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Anthocyanin tomato mutants: Overview and characterization of an anthocyanin-less somaclonal mutant

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Anthocyanin tomato mutants: Overview and characterization of an anthocyanin-less somaclonal mutant

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Abstract
Anthocyanins are secondary metabolites, which play important roles in the physiology of plants. In tomato (*Solanum lycopersicum* L.), anthocyanins are normally synthesized only in vegetative tissues. M375 is a mutant unable to produce anthocyanins in leaves and stems. In this study, we investigated the anthocyanin biosynthetic pathway in M375 and in its genetic background, Alice, in order to find out where the anthocyanin biosynthesis is blocked, along the pathway, in the mutant. Anthocyanins accumulation was enhanced by sucrose only in the wild type, even though the expression of several genes involved in anthocyanin biosynthesis was normal in both the genotypes. Genes coding for the final steps along the anthocyanin biosynthetic pathway were, however, less expressed in the M375 when compared to the wild type.

Keywords: *Anthocyanins, gene expression,* Solanum lycopersicum *L., sucrose, tomato*

Abbreviations: F3′H = flavonoid 3′-hydroxylase, F3′5′H = flavonoid 3′5′-hydroxylase, DFR = dihydroflavonol 4-reductase, FLS = flavonols synthase, ANS = anthocyanidin synthase, 3-GT = flavonoid 3-O-glucosyltransferase, 5-GT = flavonoid 5-O-glucosyltransferase, RT = flavonoid 3-O-glucoside-rhamnosyltransferase, AAC = anthocyanin acyltransferase, GST = glutathione S-transferase, PAT = putative anthocyanin transporter, NAR = naringenin, DHK = dihydrokaempferol

Introduction

Flavonoids represent a large class of plant secondary metabolites, whose presence depends by a genetic control and its interaction with the environment (Lattanzio et al. 2009). Among these naturally occurring compounds, anthocyanins are the most wide-spread, due to the wide range of chemical structures arising from their biosynthetic pathway (Gould et al. 2008; Lattanzio et al. 2009). Anthocyanins are pigments that give flowers their characteristic red, purple, and blue hues (Gould et al. 2008). In vegetative tissues, they can be synthesized in response to stressful events, such as high irradiance or low temperatures, against which they can give protection acting both as a light screen and as scavengers for radical species (Gould 2004). It has been demonstrated, through *in vitro* and *in vivo* experiments, that anthocyanins can have antiallergic, anti-inflammatory, antiviral, and antioxidant activities (Bovy et al. 2007).

The existence of such a wide range of functions of anthocyanins raises questions about how these compounds are synthesized and how their biosynthesis is modulated (Holton & Cornish 1995). Anthocyanin biosynthetic regulation has been studied in several plant species. Therefore, detailed information of the sequence of reactions are, now, available (Jaakola et al. 2002; Bovy et al. 2007) and many enzymes required for the production of different flavonoid classes have been identified (Holton & Cornish 1995; Winkel-Shirley 2001). The biosynthesis of anthocyanins and other flavonoids in plant tissues includes precursors from both the shikimate and the acetate-malonate pathways via several enzymatic steps (Dooner et al. 1991; Awad et al. 2000). Two classes of genes are required for anthocyanin biosynthesis, the structural genes encoding the enzymes that are directly involved in the production of anthocyanins and other flavonoids, and the regulatory genes that control the transcription of

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structural genes (Jaakola et al. 2002; Gould et al. 2008). All these genes influence the intensity and pattern of anthocyanin biosynthesis (Holton & Cornish 1995).

In recent years, a growing interest and an increasing number of investigations were carried out using different approaches to modulate the biosynthesis of flavonoids in plants (Schijlen et al. 2004; Tanaka & Ohmiya 2008). An excellent candidate for such an approach is tomato (*Solanum lycopersicum* L.), since it is among the most important, commonly consumed vegetables in human diets worldwide (Bovy et al. 2002; Willits et al. 2005). In tomato, the anthocyanin biosynthetic pathway has been described (Bovy et al. 2007; Gonzali et al. 2009) and most of the genes involved in the anthocyanin production have been identified and characterized (Figure 1; De Jong et al. 2004; Zuluaga et al. 2008; Gonzali et al. 2009). Tomato plants contain a variety of flavonoids in their vegetative tissues, including anthocyanins (Mes et al. 2008). In tomato fruit, however, only small amounts of flavonoid biosynthetic intermediates are accumulated, whereas anthocyanins are usually not synthesized (Torres et al. 2005; Mes et al. 2008). 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). These accessions display phenotypes characterized by intensification (Table I), partial or complete absence (Table II), or any other form of anthocyanin alteration (Table III). For instance, *Anthocyanin fruit* (*Aft*, accession LA1996) and *Aubergine* (*Abg*, accession LA3668) lines display anthocyanin accumulation in the fruit (reviewed by Gonzali et al. 2009, Table I). The recessive gene *atroviolacea* (*atv*), derived from the interspecific cross with *Solanum cheesmaniae* (L. Riley) Fosberg, has been shown to stimulate strong anthocyanin pigmentation in leaves and stems (Gonzali et al. 2009, Table I).

On the other hand, the genes anthocyanin less (a), anthocyanin absent (aa), entirely anthocyanin-less (ae), anthocyanin free (af), anthocyanin gainer (ag), incomplete anthocyanin (ai), anthocyanin loser (al), anthocyanin reduced (are), without anthocyanin (aw), and baby lea syndrome (bls), negatively regulate the production of anthocyanins in vegetative tissues (Table II). Furthermore, mutations like aw and bls, were found not only to completely inhibit anthocyanin biosynthesis but also to be associated with alterations in seed morphology and testa histochemistry (Atanassova et al. 2004).

The identity of many mutations leading to absence or reduction of anthocyanins in tomato has still to be revealed. However, mapping and candidate genes analyses, together with linkage studies, have suggested possible candidates for most mutations. De Jong et al. (2004) reported an association between the tomato *ag* locus and Petunia *anthocyanin2* (*AN2*). Similarly, the tomato *af* locus is associated with tomato *chalcone isomerase* (*CHI*), whereas the *anthocyanidin synthase* (*ANS*) gene constitutes a good

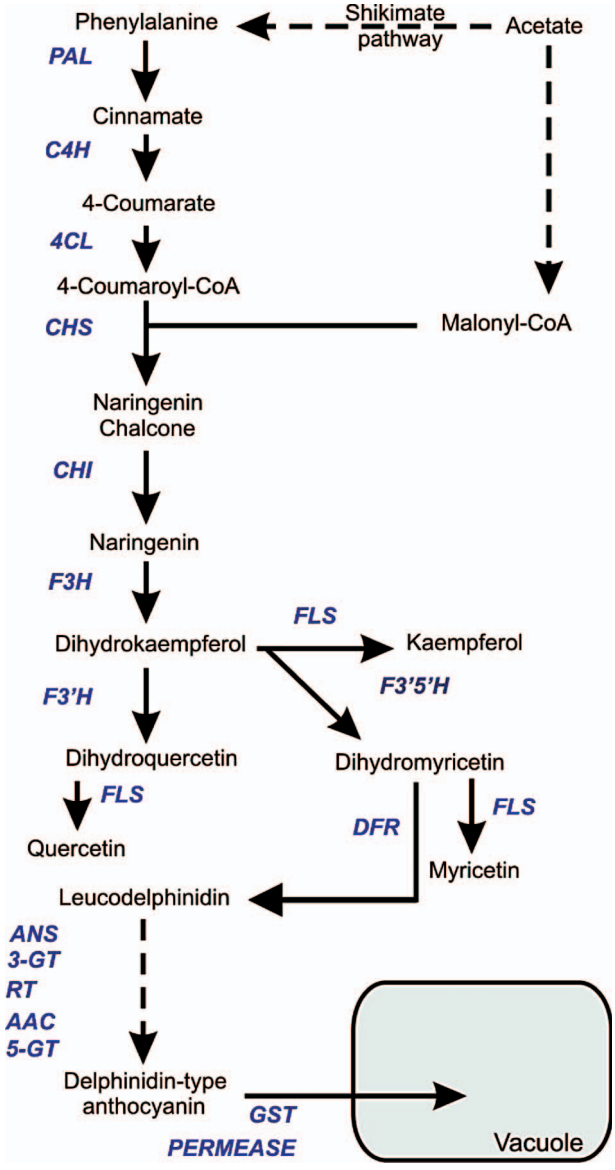

Figure 1. Schematic representation of the anthocyanin biosynthetic pathway and its regulation in tomato. PAL, phenylalanine ammonia lyase; 4CL, 4 coumarate: coenzyme A ligase; C4H, cinnamate 4-hydroxylase; C3H, 4 coumarate 3 hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3 hydroxylase; F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonolreductase; ANS, anthocyanidin synthase; 3-GT, flavonoid 3-O-glucosyltransferase; RT, flavonoid 3-O-glucoside-rhamnosyltransferase; AAC, anthocyanin acyltransferase; 5-GT, flavonoid-5 glucosyltransferase; GST, glutathione-S-transferase; PAT, putative anthocyanin transporter. (Source: Gonzali et al. 2009).

Table I. List of tomato mutants displaying intensification of anthocyanin pigmentation.

Gene	Allele	Locus name	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*	Anthocyanin in green and ripe fruit; environmentally sensitive, absent when shaded	Unknown	SPON	LA1996
atv		*atroviolacium*	Excess anthocyanin on leaves, stems, and fruits	VF-36	SPON	LA0797
atv		*atroviolacium*	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*	Much anthocyanin in hypocotyl, growth zones	Ailsa Craig	RAD	LA3170
fle		*flexifolia*	Leaves with strong anthocyanin	Ailsa Craig	RAD	LA3764
pds		*phosphorus deficiency syndrome*	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

The origin of each mutation is specified as either spontaneous (SPON) or induced by chemical treatment (CHEM) or irradiation (RAD).

candidate for the tomato *ae* mutation (De Jong et al. 2004). The tomato *are* mutation could be associated with a mutation that directly interferes with F3H function (De Jong et al. 2004). Indeed, F3H enzyme activity is abolished in the *are* mutant (Yoder et al. 1994). The tomato *a* gene is hypothesized to encode *F3′5′H*. In fact, a portion of the gene *F3′5′H* was sequenced in the *anthocyanin-less* (*a*) mutant, and a premature stop codon was observed in an *a* mutant, but not in the wild type (De Jong et al. 2004). On the other hand, up to now is not possible to make convincing predictions about the origin of the tonato *aa*, *bls*, and *al* mutations. According to De Jong et al. (2004), it is possible that tomato *bls* corresponds to Petunia *anthocyanin11* (*AN11*), and that tomato *al* codes for a bHLH protein similar to Petunia *JAF13*.

Furthermore, other tomato mutants, unable to synthesize anthocyanins, have been isolated after treatment of wild type seeds to chemical mutagenesis (Bacci et al. 1999).

In the present work, we characterize a new anthocyanin-less mutant. We studied the M375 (also called ALA in the figures of this work) somaclonal mutant obtained following *in vitro* regeneration of cotyledon explants from tomato cv. Alice seeds submitted to EMS mutagenic treatment as described by Bacci et al. (1999). The mutant line is characterized by a lower UV-B absorbing compounds content (Bacci et al. 1999). The molecular regulation of anthocyanin biosynthetic pathway in the M375 mutant was studied in order to find out where the anthocyanin biosynthesis is blocked along the anthocyanin pathway.

Materials and methods

Plant material and growth conditions

Tomato seeds of M375 mutant and its genetic background Alice were used in this study. Both genotypes have been previously described by Bacci et al. (1999). The wild type cultivar, Alice, is a processing determinate variety close to the UC-82 type and was released in Italy (ISCI-Bologna). M375 is a somaclonal mutant obtained following *in vitro* regeneration of cotyledon explants from Alice seeds submitted to EMS mutagenic treatment (6½, 24 h, 22°C in the dark). Seeds were sterilized for 13 min in 4% (v/v) sodium hypochlorite solution, rinsed seven times with sterilized, distilled water and then incubated in liquid media (full-strength, sterilized Murashige and Skoog salt solution, pH 5.7) (Murashige & Skoog 1962) with gentle shaking. We used six-well culture plates (CELLSTAR, Greiner bio-one) at 23°C ± 1 in a growth chamber under continuous light (90 μm photons m^{-2}) for germination and growth. After 6 days, the seedlings were treated adding sucrose/chemical solutions to selected wells and water to the control wells. The following concentrations of sucrose were applied to the media of selected wells: 7.5, 15, 30, 60, and 90 mM. After 4 days, seedlings were collected, immediately frozen using liquid N_2.

When used exogenous naringenin (100 μM) or dihydrokaempferol (100 μM) were added either alone or in combination with sucrose to investigate their effects.

Table II. List of tomato mutants displaying absence or reduction of anthocyanins in vegetative tissues.

Gene	Allele	Locus name	Anthocyanin modification	Background	Origin	Accession
a		*Anthocyanin less*	Stems and leaves always lack anthocyanin	Ailsa Craig	SPON	LA3263
a		*Anthocyanin less*	Stems and leaves always lack anthocyanin	Unknown	SPON	LA0291
a	*prov2*	*Anthocyanin less*	Stems and leaves always lack anthocyanin	VF36	CHEM	3–414
a	*prov3*	*Anthocyanin less*	Stems and leaves always lack anthocyanin	VF36	CHEM	3–415
aa		*Anthocyanin absent*	Complete absence of anthocyanin	Marmande	SPON	LA1194
aa		*Anthocyanin absent*	Complete absence of anthocyanin	Ailsa Craig	SPON	LA3617
ae		*Entirely anthocyanin less*	Completely free of anthocyanin	Ailsa Craig	RAD	LA3612
ae		*Entirely anthocyanin less*	Completely free of anthocyanin	Kokomo	RAD	LA1048
ae		*Entirely anthocyanin less*	Completely free of anthocyanin	Chico Grande	RAD	LA3018
ae	*2*	*Entirely anthocyanin less*	Completely free of anthocyanin	UC-82B	CHEM	3–706
ae	*afr*	*Entirely anthocyanin less*	Completely free of anthocyanin	Chatham	RAD	LA2442
ae	*prov3*	*Entirely anthocyanin less*	Lacks anthocyanin in the seedling stem	VFNT Cherry	CHEM	3–620
af		*Anthocyanin free*	Completely free of anthocyanin	Ailsa Craig	RAD	LA3610
af		*Anthocyanin free*	Completely free of anthocyanin	Red Cherry	RAD	LA1049
ag		*Anthocyanin gainer*	Anthocyanin absent except on cotyledons and lower sides of leaves	GS5	SPON	LA0177
ag		*Anthocyanin gainer*	Anthocyanin absent except on cotyledons and lower sides of leaves	Ailsa Craig	SPON	LA3163
ag	*2*	*Anthocyanin gainer*	Completely free of anthocyanin	Ailsa Craig	SPON	LA3164
ag	*2*	*Anthocyanin gainer*	Completely free of anthocyanin	*L. cheesmanii*	SPON	LA0422
ag	*k*	*Anthocyanin gainer*	As for ag, purple pigment appears on cotyledons and lower sides of leaves when growth is slow	UC-T5	SPON	LA3149
ag	*s*	*Anthocyanin gainer*	Anthocyanin absent except on cotyledons and lower sides of leaves	Unknown	SPON	LA4425
ag-2		*Anthocyanin gainer-2*	As for ag purple pigment appears on cotyledons and lower sides of leaves when growth is slow	Ailsa Craig	SPON	LA3711
ai		*Incomplete anthocyanin*	Early seedling stem has trace of anthocyanin, soon lost	Kokomo	RAD	LA1484
ai		*Incomplete anthocyanin*	Early seedling stem has trace of anthocyanin, soon lost	Ailsa Craig	RAD	LA3611
ai	*2*	*Incomplete anthocyanin*	Early seedling stem has trace of anthocyanin, soon lost	Kokomo	RAD	LA1485
al		*Anthocyanin loser*	Anthocyanin pigmentation of medium intensity appears for ten to twenty days after seedling emergence, thereafter disappearing except for islands near the leaf nodes	Ailsa Craig	SPON	LA3576
are		*Anthocyanin reduced*	Almost no anthocyanin until fruits set, then moderate amount in young foliage	VF-36	CHEM	3–073
aw		*Without anthocyanin*	Free of anthocyanin	Unknown	SPON	LA0271
aw		*Without anthocyanin*	Free of anthocyanin	Ailsa Craig	SPON	LA3281
aw	*prov3*	*Without anthocyanin*	Free of anthocyanin	VF-36	CHEM	3–121
aw	*prov4*	*Without anthocyanin*	Free of anthocyanin	VFNT-Cherry	CHEM	3–603
aw	*prov5*	*Without anthocyanin*	Free of anthocyanin	VFNT-Cherry	CHEM	3–627
bls		*Baby lea syndrome*	Anthocyaninless	Unknown	SPON	LA1004
bls		*Baby lea syndrome*	Anthocyaninless	Ailsa Craig	SPON	LA3167
bls	*prov2*	*Baby lea syndrome*	Anthocyaninless	VFNT-Cherry	CHEM	3–610

The origin of each mutation is specified as either spontaneous (SPON) or induced by chemical treatment (CHEM) or irradiation (RAD).

RNA extraction and real-time reverse transcription PCR

Seedlings were collected, immediately frozen using liquid N_2 and stored at -80°C. The frozen material was later ground to a fine powder with liquid N_2 using a mortar and pestle and undergone RNA extraction protocol. RNA was extracted from seedlings grown on Murashige and Skoog solution (control) or on the same medium supplemented with the additional treatment as indicated in figure legends. The extraction was performed using the aurintricarboxylic acid method as previously described (Perata et al. 1997). 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. To eliminate any possible DNA contamination of our RNA samples,

Table III. List of tomato mutants displaying anthocyanin alteration as a secondary phenotype.

Gene	Allele	Locus name	Anthocyanin modification	Background	Origin	Accession
cla		*Clara*	Purple veins and petioles	Lukullus	RAD	LA0540
cry-1		*Cryptochrome-1*	Anthocyanins reduced	Moneymaker	RAD	LA4359
div		*Divaricata*	Small squarrose plant with intercostally yellowish leaves and ventrally purple	Condine red	RAD	LA0671
div		*Divaricata*	Small squarrose plant with intercostally yellowish leaves and ventrally purple	Ailsa Craig	RAD	LA3818
cla		*Clara*	Purple veins and petioles	Lukullus	RAD	LA0540
civ		*Divaricata*	Small squarrose plant with intercostally yellowish leaves and ventrally purple	Condine red	RAD	LA0671
civ		*Divaricata*	Small squarrose plant with intercostally yellowish leaves and ventrally purple	Ailsa Craig	RAD	LA3818
fri	*1*	*Far red light insensitive*	Insensitive to far red light; hypocotyls elongated relative to wild type under far red light	Moneymaker	CHEM	LA3809
fri	*1*	*Far red light insensitive*	Insensitive to far red light; hypocotyls elongated relative to wild type under far red light	Moneymaker	CHEM	LA4356
hp-1		*High pigment-1*	Higher amount of anthocyanins respect to the wild type	Unknown	SPON	LA0279
hp-1		*High pigment-1*	Higher amount of anthocyanins respect to the wild type	Rukullus	SPON	LA3004
hp-1		*High pigment-1*	Higher amount of anthocyanins respect to the wild type	Ailsa Craig	SPON	LA3538
hp-1	*w*	*High pigment-1*	Higher amount of anthocyanins respect to the wild type	GT	CHEM	LA4012
hp-2		*High pigment-2*	Higher amount of anthocyanins respect to the wild type	Moneymaker	CHEM	LA4013
hp-2		*High pigment-2*	Higher amount of anthocyanins respect to the wild type	San Marzano	CHEM	LA3006
hp-2	*dg*	*High pigment-2*	Higher amount of anthocyanins respect to the wild type	Manapal	SPON	LA3005
hp-2	*dg*	*High pigment-2*	Higher amount of anthocyanins respect to the wild type	Manapal	SPON	LA2451
hp-2	*j*	*High pigment-2*	Higher amount of anthocyanins respect to the wild type	Moneymaker	SOMA	LA4014
le		*Lembiformis*	Prostrate, smaller plant, proportionately reduced; keeled or involuted yellowish pinnae, ventrally purplish	Rheinlands Ruhm	RAD	LA0956
pli		*Plicata*	All parts small; leaves dark, yellowish, plicate; strong anthocyanin	Ailsa Craig	RAD	LA3672
pli		*Plicata*	All parts small; leaves dark, yellowish, plicate; strong anthocyanin	Lukullus	RAD	LA0696
res		*Restricta*	Smaller, squarrose bush; yellowish light-green, boat-shaped pinnae, purplish ventrally	Rheinlands Ruhm	RAD	LA1085
res		*Restricta*	Smaller, squarrose bush; yellowish light-green, boat-shaped pinnae, purplish ventrally	Ailsa Craig	RAD	LA3756
sfa		*Sufflaminata*	Usually smaller, weakly branched plant; yellowish to yellow-green, involuted pinnae, purplish ventrally; slight F1 seedling heterosis	Rheinlands Ruhm	RAD	LA0862
sfa	*2*	*Sufflaminata*	Usually smaller, weakly branched plant; yellowish to yellow-green, involuted pinnae, purplish ventrally; slight F1 seedling heterosis	Condine red	RAD	LA0969
tri		*Temporarily red light insensitive*	Higher amount of anthocyanins respect to the wild type	GT	CHEM	LA3808
tri		*Temporarily red light insensitive*	Higher amount of anthocyanins respect to the wild type	Moneymaker	CHEM	LA4357

The origin of each mutation is specified as either spontaneous (SPON) or induced by chemical treatment (CHEM) or irradiation (RAD).

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). Then, the expression analysis of the genes: *LeF3′H, LeF3′5′H, LeDFR, LeFLS, LeANS , Le3-GT, Le5-GT, LeRT, LeAAC, LeGST, LePAT* was performed by real-time reverse transcription polymerase chain reaction (RT-PCR) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) and the default ABI Prism 7000 PCR program for PCR conditions. The reactions were carried out loading the primers described in Table S1.

PCR amplification was conducted using 50 ng of cDNA and SYBR GREEN Universal Master Mix (Applied Biosystems), and following the manufacturer's protocol. The gene *LeEF1A* was used as an endogenous control (*Lycopersicon esculentum* L. *elongation factor 1-alpha*, GenBank accession number X14449) (forward primer: 5′-TGCTTGCTTTC ACCCTTGGT-3′; reverse primer 5′-CGATTT CATCATACCTAGCCTTGGA-3′; TaqMan probe: 5′-CTGCTGTAACAAGATGGATGC-3′) and its relative expression was determined. Relative quantification of each single gene expression was performed using the comparative threshold cycle (C_T) method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin number 2 (Applied Biosystems).

Anthocyanins quantification

Anthocyanins extraction from tomato seedling was performed following the protocol performed by Solfanelli et al. (2006). Seedlings were collected, weighted, and immediately frozen using liquid N_2 and stored at -80°C. The frozen material was later ground to a fine powder with liquid N_2 using a mortar and pestle and undergone anthocyanins extraction protocol in which seedlings were ground in HCl 1% (v/v) in methanol with the addition of two-thirds volume of distilled water. Then, the extracts were recovered and 1 volume of chloroform was added to remove chlorophylls through mixing and centrifugation (1.5 min at 12,000*g*). Anthocyanins containing aqueous phase were recovered and the absorption was determined spectrophotometrically (at A_{535} nm). Mean values were obtained from three independent replicates.

Photography

Photos of the vegetative tissues of Alice and M375 tomato genotypes shown in this article were taken using a Nikon TMS-F microscope (type 104) connected with the NIS-elements F2.20 imaging software (Laboratory Imaging, Nikon, Tokyo, Japan).

Results and discussion

Sugars are important triggers of anthocyanin biosynthesis in Petunia, grapevine, and Arabidopsis (Boss et al. 1996; Gollop et al. 2001, 2002; Solfanelli et al. 2006; Loreti et al. 2008).

We evaluated whether the absence of anthocyanins in the M375 mutant was related to the effects of sucrose. We determined the best sucrose concentration in enhancing anthocyanin accumulation by applying different levels of this sugar to the media. Sucrose was effective only in wild type seedlings, with the best result using a 90-mM concentration (Figure 2a and c). This demonstrates that also in tomato the anthocyanin pathway is enhanced by sucrose. On the other hand, the M375 mutant has lost the potential to accumulate anthocyanins in its vegetative tissues, and is unable to produce anthocyanins, even when treated with sucrose.

Feeding naringenin, a precursors for anthocyanins biosynthesis, successfully restored the anthocyanin biosynthetic pathway in carrot (*Daucus carota* L.) cell

Table S1. List of SYBR-green primers used in gene expression analyses implementing real-time reverse transcription polymerase chain reaction (RT-PCR).

Reverse primer	Forward primer	GenBank accession number	Gene
5′-CGTTAGTACCGTCGGTCGGCGAAT-3″	5′-GCACCACGAATGCACTTGC-3′	AY547289.1	*LeF3′H*
5′-AAGGAACCTCTCGGGAGTGAA-3′	5′-GGCAATTGGACGAGATCCTG-3′	DB723744	*LeF3′5′H*
5′-TGACAAGCCAAGAGCCGATAA-3′	5′-TCCGAAGACGACAACGGTTT-3′	Z18277	*LeDFR*
5′-TGGTGGGTTGGCCTCATTAA-3′	5′-GAGCATGAAGTTGGGCCAAT-3′	FJ770475.1	*LeFLS*
5′-TTGCAAGCCAGGCACCATA-3′	5′-GAACTAGCACTTGGCGTCGAA-3′	AJ785263	*LeANS*
5′-TTTCCAAACACTTTCCACCA-3′	5′-GCACATAAGAGTGTTGGCGTTT-3′	BP893263	*Le3-GT*
5′-TCATCACTCTCAACCACACCA-3′	5′-GTGGCATTTCCTCATTGGAC-3′	CD003048	*Le5-GT*
5′-CCATCATCACCATCTCCACA-3′	5′-ATGTTGCCACAGAAAGGTGA-3′	BP890816	*LeRT*
5′-TTCAGACAACCTTCCAGCAA-3′	5′-CCCTCCAGTACCACCAGAAA-3′	EU979541	*LeAAC*
5′-TGGCTTAGATCGGCTAAGGA-3′	5′-TGGGACACAACAGTGATTTGA-3′	n.a.	*LeGST*
5′-TTGCATCTCCTTGCTGTTTG-3′	5′-CGGTGTTTCAGTCCCTCCTA-3′	AY348872	*LePAT*

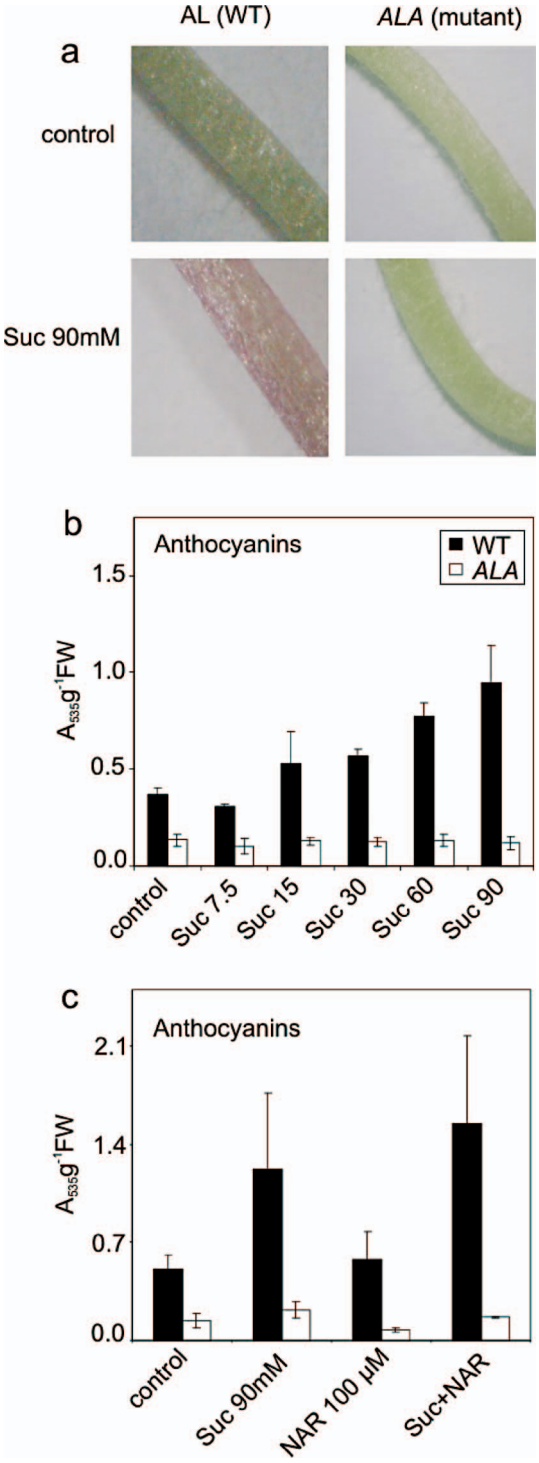

Figure 2. (a) Effect of sucrose (90 mM) on anthocyanins synthesis in *in vitro* grown tomato (data are means of three replicates ± SD). (b) Anthocyanin levels in the seedlings Alice and M375 cultured *in vitro* and treated with sucrose concentrations ranging from 7.5 to 90 mM (data are means of three replicates ± SD). (c) Effect of naringenin (NAR) on anthocyanins synthesis in *in vitro* grown tomato (data are means of three replicates ± SD).

cultures (Hinderer et al. 1984). In order to evaluate whether the anthocyanin pathway in the M375 mutant is disrupted upstream of naringenin (NAR), we treated Alice and M375 seedlings with NAR alone or together with sucrose. In Alice, naringenin alone did not result in any enhancement of anthocyanin production (Figure 2c), but when it was fed together with sucrose, anthocyanin accumulation was slightly higher (Figure 2c). In M375 mutant no effect of naringenin, either alone or in combination with sucrose, was instead observed (Figure 2c). A comparable result was obtained treating the seedlings with dihydrokaempferol (not shown), suggesting that the M375 mutation is most likely located downstream of naringenin and dihydrokaempferol synthesis.

Several genes responsible for anthocyanin biosynthesis downstream of dihydrokaempferol have been identified in tomato (Figure 1. Mathews et al. 2003; De Jong et al. 2004; Zuluaga et al. 2008). The relative expression levels of these genes involved in anthocyanin biosynthesis and accumulation were examined in the wild type and M375 mutant seedlings (Figure 3). As expected, we found that anthocyanin accumulation in the wild type was accompanied with the expression of most of the genes along the anthocyanin biosynthetic pathway (Figures 3 and 2A). Indeed, the visible accumulation of anthocyanins often reflects the activity and functioning of the biosynthetic enzymes along the pathway (Koes et al. 1994). In a study conducted on bilberry flowers, the expression of flavonoid pathway genes was accompanied by the accumulation of anthocyanins (Jaakola et al. 2002). Similarly, anthocyanin biosynthetic genes were coordinately expressed during red coloration in apple skin tissues (Honda et al. 2002).

LeDFR, LeF3′H, and *LeF3′5′H* were strongly expressed in both Alice and M375 mutant (Figure 3) upon sucrose application, suggesting that M375 is affected downstream of these genes. At the branching point between flavonols and anthocyanin along the biosynthetic pathway, we analyzed the expression of *LeFLS* and *LeANS*. Sucrose induced the expression of these genes in both wild type and the mutant (Figure 3). Again, no significant differences between the wild type and the mutant were observed. *Le3-GT* was strongly induced by sucrose when compared to *Le5-GT* in both wild type and the mutant (Figure 3). Shifting to the genes coding for transferase enzymes, only *LeAAC* was expressed in both wild type and the mutant with no significant differences between them (Figure 3). Although *LeRT* (*rhamnosyl transferase*) and *LeGST* (*glutathione S-transferase*) were expressed in both Alice and its mutant M375 (Figure 3) and their expression levels were clearly enhanced by sucrose application, the expression in the mutant line was lower than that in the wild type. Moreover, a putative gene encoding an anthocyanin transporter (*LePAT*), thought to be responsible, together with *LeGST,* for anthocyanins transport to the vacuole,

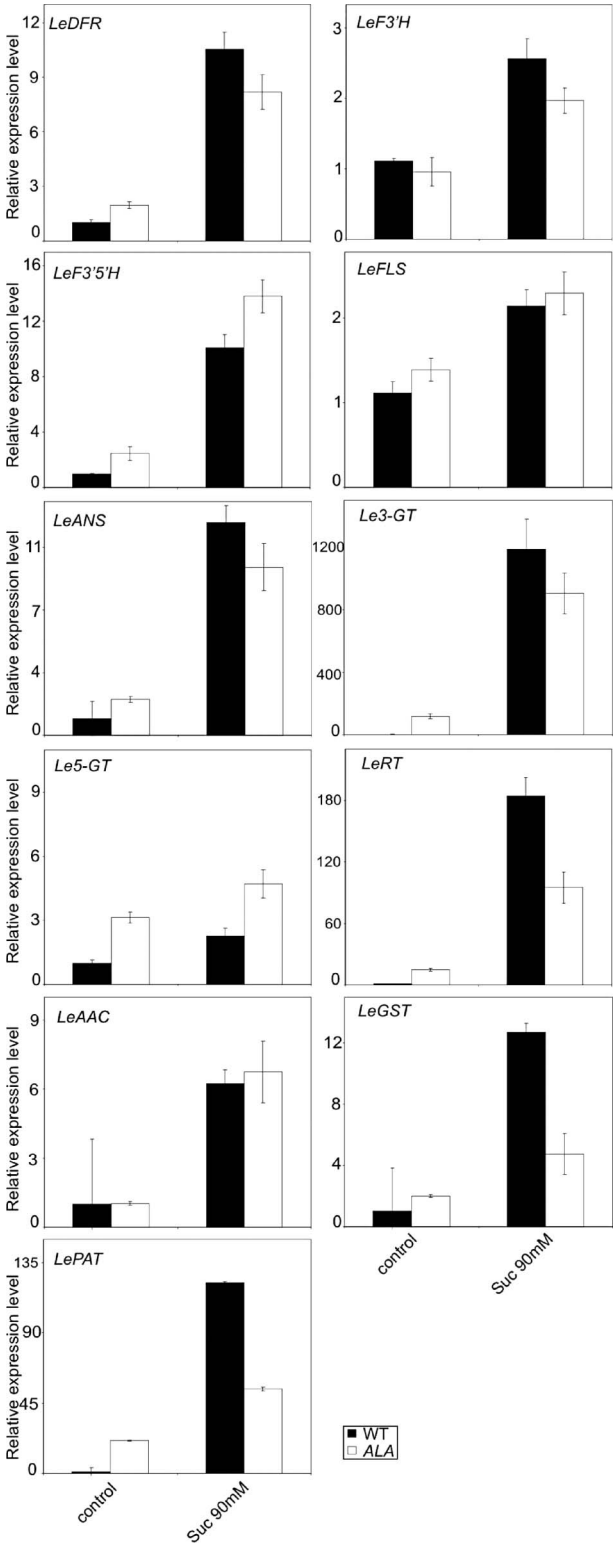

Figure 3. Expression of some genes hypothesized to be involved in anthocyanin biosynthesis and accumulation in vegetative tissues of Alice tomato in comparison with M375 mutant; control versus treatments with sucrose (90 mM). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed and relative expression levels of the following genes: *F3′H, F3′5′H, DFR, FLS, ANS, 3-GT, 5-GT, RT, AAC, GST*, and *PAT* were measured. Data are means of three replicates ± SD. The expression of *LeEF1A* gene was used as the endogenous control to normalize the data.

was only slightly upregulated by sucrose in the M375 mutant (Figure 3).

Previous studies showed that mutations that negatively affect the anthocyanin pathway in tomato, such as *a*, *aw*, *ae*, are associated with the structural genes *F3′5′H*, *DFR*, and *ANS* (De Jong et al. 2004) acting downstream of dihydrokaempferol. Our results suggest that the absence of anthocyanins in the mutant M375 is likely related to a mutation causing a lower expression of genes involved in anthocyanin transport and accumulation (Figure 1). According to this hypothesis, anthocyanins might be produced, but they cannot be compartmentalized into the vacuole, thus leading to an immediate degradation of the pigments (fading).

This research has led to the identification of candidates for the mutation affecting anthocyanin biosynthesis in M375 mutant.

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