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ORIGINAL PAPER

ANTHOCYANIN1 from *Solanum chilense* is more efficient in accumulating anthocyanin metabolites than its *Solanum lycopersicum* counterpart in association with the ANTHOCYANIN FRUIT phenotype of tomato

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Abstract Anthocyanins are flavonoid metabolites contributing attractive colors and antioxidant qualities to the human diet. Accordingly, there is a growing interest in developing crops enriched with these compounds. Fruits of the cultivated tomato, *Solanum* (S.) *lycopersicum*, do not normally produce high levels of anthocyanins. However, several wild tomato species yield anthocyanin-pigmented fruits, and this trait has been introgressed into the cultivated tomato. Two genes encoding homologous R2R3 MYB transcription factors, termed *ANT1* and *AN2*, were previously genetically implicated in anthocyanin accumulation in tomato fruit peels of the ANTHOCYANIN FRUIT (AFT) genotype originating from *S. chilense*. Here we compared transgenic tomato plants constitutively over-expressing the *S. lycopersicum* (35S*::ANT1*^{L}) or the *S. chilense* (35S*::ANT1*^{C}) allele, and show that each displayed variable levels of purple pigmentation in vegetative as well as reproductive tissues. However, 35S*::ANT1*^{C} was significantly more efficient in producing anthocyanin pigments, attributed to its gene coding-sequence rather than to its transcript levels. These results expand the potential of enhancing anthocyanin levels through engineering coding-sequence polymorphisms in addition to the transcriptional alterations commonly used. In addition, a segregating population obtained from a recombinant genotype revealed that the native *ANT1*, and not *AN2*, is fully associated with the AFT phenotype and that *ANT1* alone can generate the characteristic phenotype of anthocyanin accumulation in AFT fruits. Our results therefore provide further support to the hypothesis that *ANT1* is the gene responsible for anthocyanin accumulation in fruits of the AFT genotype.

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Introduction

Flavonoids are polyphenolic compounds occurring naturally in most plants and are classified into chalcones, flavanones, dihydroflavonols, flavonols and anthocyanins (Bovy et al. 2002). As recently reviewed (Levin 2009; Bovy et al. 2010), flavonoids are involved in multiple aspects related to proper growth and development of plants. In addition, mounting evidence suggests that flavonoids are potentially health-protecting components in the human diet, resulting from their antioxidant capacity, inhibitory effect on enzymatic activities and their interaction with genes involved in cell survival, proliferation, and apoptosis (Bovy et al. 2010). Flavonoid compounds, in particular anthocyanins, also provide attractive colors to leaves, stems, roots, flowers and fruits, and to industrial food products and beverages following their extraction from plants as natural colors. Accordingly, there is a growing interest in developing crops enriched with flavonoids.

The flavonoid biosynthetic pathway has been elucidated and comprehensively reviewed; many of the genes controlling the pathway have been cloned and classified into structural genes, encoding enzymes that directly participate in flavonoid formation, and regulatory genes controlling their expression (Winkel-Shirley 2001; Levin 2009). Flavonoids are synthesized via the phenylpropanoid pathway, starting

with a condensation reaction, catalyzed by the enzyme chalcone synthase (CHS), resulting in the yellow naringenin chalcone. Naringenin chalcone is isomerized into the flavanone naringenin either spontaneously or by the enzyme chalcone isomerase (CHI) (Holton and Cornish 1995). Flavanone 3-hydroxylase (F3H) then catalyzes the hydroxylation of flavanones to dihydroflavonols, and dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to leucoanthocyanidins, which are then converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of anthocyanins is finally catalyzed by flavonoid 3-*O*-glucosyltransferase (3-GT) (Harborne 1994; Bohm 1998). CHS, CHI, and F3H are therefore early enzymes of the anthocyanin biosynthetic pathway while DFR, ANS, and 3-GT are late enzymes of the pathway. Genes encoding these enzymes are known to be regulated by MYB and bHLH transcription factors, and WDR-type proteins, which act in a combinatorial complex to activate the pathway in tomato and other species (Bovy et al. 2002; Butelli et al. 2008; Adato et al. 2009; Levin 2009; Mahjoub et al. 2009; Ballester et al. 2010). Interestingly, a R2R3 MYB factor, termed ANTHOCYANIN1 (ANT1), was also implicated in glycosylation and transport of anthocyanin metabolites in tomato seedlings (Mathews et al. 2003).

Fruits of the cultivated tomato, *Solanum* (*S.*) *lycopersicum*, do not normally produce high levels of anthocyanins or other flavonoid compounds. However, several wild tomato species yield anthocyanin-pigmented fruits, and this trait has been introgressed into the cultivated tomato (Giorgiev 1972; Rick et al. 1994; Jones et al. 2003). The ANTHOCYANIN FRUIT (AFT) genotype, originating from the wild tomato species *S. chilense*, is characterized by anthocyanin accumulation in skin and outer pericarp tissues of its mature fruits, resulting in tomato fruits with purple skin color, highly modulated by light (Giorgiev 1972; Mess et al. 2008). The anthocyanin accumulation in fruit peels of the AFT genotype is controlled by a single dominant gene (Giorgiev 1972; Jones et al. 2003).

Fruits harvested from the AFT genotype are also typified by significantly higher levels of the flavonols quercetin and kaempferol and with transcriptional up-regulation of structural genes, affecting early and late stages of the flavonoid pathway (Sapir et al. 2008). This latter study, in agreement with that of van Tuinen et al. (2006), also demonstrates that the *HIGH PIGMENT-1* (*HP-1*) gene interacts in a synergistic manner with AFT to increase the levels of flavonol and anthocyanin compounds in fruit peels.

Two homologous genes encoding R2R3 MYB transcription factors were implicated as candidate genes causing the AFT phenotype: *ANT1*, initially discovered by Mathews et al. (2003), and *AN2* (Zuluaga et al. 2008). Both genes reside on the tomato chromosome 10 and display nucleotide and amino acid polymorphisms between AFT and red-fruited genotypes that correlated with anthocyanin accumulation. These results are in agreement with a study associating a homologous *R2R3 MYB* gene with accumulation of anthocyanin pigments in pepper fruits (Borovsky et al. 2004).

Transcript levels of *AN2* were correlated with anthocyanin accumulation in the AFT genotype (Boches 2009). However, Sapir et al. (2008) was unable to demonstrate increased transcript levels of *ANT1* in the AFT genotype, and therefore hypothesized that if *ANT1* causes anthocyanin and flavonol accumulation in the AFT genotype, sequence differences, and not transcriptional alterations of the gene might underlie these phenotypes.

This study was designed to compare phenotypic, biochemical and transcriptional effects resulting from overexpression of the *ANT1* allele originating from *S. chilense* (35S*::ANT1*^{C}) to those displayed by the allele in *S. lycopersicum* (35S*::ANT1*^{L}), both driven by the same constitutive promoter. In addition, a study initially designed to estimate the recombination distance between the native *ANT1* and *AN2* genes led to the identification of a single recombinant AFT plant, *ANT1*^{C}*/ANT1*^{C} *AN2*^{C}*/AN2*^{L}. A population derived following self-pollination of this plant led to the conclusion that *ANT1* alone can generate the characteristic phenotype of anthocyanin accumulation in the AFT genotype.

Our findings support the hypothesis that *ANT1* is the gene responsible for anthocyanin accumulation in fruits of the AFT genotype and that the functional difference between the two alleles is caused by structural rather than transcriptional alterations. This research broadens understanding of the regulation of flavonoid biosynthesis, augmenting the possibilities of enhancing flavonoid levels through engineering of structural polymorphisms in addition to the transcriptional alterations often used.

Materials and methods

Plant material and growth conditions

Seeds from a red-fruited open-pollinated tomato (*S. lycopersicum*) cv Moneymaker were obtained as previously described (Sapir et al. 2008). 15 independent T_0 Moneymaker plants, carrying 35S*::ANT1*^{C} and ten T_0 plants carrying 35S*::ANT1*^{L}, both under the control of the 35S promoter from cauliflower mosaic virus (CaMV) for whole-plant constitutive expression, were generated. These transgenic plants were identified by polymerase chain reaction (PCR) with primers specific to the *ANT1* transgene, and were each self-pollinated. Seeds acquired from each of the self-pollinated transgenic lines were germinated,

transplanted and grown in three seasons. In each season, 32 seedlings of each of the transgenic lines were genotyped and seedlings carrying the transgene were selected. During all seasons- plants were grown in a greenhouse; minimal temperature 18°C with no supplemental light. Seasons were as follows: (1) early summer 2008, sown on 22 May. Plants were transplanted in a randomized block design (two blocks with 12 plants of each line in each block). The experiment included five T_1 or T_2 lines carrying 35S*::ANT1*^{L}, five T_1 lines carrying 35S*::ANT1*^{C}, both groups representing the range of anthocyanin expression, and two control lines: the wild-type Moneymaker and azygous (null) control line (AZ), the latter derived from a T_1 plant not carrying the transgene as determined by PCR analysis with primers complementary to the CaMV 35S promoter and the *ANT1* gene; (2) winter 2008, sown on 1 September. Plants were transplanted in a randomized block design (two blocks with three plants of each line in each block). The experiment included eight T_1 or T_2 lines carrying 35S*::ANT1*^{L}, seven T_1 lines carrying the 35S*::ANT1*^{C}, both groups representing the range of anthocyanin expression, and the two control lines mentioned above; (3) spring 2009, sown on 7 April. Plants were transplanted in randomized block design (three blocks with three plants of each line in each block). The experiment included a total of five stable transgenic lines, not segregating for *ANT1*: two T_2 or T_3 lines carrying 35S*::ANT1*^{L}, three T_2 lines carrying 35S*::ANT1*^{C}, and the two control lines mentioned above. The five transgenic lines used in this season were the only stable lines obtained despite our effort to stabilize the whole collection of transgenic lines generated in this study.

Cotyledons were characterized during the early-summer and spring seasons, while fruit-peel tissues were characterized during winter 2008 and spring 2009. All of these characterizations were performed in comparison to the two control genotypes mentioned above which did not significantly differ and were therefore pooled into a single control group prior to the analyses.

In order to perform a discriminative association analysis between the native alleles of *ANT1* as well as *AN2* and the trait of anthocyanin accumulation in the AFT genotype, a segregating F_4 population resulting from self-pollination of a single recombinant F_3 plant was used. This F_3 plant was obtained from the population described by Sapir et al. (2008) and characterized as: (1) heterozygous for *AN2*; (2) homozygous for *ANT1*^{C}; (3) homozygous for the *hp-1* mutation, intensifying AFT phenotype; (4) having a determinate (*sp*) growth habit and yielding uniform-ripening (u) fruits. This plant was allowed to self-pollinate, generating F_4 seeds segregating for *AN2*^{L} and *AN2*^{C} which were sown on 27 July 2009 and transplanted a month later in a randomized block design (three blocks with three plants of each of the three *AN2* genotypes in each block), together with their control counterparts harvested from plants resulting from self-pollination of F_3 *hp-1/hp-1 ANT1*^{L}*/ANT1*^{L} *AN2*^{L}*/AN2*^{L} plants.

Plasmid construction and plant transformation

The expression cassette was constructed in pMON10098 (Monsanto Co., St. Louis, MO, USA). The coding region of each of the two *ANT1* alleles was reverse transcribed from total RNA extracted from young leaves, and amplified by the polymerase chain reaction (PCR) using the proofreading Pwo DNA polymerase (Genaxis Biotechnology, Kronberg/Taunus, Germany). The *ANT1*^{L} allele was amplified from cDNA of the open-pollinated tomato cv Ailsa Craig (GenBank accession EF433416.1) and was identical to the *ANT1* sequence originally isolated from cv. Micro-Tom (Mathews et al. 2003). The *ANT1*^{C} allele was amplified from cDNA of the AFT genotype LA1996, originated from *S. chilense* (GenBank accession EF433417.1). *ANT1*^{C} and *ANT1*^{L} were cloned separately into the pCRII-TOPO (Invitrogen, Carlsbad, CA, USA). Sequence-validated *ANT1* clones were inserted, following digestion with *EcoRI*, into pMON10098, forming a transcriptional fusion between the CaMV 35S promoter and the E9 terminator. The transgenic tomato plants were generated by *Agrobacterium tumefaciens*-mediated transformation of cotyledons from 10-day-old seedlings (McCormick 1991).

Tissue sampling for biochemical and transcriptional characterizations

Tissues were sampled for both RNA and pigment extraction in paired observations, allowing coupling between the transcriptional and the biochemical analyses. In seedlings, a fully-expanded cotyledon was harvested from each of six randomly selected seedlings of each transgenic line for transcriptional profiling while opposite cotyledons were harvested for biochemical analyses. For analysis of fruit peels, a total of three ripe-mature fruits were harvested from single plants of each transgenic line in each block. From each fruit three discs of fresh tomato peels (0.1–0.2 g) were sampled and pooled for metabolic characterization and a pool of three squares (1 cm^2) were taken for transcriptional profiling.

Source and design of polymerase chain reaction (PCR) primers

All PCR primers used throughout this study were purchased from Syntezza Bioscience Ltd. (Jerusalem, Israel). Sequence analysis and primer design were carried out with the DNAMAN sequence analysis software v4.1 (Lynnon BioSoft, Pointe-Claire, Quebec, Canada). Sequences for quantitative Real-Time PCR (qRT-PCR) analyses were

obtained based on Bovy et al. (2002) and Sapir et al. (2008), followed validation with current NCBI and DFCI gene databases at http://www.ncbi.nlm.nih.gov/ and http://compbio.dfci.harvard.edu/tgi/tgipage.html, respectively.

Transgenic characterization of plants and genotyping *ANT1* and *AN2*

Genomic DNA was extracted from young leaves of individual plants according to Fulton et al. (1995). To validate the incorporation of the *ANT1* transgenes, DNA samples extracted from the transgenic lines served as templates in PCRs with primers complementary to the CaMV 35S promoter and the *ANT1* gene (5′→3′): CCTTCGCAAG ACCCTTCCTCT and GTTGCATGGGTGGTAAATTA AG. Genotyping analyses of the native *ANT1* and *AN2* alleles were also carried out by PCR. The primers used in these PCRs were: *ANT1* (5′→3′): GGAAGGACAGCT AACGATGTG and GTTGCATGGGTGGTAAATTAAG (Sapir et al. 2008); *AN2* (5′→3′): GCATCGTTGGGAGT TAGGAA and AACGAGGACGAGAATGAGGA (Boches 2009).

The PCRs were performed in a T-GRADIENT thermal cycler (Biometra, Analytik Jena, Gottingen, Germany) in a volume of 25 µl containing 15 ng of template DNA, 10 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 U of Taq DNA polymerase, and 1× PCR buffer. The PCRs consisted of an initial incubation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, and annealing at 58°C for 40 s and 72°C for 40 s. Final elongation, at 72°C, was carried out for 5 min.

PCR products were visualized by electrophoresis in 1.0% agarose gels stained with ethidium bromide. PCR amplification products were visualized by electrophoresis in 1.0% agarose gels stained with ethidium bromide.

qRT-PCR analyses

Total RNA was extracted from cotyledons and fruit peels of ripe tomato fruits using the TRIzol reagent system (Invitrogen Corp., Carlsbad, CA, USA), and genomic DNA contaminants were digested with TURBO DNA-free DNAase (Ambion Inc., Austin, TX, USA). The remaining RNA was then used as the template for cDNA synthesis using the Masterscript cDNA synthesis kit with random hexamer primers (KAPA Biosystems, Woburn, MA, USA.

The qRT-PCR analysis was performed using the KAPA SYBER®FAST Master Mix (KAPA Biosystems, Woburn, MA, USA): initial incubation at 95°C for 3 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and polymerization at 72°C for 20 s. A melting curve method was carried out by elevating the temperature 1°C every 5 s, ranging from 72 to 99°C in order to corroborate acceptance of one gene product and confirm a specific amplification. DNA sequences complementary to *18S RIBOSOMAL RNA* were used as reference and the primers designed for it, as well as for the other genes analyzed by qRT-PCR are presented in Table S1. The primers used for the analysis of *ANT1* expression were designed to avoid sequence differences between the $ANT1^L$ and $ANT1^C$ alleles.

Four technical replicates were performed for every biological repeat. The qRT-PCR analyses were performed using the Rotor-Gene Q detection system and data was collected and analyzed with the Rotor-Gene 6000 software version 1.7.28 (Qiagen Corbett Life Science, Duesseldorf, Germany). The relative abundance of the examined gene transcripts was calculated by the formula: $2^{-(\text{CT\_examined gene-CT\_reference gene})}$, where CT represents the fractional cycle number at which the fluorescence crosses a fixed threshold (usually set on 0.1).

Extraction and quantification of major flavonoids

All chemicals and solvents were purchased from Sigma (St Louis, MO, USA). Samples were ground in liquid nitrogen, and pigments were extracted in the dark with 2 ml of cold methanol:water:acetic acid (11:5:1; Markham and Ofman 1993). Extracts were spun for 10 min at 20,800*g* (14,000 rpm), leaving the anthocyanins in the supernatant. Anthocyanins were initially determined by measuring the OD at 530 nm by spectrophotometer in a UV-2401 PC (Shimadzu Co., Kyoto, Japan). Anthocyanins were hydrolyzed in acids and the resultant anthocyanidins were determined in high performance liquid chromatography (HPLC). The purifications and HPLC quantification of anthocyanidins and other major flavonoid compounds were carried out by as previously described (Sapir et al. 2008), and expressed as total peak area per g fresh weight (FW) (peak area/gFW). These total peak area values are fully correlated with the quantity of these flavonoid compounds.

Statistical analyses

The following variables were analyzed: (1) OD_{530}/gFW of fruit peel, anthers, leaves and cotyledons of 11-d-old seedlings; (2) Anthocyanidin and flavonol content in ripe-fruit peels and cotyledons; (3) transcript levels of genes, following transformation to their natural logarithm, in fruit peels and cotyledons; (4) fold increase of *ANT1* transcript levels in transgenic plants relative to the control genotypes; (5) phenotype to transcript level ratios in fruit peels and cotyledons, calculated as the average total anthocyanidin levels obtained for each genotype divided by its average *ANT1* transcript level.

Analyses of variance (ANOVA), linear regressions and estimation of correlations were carried out with the JMP Statistical Discovery Software (SAS Institute Inc., Cary, NC, USA). The ANOVA models included effects related to block and genotype although no statistically significant block effects were obtained in any of our experiments. Due to heterogeneity of variances, resulting from scale effects, data were transformed to their natural logarithm prior to the analyses. Differences among means were calculated based on the Tukey–Kramer honestly significant difference (HSD) test (Kramer 1956).

Results

Phenotypes of tomato plants over-expressing *ANT1*

The transgenic lines displayed variable levels of purple pigmentation in vegetative and reproductive tissues, irrespective of the specific *ANT1* allele transformed (Fig. 1). Spectrophotometric quantification showed that 35S*::ANT1*^{C} plants displayed higher average anthocyanin levels than their 35S*::ANT1*^{L} counterparts that were, with the exception of fruit peels in spring 2009, also statistically significant (Fig. 2). Noteworthy, 4 of the 15 transgenic T_0 35S*::ANT1*^{C} plants, displaying exceptionally strong anthocyanin accumulation in fruits and leaves, were unable to pass the transgene to their T_1 descendents suggesting that 35S*::ANT1*^{C} may be more embryo-lethal to tomato compared to 35S*::ANT1*^{L}. Furthermore, only five stable transgenic lines were finally obtained despite our efforts to stabilize the whole collection of transgenic lines generated in this study. This can suggest again that higher expression of the gene, due to higher copy number, is also lethal.

Biochemical analysis of cotyledons

HPLC analysis of cotyledon extracts showed that total anthocyanin levels were highly and significantly correlated with results obtained by spectrophotometer [$r = 0.72$ ($P = 7.3 \times 10^{-5}$) and $r = 0.88$ ($P = 1.0 \times 10^{-13}$) in early summer 2008 and spring 2009, respectively]. The HPLC analysis preformed in early summer 2008 showed that the purple pigmentation observed was mainly due to petunidin, malvidin and delphinidin, commonly accumulating in vegetative tomato tissuces (Fig. 3). Over-expression of *ANT1* resulted in significantly higher levels of anthocyanins in both transgenic genotypes compared to their control counterparts. However, 35S*::ANT1*^{C} plants accumulated significantly more anthocyanins than their 35S*::ANT1*^{L} counterparts. The flavonols quercetin and kaempferol showed a similar trend of accumulation and their total level was significantly higher in the 35S*::ANT1*^{C} genotypes. The transgenic cotyledons also had significantly higher caffeic-acid levels, but no statistical difference was detected between the two alleles. In spring 2009 (Fig. 3), the effect on anthocyanin levels was in agreement with the results obtained in early summer 2008, but the average levels of flavonols and caffeic-acid did not differ statistically between the two transgenic genotypes and their control counterparts.

Biochemical analysis of fruit peels

Fruits of the control plants never had anthocyanin pigmentation while either 35S*::ANT1*^{C} or 35S*::ANT1*^{L} plants displayed variable levels of purple pigmentation in fruit peels and flesh. Furthermore, several transgenic lines, particularly those over-expressing *ANT1*^{C}, displayed an exceptionally strong purple pigmentation in their fruit flesh (Fig. S1). We focused on fruit peel because anthocyanin accumulation in the AFT genotype is mainly restricted to this tissue.

HPLC analysis of the fruit peel extracts showed that total anthocyanin levels were highly and significantly correlated with the spectrophotomer measurements [$r = 0.96$ ($P = 1.3 \times 10^{-17}$) and $r = 0.85$ ($P = 1.2 \times 10^{-6}$), in winter 2008 and spring 2009, respectively]. In winter 2008, one block consisting of all lines was subjected to HPLC analysis (Fig. 4). As expected, tomato fruit peels of non-transgenic lines had no measurable levels of anthocyanins. In contrast, fruit peels of the transgenic genotypes had high levels of anthocyanins and significantly higher levels were obtained in 35S*::ANT1*^{C} plants. The major anthocyanin metabolites accumulating in fruit peels, malvidin, petunuidin and delphinidin, were identical to those obtained in the cotyledons. Flavonols showed the same trend of accumulation with significantly higher average levels in the 35S*::ANT1*^{C} genotypes. Levels of the flavanone naringenin in the transgenic fruit peels were not statistically different in comparison to their control counterparts.

In spring 2009, anthocyanin accumulation in fruit peels was generally in agreement with the results obtained in winter 2008; however, no statistical difference was obtained between 35S*::ANT1*^{C} and 35S*::ANT1*^{L} genotypes (Fig. 4). In addition, no statistically significant differences were observed between the average levels of flavonols accumulating in fruit peels of transgenic fruits in comparison to the control genotypes, although 35S*::ANT1*^{C} lines exhibited a quantitative advantage. Levels of the flavanone naringenin in the transgenic fruit peels did not differ statistically from their control counterparts.

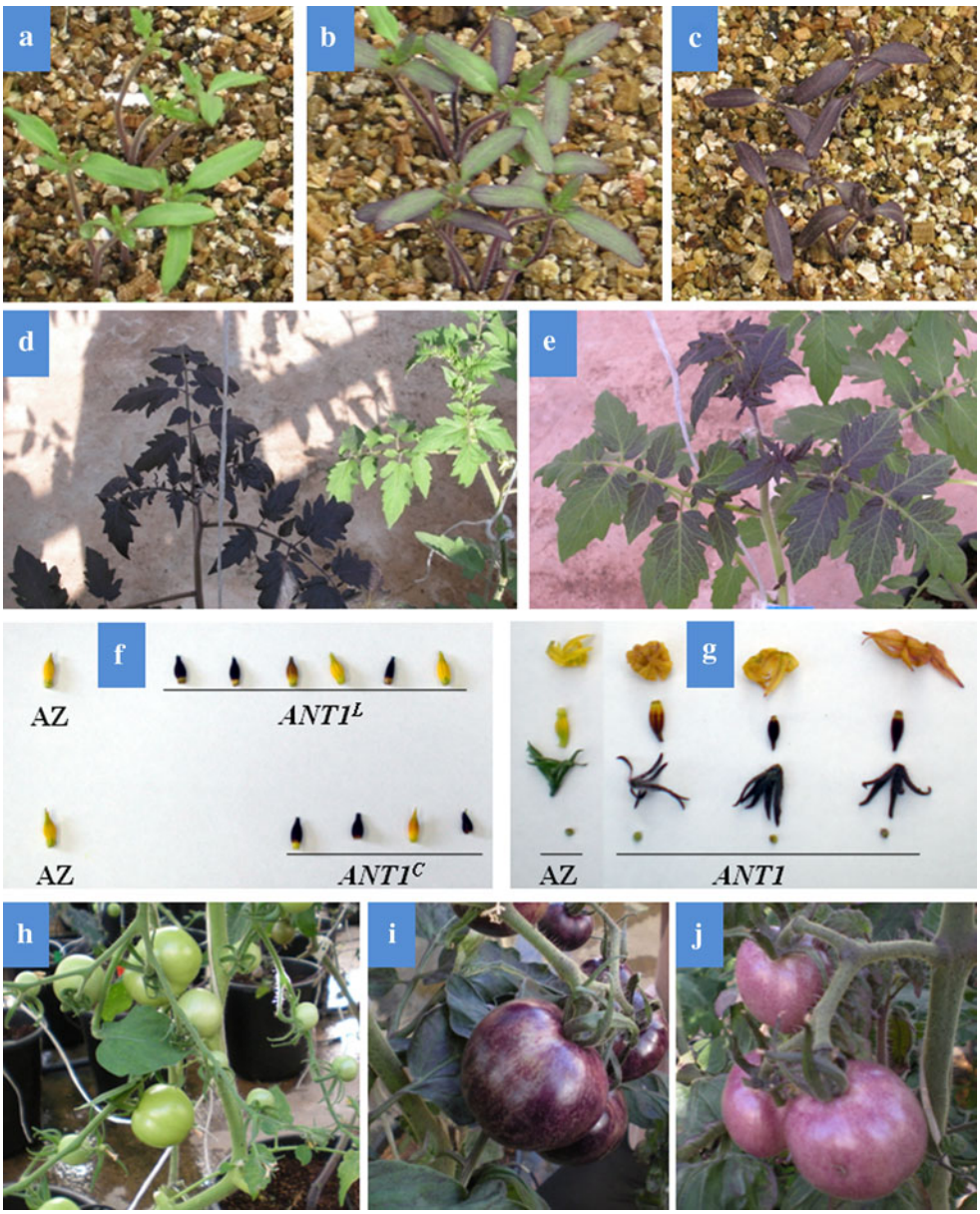

Fig. 1 Phenotypes of transgenic tomato plants over-expressing *ANT1* [cotyledons of azygous control (**a**) and transgenic (**b**, **c**) plants; leaves of azygous control (right plant in **d**) and transgenic (left plant in **d** and **e**) plants; anthers of azygous control (*AZ* in **f**) and transgenic 35S::$ANT1^L$ or 35S::$ANT1^C$ plants ($ANT1^L$ and $ANT1^C$ in **f**); flower petals, anthers, sepals and ovaries of azygous (left panel in **g**) and transgenic plants (right 3 panels in **g**); fruits of azygous control (**h**) and transgenic 35S::$ANT1^C$ (**i**) or 35S::$ANT1^L$ (**j**) plants]

Transcript levels of the two 35S::*ANT1* alleles and their effect on flavonoid biosynthesis genes

As shown in Table 1, over-expression of either $ANT1^C$ or $ANT1^L$ resulted in a highly significant transcript level increase in the two tissues examined. However, no statistically significant differences in the average transcript levels of *ANT1* were obtained between 35S::$ANT1^C$ and 35S::$ANT1^L$ lines except in winter 2008, when fruit peels of 35S::$ANT1^L$ lines displayed statistically significant higher levels of *ANT1* expression (Table 1B).

To establish a link between the over-expression of each of the two *ANT1* alleles and the flavonoid pathway, transcript levels of selected structural genes of the pathway were analyzed in cotyledons and fruit peels of lines that were subjected to biochemical analyses during spring 2009 (Table 2). In cotyledons (Table 2A), all tested genes, except *CHI*, displayed higher transcript levels, which were usually statistically significant, in plants over-expressing *ANT1*, but there were no differences between lines carrying the 35S::$ANT1^C$ or the 35S::$ANT1^L$ allele. Although no statistical difference was obtained between the average

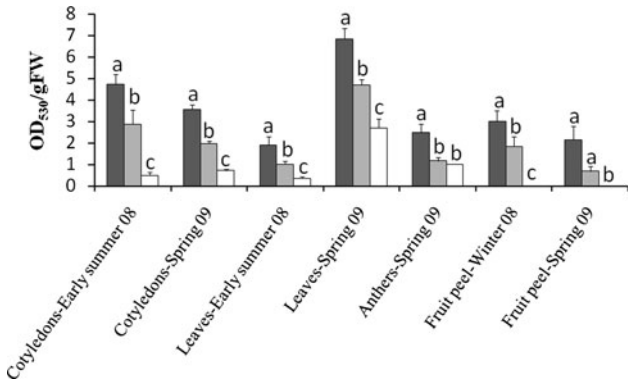

Fig. 2 Spectrophotometric quantification of anthocyanin levels in tissues harvested from lines over-expressing *ANT1* (*black columns*, 35S*::ANT1*^{C} lines; *grey columns*, 35S*::ANT1*^{L} lines; *white columns*, pooled wild-type and azygous control lines; *different letters* above columns indicate statistically significant differences, $P < 0.05$, between genotypes within each season and for each tissue separately; bars above columns represent the standard error, SE, of the mean)

transcript levels of *CHI* in cotyledons of the two groups of transgenic lines, transcript levels of both transgenic lines were significantly lower in comparison to their control counterparts. In fruit peels (Table 2B), no significant up-regulation of the early structural genes of the flavonoid biosynthetic pathway, *CHS1*, *CHS2* and *CHI*, was obtained in the two groups of the transgenic lines. However, with the exception of *FLS*, all other structural genes analyzed, in particular those operating at the late stages of the pathway, including *F3H*, *DFR*, *ANS* and *3-GT*, displayed higher average transcript levels in transgenic lines carrying the 35S*::ANT1*^{C} allele than in those carrying the 35S*::ANT1*^{L} allele or in the non-transgenic counterparts. Of these four genes, *DFR*, *ANS* and *3-GT* displayed average transcript levels that were significantly higher in the transgenic lines carrying the 35S*::ANT1*^{C} allele than in the other two groups. Interestingly, the average transcript levels of *FLS*, which participates in flavonol biosynthesis, did not differ

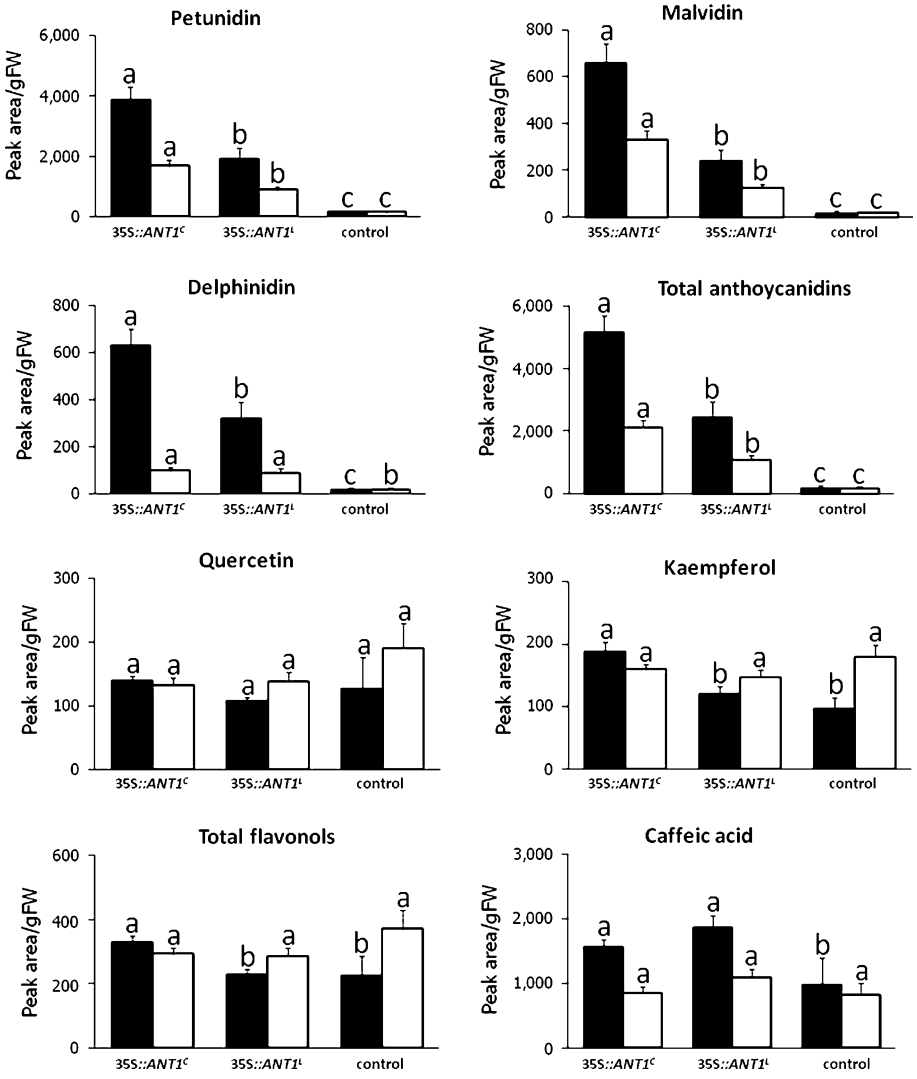

Fig. 3 HPLC analysis of cotyledons harvested from plants over-expressing *ANT1* in comparison to their control lines in early summer 2008 and spring 2009 [*black columns*, summer 2008; *white columns*, spring 2009; the *control columns* represent pooled wild-type and azygous lines; *peak area* was divided by 10^{6} prior the analyses; *different letters* above columns indicate statistically significant differences, $P < 0.05$, between genotypes for each metabolite and season separately; *bars above columns* represent the standard error, SE, of the mean]

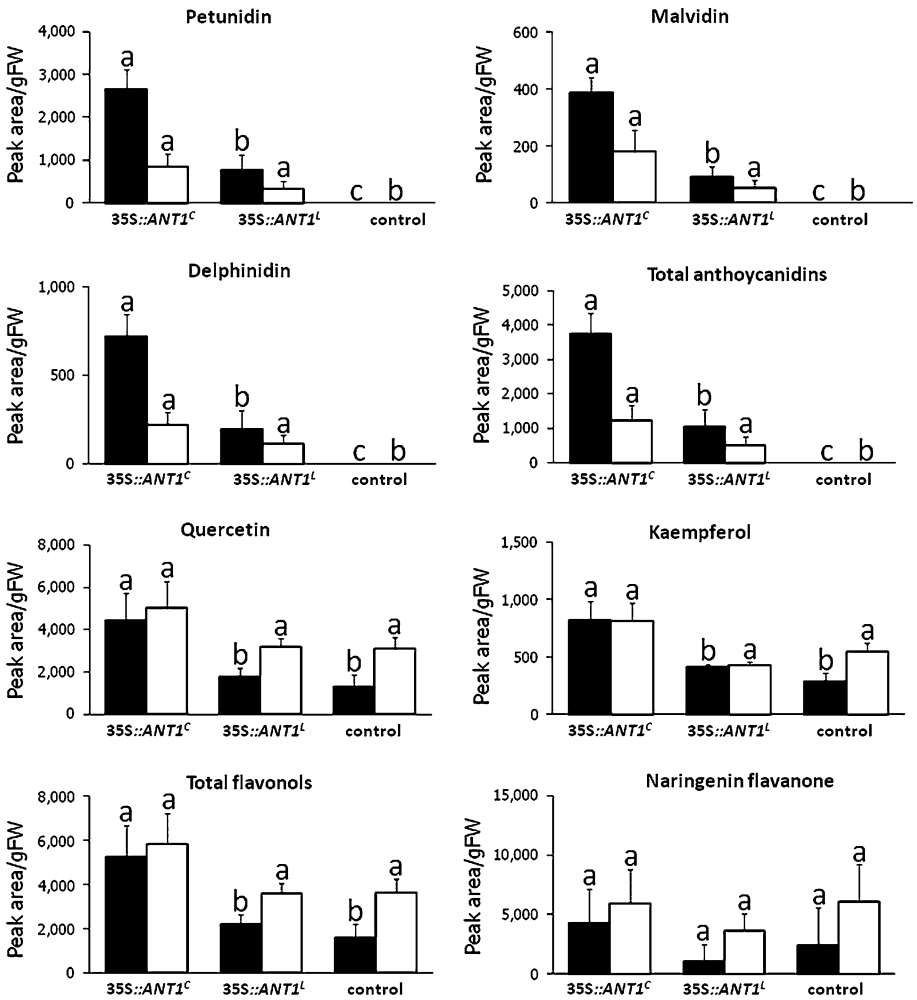

Fig. 4 HPLC analysis of fruit peels of ripe tomato fruits over-expressing *ANT1* in comparison to their control lines in winter 2008 and spring 2009 [*black columns*, winter 2008; *white columns*, spring 2009; the *control columns* represents pooled wild-type and azygous lines; *peak area* was divided by 10^6 prior the analyses; *different letters* above columns indicate statistically significant differences, $P < 0.05$, between genotypes for each metabolite and season separately; *bars* above columns represent the standard error, SE, of the mean]

Table 1 Average transcript levels of *ANT1* in cotyledons (A) and fruit peels (B) of transgenic and non-transgenic control lines according to season

Genotype	Early summer 2008			Spring 2009		
	N	Ln (transcript level)	Fold increase	*N*	Ln (transcript level)	Fold increase
A						
35S::*ANT1*^{C}	5	$-2.8^a \pm 0.2$	365.0	3	$-3.7^a \pm 0.1$	54.6
35S::*ANT1*^{L}	5	$-2.4^a \pm 0.3$	544.6	2	$-3.7^a \pm 0.2$	54.6
Control	2	$-8.7^b \pm 0.5$	–	2	$-7.7^b \pm 0.2$	–
P(F)		6.3×10^{-12}			1.9×10^{-22}	
Genotype	Winter 2008			Spring 2009		
	N	Ln (transcript level)	Fold increase	*N*	Ln (transcript level)	Fold increase
B						
35S::*ANT1*^{C}	7	$-4.3^b \pm 0.17$	36.6	3	$-8.7^a \pm 0.3$	16.4
35S::*ANT1*^{L}	8	$-3.3^a \pm 0.25$	99.5	2	$-8.2^a \pm 0.5$	27.1
Control	2	$-7.9^c \pm 0.47$	–	2	$-11.5^b \pm 0.2$	
P(F)		2.1×10^{-14}			1.6×10^{-5}	

Average transcript levels were obtained by qRT-PCR and analyzed following transformation to their natural logarithm values; fold increase values were calculated relative to the control genotypes; *N* number of independent lines; values represent mean ± SE; different lower-case superscript letters indicate statistically significant differences, $P < 0.05$, between genotypes in each season separately]

Table 2 Average transcript levels of selected structural genes of the flavonoid biosynthetic pathway in cotyledons (A) and fruit peels (B) harvested from transgenic and non-transgenic control lines in spring 2009

Genotype	*N*	Ln(transcript level)						
		CHS1	*CHS2*	*CHI*	*F3H*	*DFR*	*3-GT*	
A								
35S::$ANT1^C$	3	$-4.1^a \pm 0.2$ (3.0)	$-1.8^a \pm 0.2$ (8.2)	$-9.1^b \pm 0.2$ (0.3)	$-4.7^a \pm 0.4$(3.7)	$-3.8^a \pm 0.3$ (16.4)	$-6.5^a \pm 0.3$ (5.5)	
35S::$ANT1^L$	2	$-3.5^a \pm 0.2$ (5.5)	$-2.1^a \pm 0.2$ (6.0)	$-9.5^b \pm 0.2$ (0.2)	$-5.3^{ab} \pm 0.4$ (2.0)	$-4.4^a \pm 0.2$ (9.0)	$-7.0^a \pm 0.3$ (3.3)	
Control	2	$-5.2^b \pm 0.2$	$-3.9^b \pm 0.2$	$-8.0^a \pm 0.2$	$-6.0^b \pm 0.3$	$-6.6^b \pm 0.3$	$-8.2^b \pm 0.3$	
P(F)		2.3×10^{-6}	1.1×10^{-8}	5.3×10^{-4}	4.7×10^{-3}	3.7×10^{-7}	2.4×10^{-4}	
Genotype	*N*	Ln (transcript level)						
		CHS1	*CHS2*	*CHI*	*F3H*	*FLS*	*DFR*	*ANS*	*3-GT*
B									
35S::$ANT1^C$	3	$-5.8^a \pm 0.3$ (0.9)	$-2.1^a \pm 0.3$ (0.4)	$-14.9^a \pm 0.2$ (1.2)	$-7.4^a \pm 0.3$ (3.7)	$-3.4^a \pm 0.3$ (0.7)	$-6.5^a \pm 0.3$ (298.9)	$-9.6^a \pm 0.7$ (44.7)	$-13.7^a \pm 1.9$ (134.3)
35S::$ANT1^L$	2	$-5.8^a \pm 0.5$ (0.9)	$-1.9^a \pm 0.7$ (0.5)	$-15.0^a \pm 0.2$ (1.1)	$-8.3^{ab} \pm 0.4$ (1.5)	$-3.3^a \pm 0.4$ (0.8)	$-11.7^b \pm 0.4$ (1.6)	$-12.4^b \pm 1.0$ (2.7)	$-15.9^b \pm 1.8$ (14.9)
Control	2	$-5.7^a \pm 0.3$	$-1.3^a \pm 0.3$	$-15.1^a \pm 0.3$	$-8.7^b \pm 0.2$	$-3.1^a \pm 0.3$	$-12.2^b \pm 0.2$	$-13.4^b \pm 0.3$	$-18.6^b \pm 1.4$
P(*F*)		0.920	0.267	0.891	0.009	0.618	0.007	0.005	0.045

Average transcript levels were obtained by qRT-PCR and analyzed following transformation to their natural logarithm values

N number of independent lines; *CHS1, CHALCONE SYNTHASE1; CHS2, CHALCONE ISOMERASE, F3H, FLAVANONE 3-HYDROXYLASE; FLS, FLAVANOL SYNTHASE, DFR, DIHYDROFLAVONOL REDUCTASE; ANS, ANTHOCYANIDIN SYNTHSE; 3-GT, FLAVONOID 3-O-GLUCOSYLTRANSFERASE*; values represent mean ± SE; different lower-case superscript letters indicate statistically significant differences, $P < 0.05$, between genotypes for each gene separately; in parentheses: the fold increase of transcript levels in the transgenic lines relative to the control genotypes

between the three groups in this study. This may partially explain why flavonol levels did not differ statistically between the transgenic and the control genotypes in spring 2009 (Fig. 4).

Efficiencies of the *ANT1* alleles

Integration of biochemical and transcriptional results show that plants over-expressing $ANT1^C$ share higher average anthocyanin levels than plants over-expressing $ANT1^L$, although *ANT1* transcript levels among the 35S*::*$ANT1^C$ genotypes were either similar or lower than their 35S*::*$ANT1^L$ counterparts. This suggests that plants carrying 35S*::*$ANT1^C$ are more efficient in accumulating anthocyanin compounds than those with 35S*::*$ANT1^L$. To demonstrate the higher efficiency attributed to 35S*::*$ANT1^C$, we analyzed the ratio between the levels of anthocyanins, obtained by HPLC or spectrophotometer quantification, and their respective transcript levels. This ratio represents the amount of anthocyanin metabolites accumulating per one transcriptional unit of *ANT1*. The results show that in both tissues analyzed this ratio was significantly higher in 35S*::*$ANT1^C$ than in 35S*::*$ANT1^L$ plants (Table 3). In cotyledons, the 35S*::*$ANT1^C$ allele displayed 3.6 and 1.9 more anthocyanins per one transcriptional unit than 35S*::*$ANT1^L$ in early summer 2008 and spring 2009, respectively (Table 3A). Tomato fruit peels showed a similar trend: the 35S*::*$ANT1^C$ transgene displayed 4.9 and 3.9 more anthocyanins per one transcriptional unit than 35S*::*$ANT1^L$ in winter 2008 and spring 2009, respectively.

Our results suggest that anthocyanin accumulation is due to up-regulation of *ANT1*. To better demonstrate the involvement of *ANT1* in anthocyanin accumulation, we preformed a comparative association analysis between of anthocyanin levels and 35S*::ANT1* transcript levels in cotyledons and fruit peels (Fig. 5a, b, respectively). For this purpose, results obtained in spring 2009 were chosen because in this season both tissues were tested. In cotyledons, statistically significant correlations of similar magnitude were obtained between *ANT1* transcript and anthocyanin levels for both transgenic genotypes [$r = 0.50$ ($P = 0.0421$) and $r = 0.58$ ($P = 0.0455$) for 35S*::*$ANT1^C$ and 35S*::*$ANT1^L$ genotypes, respectively]. In fruit peels, however, a much higher correlation was found for the 35S*::*$ANT1^C$ [$r = 0.87$ ($P = 0.002$)] than for the 35S*::*$ANT1^L$ genotypes [$r = 0.32$, ($P = 0.54$)], the latter being statistically insignificant. Analysis of the respective linear regression coefficients, *b*, of the two alleles in the two tissues displayed a similar trend. The *b* value obtained for the 35S*::*$ANT1^C$ genotypes in cotyledons was 2.5-fold higher and statistically significant ($P < 0.05$) than for 35S*::*$ANT1^L$ genotypes and also slightly higher but in agreement with the results presented in Table 3A. The

Table 3 Analysis of allele efficiencies in cotyledons (A) and fruit peels (B) of transgenic plants over-expressing *ANT1*

Genotype	Early summer 2008			Spring 2009		
	Total anthocyanidins (Peak area/gFW)	Transcript level	Total anthocyanidins / Transcript level$_{\times 10^3}$	Total anthocyanidins (Peak area/gFW)	Transcript level	Total anthocyanidins / Transcript level$_{\times 10^3}$
A						
35S::*ANT1*^{C}	$5144^a \pm 541$	$0.07^a \pm 0.01$	$89^a \pm 13$	$2103^a \pm 244$	$0.03^a \pm 0.00$	$83^a \pm 10$
35S::*ANT1*^{L}	$2443^b \pm 494$	$0.11^a \pm 0.03$	$24^b \pm 3$	$1091^b \pm 140$	$0.03^a \pm 0.01$	$44^b \pm 9$
P(*F*)	5.2×10^{-9}	0.2	1.4×10^{-7}	7.9×10^{-4}	0.7	6.7×10^{-3}
Genotype	Winter 2008			Spring 2009		
	Total anthocyanidins (OD_{530}/gFW)	Transcript level	Total anthocyanidins / Transcript level	Total anthocyanidins (Peak area/gFW)	Transcript level	Total anthocyanidins / Transcript level$_{\times 10^3}$
B						
MM 35S::*ANT1*^{C}	$2.4^a \pm 0.4$	$0.02^b \pm 0.00$	$249^a \pm 57$	$1245^a \pm 439$	$0.0002^a \pm 0.0000$	$4773^a \pm 920$
MM 35S::*ANT1*^{L}	$1.9^a \pm 0.5$	$0.07^a \pm 0.01$	$51^b \pm 13$	$509^a \pm 240$	$0.0005^a \pm 0.0002$	$1227^b \pm 467$
P(*F*)	0.4	1.9×10^{-6}	2.6×10^{-3}	0.20	0.19	0.01

Average transcript levels were obtained by qRT-PCR; values are displayed as mean ± SE; peak area was divided by 10^6 before analyses; different lower-case superscript letters indicate statistically significant differences, $P < 0.05$, between genotypes)

b value obtained for the 35S::*ANT1*^{C} genotypes in fruit peels was 21-fold higher and statistically significant ($P < 0.05$) than for the 35S::*ANT1*^{L} genotypes and also much higher but generally in agreement with the results presented in Table 3B. It is noteworthy, unlike the 35S::*ANT1*^{C} genotypes, that the regression coefficient, *b*, obtained between *ANT1* transcript and anthocyanin levels in fruit peels of 35S::*ANT1*^{L} genotypes was statistically insignificant from 0.

Linkage between the native *ANT1* and *AN2* genes and their association with anthocyanin accumulation

A total of 160 F_3 plants were used to analyze linkage between the native *ANT1* and *AN2*. Among these plants, a single recombinant AFT plant, *ANT1*^{C}/*ANT1*^{C} *AN2*^{C}/*AN2*^{L} (Fig. S2), was obtained, indicating a recombination distance of ~0.3 centiMorgan (cM) between the two genes. A population derived following self-pollination of this plant was confirmed to be stable for the *ANT1*^{C} allele but segregating for the two *AN2* alleles. Visual inspection of mature-unripe (Fig. S3) and mature-ripe fruits (not presented), showed that all fruits displayed the characteristic phenotype of AFT, irrespective of the *AN2* genotype.

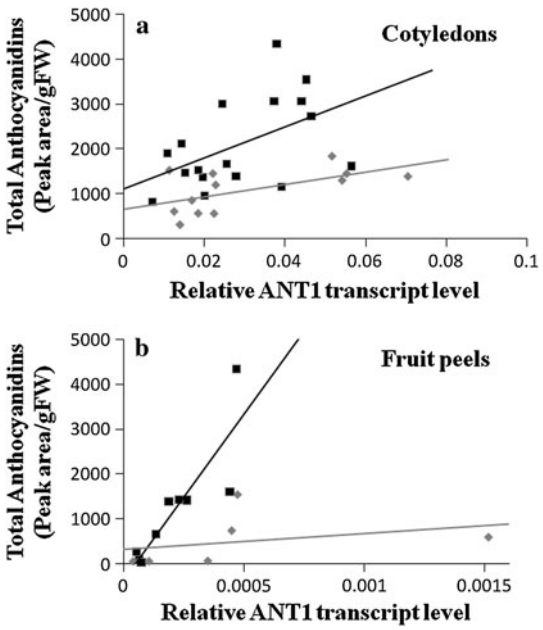

Fig. 5 Association between *ANT1* transcript levels and total anthocyanin accumulated in cotyledons (**a**) and fruit peels (**b**) of plants over-expressing *ANT1* in spring 2009 (each point in **a** represents an individual seedling while each point in **b** represents an average of three plants comprising a plot in a block: *black squares* and regression slop, 35S::*ANT1*^{C} lines; *grey diamonds* and regression slop, 35S::*ANT1*^{L} lines; *peak area* was divided by 10^6 prior the analyses; each data points in **a** is based on one biological and four technical repeats, while each data point in **b** is based on three biological and four technical repeats)

Discussion

We performed a comparative transgenic analysis of two alleles of the tomato *ANT1* gene. *ANT1* and its homolog, *AN2*, were mapped to tomato chromosome 10 as candidate genes controlling anthocyanin and flavonol accumulation in fruit peels of the AFT genotype (Sapir et al. 2008; Zuluaga et al. 2008). The draft tomato genome-sequence

positions *AN2* ~2.7 Kb downstream from *ANT1* on scaffold01162 (http://solgenomics.net/).

The predicted protein sequence of ANT1^{C} differs from ANT1^{L}, but no differences were obtained in the average transcript levels of *ANT1* in fruit peels of the AFT genotype compared to red-fruited genotypes (Sapir et al. 2008). Borovsky et al. (2004) reports an association between anthocyanin accumulation in immature fruits of the *A* genotype of pepper and *ANT1* homolog on chromosome 10. In pepper, this gene is transcribed throughout immature-fruit development in the purple-fruited genotype whereas in its green-fruited counterpart it is not transcribed at all. This transcriptional difference is attributed to the promoter because the coding region of the gene does not differ between these two pepper genotypes (Borovsky et al. 2004). Sapir et al. (2008) therefore hypothesizes that if *ANT1* causes the AFT phenotypes in the tomato, its effects should be ascribed to sequence rather than transcriptional alterations. To validate this, we compared the phenotypes resulting from over-expression of *ANT1^{C}* and *ANT1^{L}*, both driven by the same promoter. The use of the constitutive promoter also enabled the analysis of plant tissues other than fruit peels.

Constitutive over-expression of *ANT1* led to accumulation of anthocyanin pigments in vegetative and reproductive tissues. Surprisingly, significant accumulation of anthocyanins was also observed in the entire flesh of the transgenic fruits whereas in the AFT genotype anthocyanin accumulation is mainly restricted to peels (Jones et al. 2003; Mes et al. 2008). Moreover, a few 35S*::ANT1^{L}* lines also displayed high levels of anthocyanins in fruit peels and flesh in contrast to Mathews et al. (2003) where over-expression *ANT1^{L}* from cv Micro-Tom in cv Micro-Tom plants resulted in scattered purple spots on fruit surface. Sapir et al. (2008) found no sequence differences in *ANT1* between cv Micro-Tom and cv Ailsa Craig. Therefore, the much stronger anthocyanin accumulation in fruit flesh and peels obtained in this study among 35S*::ANT1^{L}* genotypes is likely attributed to the differences in the genetic backgrounds used for transformation.

Transcript levels of *ANT1* in 35S*::ANT1^{C}* plants did not usually differ significantly from their 35S*::ANT1^{L}* counterparts in cotyledons and fruit peels. However, anthocyanin levels in both tissues were usually significantly higher in 35S*::ANT1^{C}* than in 35S*::ANT1^{L}* plants. These results indicate that *ANT1^{C}* can induce higher anthocyanin pigmentation under equivalent transcript levels. This is supported by the statistically significant higher average anthocyanidin to transcript-level ratio obtained for 35S*::ANT1^{C}* in both cotyledons and fruit peels. *ANT1^{C}* is thus more efficient than *ANT1^{L}* in both tissues providing support to the hypothesis that *ANT1* is the gene controlling anthocyanin accumulation in AFT fruits.

In cotyledons, a significant association was obtained between anthocyanin accumulation and *ANT1* transcript levels, irrespective of the specific transgenic allele examined. However, anthocyanin accumulation was significantly more associated with transcript levels of 35S*::ANT1^{C}* than with transcript levels of 35S*::ANT1^{L}*. This difference was much more pronounced in fruit peels indicating that anthocyanin accumulation in 35S*::ANT1^{L}* plants is more dependent upon other factors than in their 35S*::ANT1^{C}* counterparts particularly in fruit peels. Such factors may include bHLH transcription factors, WDR-type or other proteins unable to display their effects in tissues which do not normally produce anthocyanins, such as fruit peels of cv Moneymaker. Despite this notable difference, both alleles similarly activated transcript levels of selected structural genes of the flavonoid biosynthetic pathway. In cotyledons, 35S*::ANT1^{L}* and 35S*::ANT1^{C}* did not significantly differ in their effect on these genes, and over-expression of both alleles usually induced transcript levels of early (*CHS1* and *CHS2*) and late (*DFR* and *3-GT*) structural genes of the pathway, similarly to Mathews et al. (2003). It is noteworthy that both alleles significantly down-regulated transcript levels of *CHI*, suggesting a negative transcriptional feedback of *ANT1* on *CHI* and that *CHI* transcription is not limiting to anthocyanin accumulation in tomato cotyledons.

Over-expression of the two *ANT1* alleles in fruit peels did not yield significantly higher transcript levels of the early structural genes (*CHS1*, *CHS2* and *CHI*) while the late structural genes analyzed displayed higher transcript levels in both transgenic genotypes. However, only in 35S*::ANT1^{C}* genotypes was this increase statistically significant. These differences between the two types of transgenic genotypes are intriguing because they raise the question of how anthocyanin metabolites can be significantly accumulating in fruit peels of the 35S*::ANT1^{L}* genotypes without a significant collateral effect on transcript levels of the structural genes of the pathway. This discrepancy could be explained by possible effects of *ANT1* on genes controlling the fate of anthocyanins, particularly those implicated in their glycosylation and transport (Mathews et al. 2003), and possibly others related to their degradation and sequestration.

We have previously shown that fruit peels of the AFT genotype and its descendants carrying the native *ANT1^{C}* allele are also typified by significantly higher levels of the flavonols quercetin and kaempferol than in control genotypes (Sapir et al. 2008). A similar effect was obtained in transgenic plants carrying the 35S*::ANT1^{C}* allele in the winter and spring. However, only in spring this increase was statistically significant. This difference, observed to a lesser extent in cotyledons, may represent seasonal fluctuations in metabolite flux through the flavonoid pathway

or its redirection. Indeed, flavonol synthesis is a side branch of anthocyanin biosynthesis. Therefore, a stronger flux towards anthocyanin biosynthesis due to over-expression of *ANT1* may lower flavonol levels resulting in stronger seasonal differences. This variation in flavonol accumulation may also imply that an additional transcription factor genetically linked to *ANT1*, such as *AN2* (Boches 2009) or a 3′ truncated *ANT1*-homolog which maps ~6 Kb upstream of *ANT1* on scaffold01162, may participate in the control of flavonol synthesis in the AFT genotype. This latter explanation is consistent with species variation noted in the control of the early and late phases of the flavonoid pathway (Schijlen et al. 2004). Therefore, ANT1 activity in fruit peels may be restricted to later phases of the flavonoid pathway and a different MYB may regulate its early phases. It is noteworthy that transcriptional analysis of the AFT genotype (Sapir et al. 2008) agrees with Borovsky et al. (2004) and shows that in this genotype the early *CHS1* and *CHS2* genes, as well as the late gene, *DFR*, are transcriptionally up-regulated in fruit peels. In fruit peels of the transgenic lines of this study, *CHS1* and *CHS2* were not transcriptionally up-regulated and only genes representing the late stages of the pathway were up-regulated in agreement with the analysis of the AFT genotype by Boches (2009). The differences between Sapir et al. (2008) and Boches (2009) can be attributed to the different non-isogenic control lines used in these two studies and thus reinforce the inconsistent effect of the gene controlling the AFT phenotype on the early genes of anthocyanin biosynthesis pathway in fruit peels. Alternatively, this may imply that in the AFT genotype different, genetically linked, MYB genes control the early and the late genes of the pathway.

A recombination distance of 0.3 cM was obtained between the native *ANT1* and *AN2* in our linkage analysis. A segregating population obtained from the recombinant genotype $ANT1^{C}/ANT1^{C}$ $AN2^{L}/AN2^{C}$, showed that *ANT1* alone can generate the characteristic phenotype of anthocyanin accumulation in AFT fruits, strongly implicating *ANT1* as the determinant of the AFT phenotype. Nevertheless, we cannot completely rule out that *AN2* has an effect on other components of the flavonoid biosynthesis pathway or that it may exert a non-additive effect on anthocyanin accumulation, similar to *ANT1*.

Most studies designed to genetically engineer *MYB* genes in tomato fruits resulted in increased flavonols levels, while anthocyanin pigmentation was unchanged or restricted to peel tissues (Goldsbrough et al. 1996; Mathews et al. 2003; Schijlen 2007; Luo et al. 2008; Adato et al. 2009). A recent study (Butelli et al. 2008), however, demonstrated a strong anthocyanin accumulation in fruit peel and flesh of Micro-Tom tomato plants following over-expression of two transcription factors from snapdragon, ROSEA1 and DELILA (MYB and bHLH, respectively). In our study, strong anthocyanin accumulation was obtained in fruit peels and flesh by over-expression of a single endogenous MYB. This research therefore augments the possibilities of enhancing flavonoid levels in tomato fruits.

Finally, Sapir et al. (2008) proposes that coding-sequence differences of $ANT1^{C}$ are causing anthocyanin accumulation in the AFT genotype in contrast to the dogma stating that regulatory genes controlling the flavonoid pathway exert their effect through transcription. Our results support this proposition and thus expand the potential of enhancing flavonoid levels through engineering coding-sequence polymorphisms in addition to the transcriptional alterations.

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References

Adato A, Mandel T, Mintz-Oron S, Venger I, Levy D, Yativ M, Domínguez E, Wang Z, De Vos RC, Jetter R, Schreiber L, Heredia A, Rogachev I, Aharoni A (2009) Fruit-surface flavonoid accumulation in tomato is controlled by a *SlMYB12*-regulated transcriptional network. PLoS Genet 5:e1000777

Ballester AR, Molthoff J, de Vos R, Hekkert BL, Orzaez D, Fernández-Moreno JP, Tripodi P, Grandillo S, Martin C, Heldens J, Ykema M, Granell A, Bovy A (2010) Biochemical and molecular analysis of pink tomatoes: deregulated expression of the gene encoding transcription factor SlMYB12 leads to pink tomato fruit color. Plant Physiol 152:71–84

Boches P (2009) Breeding tomato for increased fruit phenolics. Ph.D. thesis submitted to the senate of Oregon State University

Bohm BA (1998) Chemistry and biochemistry of organic natural products. In: *Introduction to Flavonoids*. Harwood Academic Publishers, Amsterdam

Borovsky Y, Oren-Shamir M, Ovadia R, De Jong W, Paran I (2004) The *A* locus that controls anthocyanin accumulation in pepper encodes a *MYB* transcription factor homologous to *Anthocyanin2* of *Petunia*. Theor Appl Genet 109:23–29

Bovy AG, Gomez-Roldan V, Hall RD (2010) Strategies to optimize the flavonoid content of tomato fruit. In: Santos-Buelga C, Escribano-Bailon MT, Lattanzio V (eds) Recent Advances in Polyphenols Research, Vol II. Wiley, Oxford, pp 138–162

Bovy A, de Vos R, Kemper M, Schijlen E, Almenar Pertejo M, Muir S, Collins G, Robinson S, Verhoeyen M, Hughes S, Santos-Buelga C, van Tunen A (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell 14:2509–2526

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat Biotechnol 26:1301–1308

Fulton TM, Chunwongse J, Tanksley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13:207–209

Giorgiev C (1972) Anthocyanin fruit tomato. Rep Tomato Genet Coop 22:10

Goldsbrough AP, Tong Y, Yoder JI (1996) *Lc* as a non-destructive visual reporter and transposition excision marker gene for tomato. Plant J 9:927–933

Harborne JB (1994) The flavonoids: advances in research since 1986. Chapman and Hall, London

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7:1071–1083

Jones CM, Mes P, Myers JR (2003) Characterization and inheritance of the *Anthocyanin fruit* (*Aft*) tomato. J Hered 94:449–456

Kramer CY (1956) Extension of multiple range tests to group means with unequal number of replications. Biometrics 12:309–310

Levin I (2009) Regulating phytonutrient levels in plants—toward modification of plant metabolism for human health. In: Kaufman PB, Kirakosyan A (eds) Recent Advances in Plant Biotechnology. Springer, Dordrecht, pp 289–330

Luo J, Butelli E, Hill L, Parr A, Niggeweg R, Bailey P, Weisshaar B, Martin C (2008) AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol. Plant J 56:316–326

Mahjoub A, Hernould M, Joubès J, Decendit A, Mars M, Barrieu F, Hamdi S, Delrot S (2009) Overexpression of a grapevine R2R3-MYB factor in tomato affects vegetative development, flower morphology and flavonoid and terpenoid metabolism. Plant Physiol Biochem 47:551–561

Markham KR, Ofman DJ (1993) Lisianthus flavonoid pigments and factors influencing their expression in flower colour. Phytochemistry 34:679–685

Mathews H, Clendennen SK, Caldwell CG, Liu XL, Connors K, Matheis N, Schuster DK, Menasco DJ, Wagoner W, Lightner J, Wagner DR (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703

McCormick S (1991) Transformation of tomato with *Agrobacterium tumefaciens*. In: Lindsey K (ed) Plant tissue culture manual: fundamentals and applications. Kluwer, Dordecht, pp 1–9

Mes PJ, Boches P, Myers JR (2008) Characterization of tomatoes expressing anthocyanin in the fruit. J Am Soc Hortic Sci 133:262–269

Rick CM, Cisneros P, Chetelat RT, DeVerna JW (1994) *Abg*—a gene on chromosome 10 for purple fruit derived from *S. lycopersicoides*. Rep Tomato Genet Coop 44:29–30

Sapir M, Oren-Shamir M, Ovadia R, Reuveni M, Evenor D, Tadmor Y, Nahon S, Shlomo H, Chen L, Meir A, Levin I (2008) Molecular aspects of *Anthocyanin fruit* tomato in relation to *high pigment-1*. J Hered 99:292–303

Schijlen EG, Ric de Vos CH, van Tunen AJ, Bovy AG (2004) Modification of flavonoid biosynthesis in crop plants. Phytochemistry 65:2631–2648

Schijlen E (2007) Genetic engineering of flavonoid biosynthesis in tomato. Ph.D. thesis submitted to the senate of wageningen UR, The Netherlands

van Tuinen A, de Vos CHR, Hall RD, Linus HW, van der Plas LHW, Bowler C, Bino RJ (2006) Use of metabolomics for identification of tomato genotypes with enhanced nutritional value derived from natural light-hypersensitive mutants. In: Jaiwal PK (ed) Plant genetic engineering, vol 7: metabolic engineering and molecular farming-1. Studium Press, LLC. Huston, pp 240–256

Winkel-Shirley B (2001) Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126:485–493

Zuluaga DL, Gonzali S, Loreti E, Pucciariello C, Degl'Innocenti E, Guidi L, Alpi A, Perata P (2008) *Arabidopsis thaliana MYB75/PAP1* transcription factor induces anthocyanin production in transgenic tomato plants. Funct Plant Biol 35:606–618