AC)

Chemical	Composition for 100 liters
Stock solution A	
$Ca(NO_3)_2$	10,79 kg
KNO_3	4,29 kg
Iron chelates (EDDHA)	140 g
Stock solution B	
$MgSO_4$	2,12 kg
KH_3PO_4	3,54 kg
KNO_3	8,88 kg
K_2SO_4	0,36 kg
Chelamix 5 WG®	300 g
Borofast® (A source of Boron)	30 g
H_2SO_4	0,76 L