



Transcriptional analysis in high-anthocyanin tomatoes reveals synergistic effect of *Aft* and *atv* genes

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ABSTRACT

Anthocyanins are high value plant antioxidants, which are not present in the fruits of the cultivated tomato. However, both the dominant gene *Anthocyanin fruit* (*Aft*) and the recessive gene *atroviolacea* (*atv*), when introgressed into the domesticated tomato from two different wild *Solanum* species, stimulate a limited anthocyanin pigmentation. Surprisingly, the double mutant *Aft/Aft atv/atv* gives rise to intensely purple pigmented tomatoes.

A transcript profiling analysis was carried out using quantitative RT-PCR and GeneChip[®] Tomato Genome Arrays to identify differentially expressed genes when comparing Ailsa Craig, *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* fruits. Anthocyanin levels and the expression of the genes involved in anthocyanin production and compartmentalization were higher in the peel of *Aft/Aft atv/atv* fruits than in the individual parental lines. Moreover, a synergistic effect of the two alleles *Aft* and *atv* on the transcription of specific anthocyanin genes and the activation of the whole anthocyanin pathway was observed. Among the differentially expressed transcripts, genes involved in the phenylpropanoid pathway, biotic and abiotic stress responses, cell wall and hormone metabolism were over-represented in *Aft/Aft atv/atv* fruit peel. Transcriptomic analyses thus revealed that the activation of anthocyanin synthesis in the peel of tomato fruit was accompanied by a complex remodulation of gene expression.

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Introduction

Anthocyanins represent an important group of polyphenolic pigments deriving from the phenylpropanoid biochemical pathway. They belong to the class of flavonoids, of which they are the most conspicuous, owing to the wide range of chemical structures that derive from their synthesis (Holton and Cornish, 1995). In addition to providing attractive colors in flowers, fruits, seeds and leaves, anthocyanins have other important functions in plants. They can be synthesized in response to stressful events, such as high irradiance or low temperatures, against which they can protect the plant since they act both as a light-screen and as scavengers for radical species (Gould, 2004).

Due to their ubiquitous presence in plants, anthocyanins are important components in the human diet. Recent evidence suggests that anthocyanins and other flavonoids represent potent biomolecules with beneficial effects for human health. They act as

anticancer agents, cardioprotectants, and inhibitors of neurodegeneration, as a result of their antioxidant activity and their ability to induce protective enzymes (Levin et al., 2006).

Unfortunately, anthocyanins are not present in the edible products of some important crop plants, such as tomato, whose fruit is one of the most consumed vegetables worldwide. Cultivated tomatoes (*Solanum lycopersicum* L.) produce anthocyanins in vegetative tissues, but only small amounts of other flavonoids, such as naringenin chalcone and flavonols, can be found in the fruit (Muir et al., 2001; Torres et al., 2005; Bovy et al., 2007). As a consequence, tomato is considered an excellent candidate for an enhancement of the flavonoid and anthocyanin contents through transgenic approaches (Gonzali et al., 2009). Recently, Butelli et al. (2008) expressed, in tomato, *Delila* and *Rosea1*, two genes coding for transcription factors involved in anthocyanin production in snapdragon. The fruits of the engineered tomato plants displayed strong anthocyanin accumulation both in the peel and flesh, thus demonstrating that the anthocyanin biosynthetic pathway is fully present and functional in the fruit of this species if activated appropriately. However, consumers are often reluctant to accept genetically modified fruits and vegetables. As a consequence, there is ongoing interest in non-transgenic tomato lines producing anthocyanins in the fruit. Indeed, some wild species accumulate anthocyanins in the peel of the fruit, and this trait has been transferred into

Abbreviations: AC, Ailsa Craig; *Aft*, *Anthocyanin fruit*; *atv*, *atroviolacea*; DPA, days post anthesis; dS, deciSiemens; EBGs, early biosynthetic genes; G, mature green; LBGs, late biosynthetic genes; MJ, megajoule; R, red; T, turning red.

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the cultivated tomato by interspecific crosses (Gonzali et al., 2009).

The dominant gene *Aft* (*Anthocyanin fruit*) was introgressed into domesticated tomato plants by a cross with *S. chilense* (Jones et al., 2003). *Aft* triggers anthocyanin accumulation in immature green fruit upon stimulation by high light. Subsequently, pigments are produced continuously throughout development (Mes et al., 2008). The *Aft* gene identity has still to be revealed. Recent linkage analyses showed that the *Aft* locus co-segregates with two different MYB transcription factor genes located on chromosome 10, *SIAN2* (Mes et al., 2008; Boches et al., 2009) and *Anthocyanin 1* (*SIANT1*) (Sapir et al., 2008), both involved in anthocyanin synthesis in tomato (Mathews et al., 2003; Mes et al., 2008).

A recessive gene, *atv* (*atroviolacea*), derived from the interspecific cross with *S. cheesmaniae* (L. Riley) Fosberg, has been shown to influence anthocyanin pigmentation in the entire tomato plant, particularly in the vegetative tissues (Mes et al., 2008). The *Atv* gene has been located to chromosome 7 (Rick et al., 1968) and previous studies indicated that its mutation may affect phytochrome responses, since *atv* plants exhibit an exaggerated response to red light in terms of anthocyanin production (Kendrick et al., 1997).

Tomato plants with both *Aft* and *atv* alleles have been produced (Mes et al., 2008; Gonzali et al., 2009). They can be distinguished by the presence of intensely pigmented fruits. In these double mutants, hereafter called *Aft/Aft atv/atv*, anthocyanin production initiates in the skin of immature green fruit with continued accumulation until ripening, and is strongly stimulated by light (Mes et al., 2008) and low temperature (our unpublished data). An accurate metabolic characterization of *Aft*, *atv*, and *Aft/Aft atv/atv* fruits was performed by Mes et al. (2008), who analyzed the anthocyanidin profile and content of the various genetic combinations. In the fruits of the double mutant line, the primary anthocyanidin accumulated was petunidin, with malvidin and delphinidin present at lower levels (Mes et al., 2008). Different glycosyl and acyl moieties were identified. However, the anthocyanidin profile of *Aft/Aft atv/atv* fruits was consistent with results from previous analyses on high anthocyanin tomatoes (Jones et al., 2003; Mathews et al., 2003), indicating that *Aft* and *atv* alleles likely do not affect structural genes of the biosynthetic pathway. No influence of anthocyanin accumulation on carotenoid levels was detected (Mes et al., 2008).

Anthocyanin gene expression patterns for these single and double mutant lines are still incomplete, and possible transcriptomic changes associated with anthocyanin production are not known. In this study, a detailed transcript profiling analysis of the anthocyanin biosynthetic pathway was carried out in these tomato fruits, revealing sets of differentially regulated genes and synergistic effects of *Aft* and *atv*. Our results demonstrate that the regulation of transcripts involved in phenylpropanoid metabolism is tightly linked to the anthocyanic phenotypes of tomato fruits, and is also accompanied by changes in other structural and metabolic traits.

Materials and methods

Plant material and growth conditions

Ailsa Craig (AC) (accession number LA2838A), *Aft/Aft* (accession number LA1996) and *atv/atv* (accession number LA0797) seeds were provided by the Tomato Genetic Resource Center (TGRC, University of California, Davis). Seeds from the double mutant *Aft/Aft atv/atv* were obtained by G.P. Soressi (Department of Agrobiology and Agrochemistry, University of Tuscia, Viterbo, Italy) by crossing the single mutants *Aft* and *atv*. Since nearly isogenic lines for both *Aft* and *atv* mutations were not available among the *S. lycopersicum* varieties, we chose the cv. AC as a control tomato line for our analyses. This choice was made considering that, unlike the mutant lines

selected for this study, AC does not produce anthocyanins in the fruit skin. We also considered other parameters, such as the morphology of the plant and the fruit, the size of mature tomatoes, and their ripening time (time from anthesis to the different ripening stages). All of these characteristics are quite similar between AC and the mutant lines, creating conditions to perform valid comparisons.

Plants were grown during the winter in a controlled heated greenhouse with a density of 3 plants m⁻². Drip irrigation was conducted by using a nutrient solution with electrical conductivity of 3.5 dS m⁻¹ and pH 6.5. The composition of the nutrient solution was as follows (concentrations are expressed in mM): 12 N-NO₃⁻, 1.3 P-PO₄⁻, 8 K⁺, 4 Ca²⁺, 1.2 Mg²⁺, 9 Na⁺, 1.5 S-SO₄²⁻. Micronutrients were added at Hoagland's concentration (in μmol L⁻¹: 40 B, 40 Fe, 1 Cu, 5 Zn, 10 Mn). The minimum temperature and ventilation air temperature inside the glasshouse were 13 °C and 27 °C, respectively. The maximum photosynthetic photon flux density ranged from 500 to 700 μmol m⁻² s⁻¹; the mean value of daily global radiation was 5.1 MJ m⁻².

Ailsa Craig control plants and the different mutants were planted in a randomized block design (three blocks) and grown vertically with single stems (only five trusses were left). Fruits were collected at mature green [corresponding to 40 days post anthesis (DPA) for AC, 44 DPA for *Aft/Aft* and the double mutant *Aft/Aft atv/atv*, and 50 DPA for *atv/atv*], turning red (49 DPA for AC, 54 DPA for *Aft/Aft* and the double mutant, and 60 DPA for *atv/atv*), and red (60 DPA for AC, 64 DPA for *Aft/Aft* and the double mutant, and 70 DPA for *atv/atv*) stages of development and ripening. These stages correspond to mature green, breaker and red ripe, respectively, in the classification of Giovannoni (2004). At each of the three stages of development, a single fruit was harvested from a randomly chosen plant of each genotype within each block. Subsequently, equal peel samples were removed with a scalpel from each fruit. These samples were then pooled according to genotype prior anthocyanin and RNA extraction, allowing equal representation of each block in the final sample. A total of three harvests taken from each genotype were analyzed, with each harvest representing a biological replicate.

Anthocyanin quantification

Anthocyanin extraction from the skin of tomato fruits was performed as described by Solfanelli et al. (2006). The amount of anthocyanins was determined spectrophotometrically (*A*₅₃₅) and expressed as mg of petunidin-3-(*p*-coumaroyl rutinoside)-5-glucoside per g, based on an extinction coefficient of 17,000 and a molecular weight of 934 (Butelli et al., 2008). Mean values were obtained from three independent replicates.

Expression analysis by quantitative RT-PCR

Total RNA was extracted from fruit peel samples using a "Spectrum™ Plant Total RNA Kit" (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. The RNA was subjected to DNase treatment using a "TURBO DNA free Kit" (Ambion, Austin, TX, USA). One micrograms of each sample was reverse transcribed into cDNA with an "iScript cDNA Synthesis Kit" (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative RT-PCR amplification (qPCR) was carried out using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers used for the amplification of the regulatory and the structural genes analyzed, with relative GeneBank accession numbers, are listed in Table S1. *LeEF1A* (*Lycopersicon esculentum elongation factor 1-alpha*) was used as an endogenous control. qPCR reactions were carried out using a SYBR Green PCR Master Mix (Applied Biosystems), 10 ng of cDNA template, and gene-specific primers in a final reaction volume of 15 μL. The relative quantitation of each individual gene expression was performed using

the geometric averaging method (geNorm) (Vandesompele et al., 2002).

RNA isolation, cRNA synthesis, and hybridization to Affymetrix GeneChips

Total RNA was extracted from the peel of the mature green fruits using a “Spectrum™ Plant Total RNA Kit” (Sigma–Aldrich). RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on GeneChip® Tomato Genome Arrays (http://www.affymetrix.com/products_services/arrays/specific/tomato.affx). Hybridization, washing, staining, and scanning procedures were performed by Genopolis (University of Milano-Bicocca), as described in the Affymetrix technical manual. Microarray data analysis was performed using an R/Bioconductor (Gentleman et al., 2004). Expression measures were obtained using a GeneChip Robust Multi-Array (Wu and Irizarry, 2005), a multi-array analysis method estimating probe set signals, taking into account the physical affinities between probes and targets. Normalization was performed using a quantile method (Bolstad et al., 2003). To reduce the number of non-informative genes, we applied an interquartile filter (interquartile range, IQR=0.25). To identify a statistically reliable number of differentially expressed genes among the genotypes, a linear model was applied (Wettenhall and Smyth, 2004). To assess differential expression, an empirical Bayesian method (Smyth and Gordon, 2004) was used to moderate the SE of the estimated log-fold changes. To control *P* values in the context of multiple testing problems, a Benjamini-Hochberg correction of the false discovery rate (Reiner et al., 2003) was applied (adjusted *P*-value ≤ 0.001), leading to a list of differentially expressed probe sets. Microarray datasets were deposited in a public repository with open access (accession number GSE19792; <http://www.ncbi.nlm.nih.gov/projects/geo>).

Results and discussion

Aft/Aft atv/atv fruits show intense anthocyanin pigmentation

Tomato fruits were collected from AC plants and from plants carrying the *Aft* and *atv* genes and their stable combination *Aft/Aft atv/atv*. For phenotypic and molecular analyses, we selected three representative stages of fruit development and ripening, namely mature green (G), turning red (T) and red (R). Anthocyanins were not observed in AC or *atv/atv* fruits (Fig. 1A and B), whereas *Aft/Aft* fruits showed purple spots in their peel beginning at the G stage (Fig. 1A and B). As expected, very strong anthocyanin pigmentation was displayed by *Aft/Aft atv/atv* fruits (Fig. 1A and B). The presence of anthocyanins was evident starting at the G stage and was limited to the fruit peel (Fig. 1A and B), while anthocyanins were not produced in the flesh (Fig. S1). Anthocyanin quantification confirmed the constant accumulation of pigments during fruit ripening in *Aft/Aft* and, especially, in *Aft/Aft atv/atv* (Fig. 1C). Very small amounts of anthocyanins, detectable only by spectrophotometric analysis, were also measured in *atv/atv* fruits (Fig. 1C). On the other hand, no significant anthocyanin production was measured in AC tomatoes (Fig. 1C), or in the flesh of the fruit from any of the genotypes analyzed (data not shown).

The strong anthocyanin accumulation in *Aft/Aft atv/atv* fruits (Fig. 1A and B) was the result of a synergism between the *Aft* and *atv* genes. It seems that the co-presence of these two alleles strongly reduced the high light requirement for the production of anthocyanins. As a consequence, pigments were produced in *Aft/Aft atv/atv* fruit from the G stage onwards and accumulated quite homogeneously throughout the skin, conferring a purple color to the epidermis of the fruit (Fig. 1A and B).

SIANT1 and *SIAN2* are up-regulated in genotypes producing anthocyanins

In higher plants, the variety of anthocyanin pigmentation patterns is mainly based on differences in the regulatory mechanisms that control the expression of the structural genes of the pathway (Quattrocchio et al., 2006). In order to gain additional insight into the transcriptional regulation of anthocyanin synthesis in tomato, the mRNA levels of *SIANT1* and *SIAN2* were evaluated during fruit ripening. These two genes share high homology and encode two MYB transcription factors known to be involved in anthocyanin biosynthesis in tomato (Mathews et al., 2003; Boches et al., 2009). They are also considered as possible candidates for *Aft* mutation (Sapir et al., 2008; Boches et al., 2009).

A peak of expression for *SIANT1* and for *SIAN2* was observed in *Aft/Aft*, *atv/atv* and *Aft/Aft atv/atv* fruits at the G stage (Fig. 1D). On the other hand, *SIAN2* and *SIANT1* expression was negligible in the peel of AC (Fig. 1D) and in the flesh of all the genotypes (data not shown), where anthocyanins were not produced. The relative expression levels of *SIAN2* and *SIANT1* during the G stage were higher in *Aft/Aft* with respect to *atv/atv* and even *Aft/Aft atv/atv*. Taken together, these data support the hypothesis that *SIANT1* and/or *SIAN2* genes might be candidates for the *Aft* mutation (Sapir et al., 2008; Boches et al., 2009). Both *SIANT1* and *SIAN2* were expressed in all the genotypes that produce anthocyanins in the fruit skin. Their expression declined during the T and R phases (Fig. 1D). Anthocyanin content, instead, increased throughout ripening in *Aft/Aft atv/atv* fruits (Fig. 1C). As a whole, our data suggest that *SIANT1* and *SIAN2* might act as early triggers of anthocyanin production, particularly in green fruit.

Several genes of the flavonoid pathway are up-regulated in *atv/atv* and in *Aft/Aft atv/atv* during ripening

To better understand the molecular regulation of the anthocyanin pathway in tomato, the expression of the genes coding for enzymes in the biosynthetic pathway was studied. Anthocyanins are synthesized through a branch of the flavonoid biosynthetic pathway (Winkel-Shirley, 2001). Genes encoding enzymes of the pathway are classically divided into two groups: early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs), showing independent activation mechanisms in dicotyledonous species (Martin and Gerats, 1993; Quattrocchio et al., 2006). While EBGs are involved in the synthesis of precursors and final products of different classes of flavonoids, such as chalcones, dihydroflavonols and flavonols (Fig. 2A), LBGs are more specific to a restricted number of groups, including anthocyanins (Fig. 3A). The analysis of the biosynthetic pathway in tomato fruits was therefore subdivided into these two parts.

The analysis of the expression pattern of EBGs, leading to dihydroflavonols and flavonols, showed that this part of the pathway was active in all the genotypes analyzed (Fig. 2B). Previous analyses indicated that a major limitation in the flavonoid biosynthetic pathway in tomato fruit was the lack of expression of the *chalcone isomerase* (*CHI*) gene (Muir et al., 2001; Bovy et al., 2007). However, in this study, transcripts for *CHI* and *CHI*-like genes were found in the control line AC, at the G and T maturity stages, respectively (Fig. 2B). *Aft/Aft* fruits were not significantly different from AC, while *atv/atv* fruits showed a higher level of expression of some EBGs, particularly *phenylalanine ammonia-lyase 5* (*PAL5*), *chalcone synthase* (*CHS*), *CHI*-like and *flavanone-3-hydroxylase* (*F3H*) (Fig. 2B). It therefore appears that the mutation in the *Atv* gene might up-regulate some steps in the early phase of the flavonoid biosynthesis. In a previous study, the amount of flavonols measured in *atv* fruits turned out to be similar to the wild type tomatoes (Torres et al., 2005). Further analyses are necessary to verify if other flavonoids

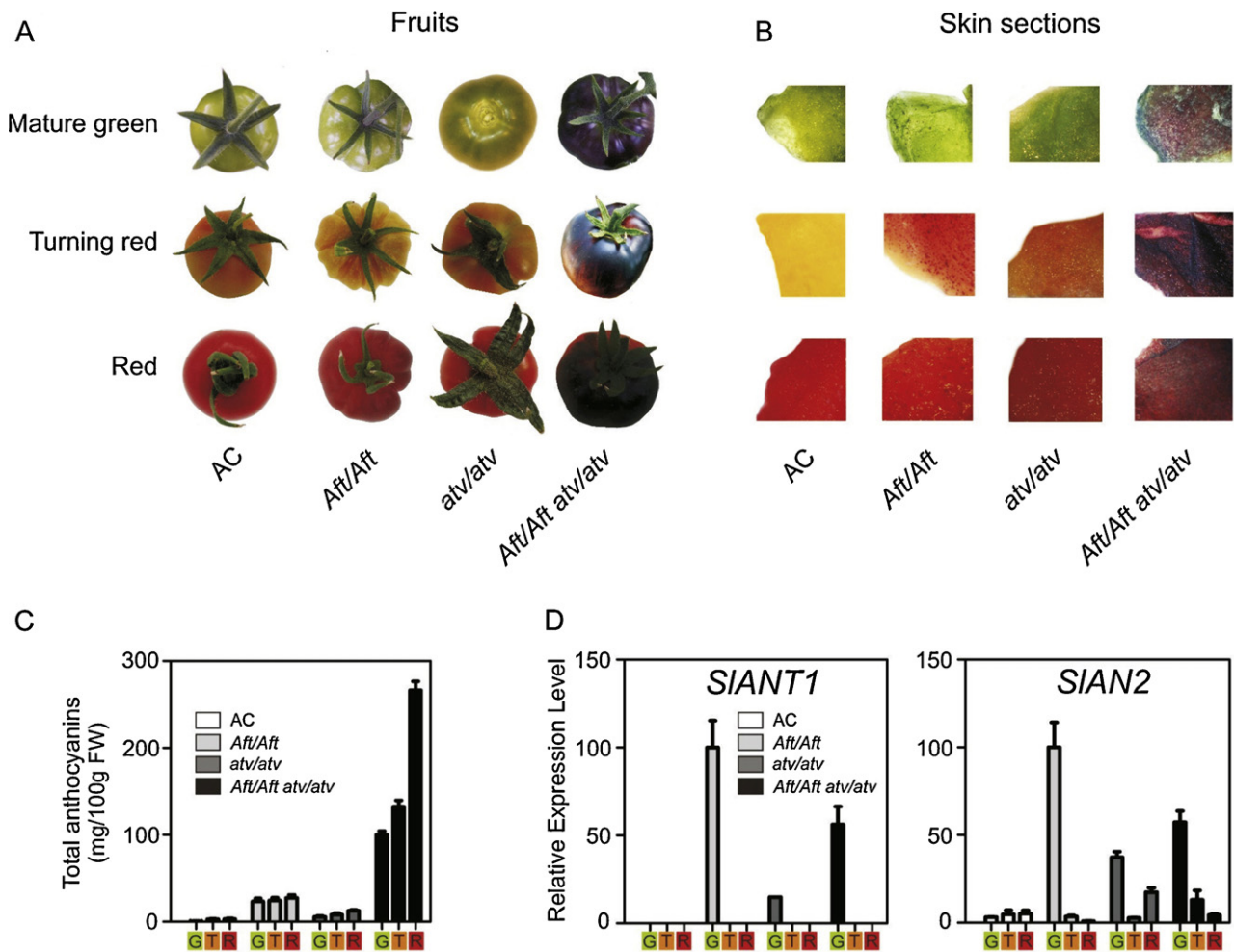


Fig. 1. Anthocyanin production in different tomato genotypes. Photographs of (A) mature green, turning red and red tomato fruits and (B) fruit skin sections taken from Ailsa Craig (AC), *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* plants. (C) Anthocyanin levels in tomato peel from AC, *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* fruits (data are means of three replicates \pm SD). For each genotype, anthocyanin levels measured in mature green (G, green box), turning red (T, orange box) and red (R, red box) stages of ripening are shown. (D) *SIAN2* and *SLANT1* mRNA levels in tomato peel from AC, *Aft/Aft*, *atv/atv*, and *Aft/Aft atv/atv* fruits. Ripening stages were as follows. G: mature green (green box); T: turning red (orange box); R: red (red box). Relative expression levels of *SIAN2* and *SLANT1* were measured by quantitative RT-PCR, assuming the highest level of expression as 100 in each dataset. Data are means of three replicates \pm SD.

are produced at higher rates in *atv*. Interestingly, a significant effect on the activation of the flavonoid pathway was observed after introgression of another recessive mutation affecting photomorphogenesis, the *high pigment* (*hp*) (van Tuinen et al., 2006). Moreover, it was demonstrated that several phenolic compounds are new or increased in fruits of a double mutant *Aft/Aft hp/hp*, as compared with fruits of single-mutant parents (van Tuinen et al., 2006). In the double mutant *Aft/Aft atv/atv* fruits, many of the flavonoid genes exhibited a higher or a more prolonged activation, as in the case of *PAL*, *PAL5*, *4-coumarate-CoA ligase* (*4CL*), *CHI*, *CHI-like* and *F3H* (Fig. 2B). *PAL* and *PAL5* were strongly expressed, especially at the T and R maturity stages (Fig. 2B). *PAL* induction is necessary to ensure the flux through the general phenylpropanoid metabolism in order to feed flavonoid biosynthesis (Bate et al., 1994). This seems to be particularly important to obtain very high levels of anthocyanins, as previously observed in *Delila-Rosea1* transgenic purple tomatoes (Butelli et al., 2008).

mRNA levels of specific anthocyanin biosynthetic genes are positively affected in Aft/Aft and Aft/Aft atv/atv

Delphinidin-types represent the major class of anthocyanins observed in tomato (Bovy et al., 2007). They are formed by

the concerted action of flavonoid 3'-hydroxylase (F3'5'H), dihydroflavonol reductase (DFR), and leucoanthocyanidin dioxygenase (ANS) (Fig. 3A). Modification with hydroxyl, methyl, glycosyl and acyl groups by the action of specific enzymes results in the final different anthocyanin structures (Fig. 3A). When synthesized, anthocyanins are compartmentalized into the vacuole. Different and not completely characterized mechanisms of anthocyanin transport into the vacuole take place in plants (Zhao and Dixon, 2010). However, the action of different enzymes, such as a glutathione S-transferase (GST), and an anthocyanin permease (PAT) has been shown to be associated with the anthocyanin accumulation in tomato fruits (Mathews et al., 2003; Butelli et al., 2008) (Fig. 3A). The genes encoding for these proteins were therefore analyzed in this study.

The analysis of the expression pattern of most of the genes that act in this second part of the biosynthetic pathway highlights that they were all strongly up-regulated in the *Aft/Aft atv/atv* fruits when compared to the other genotypes (Fig. 3B). The genes *F3'5'H*, *DFR*, *ANS*, together with those encoding an acyltransferase (*AAC*), a rhamnosyltransferase (*RT*), a glucosyltransferase (*3GT*), putatively involved in anthocyanin metabolism, *PAT* and *GST*, were all strongly up-regulated in double mutant fruits (Fig. 3B). A very slight induction of some of these genes was also observed in *Aft/Aft* green

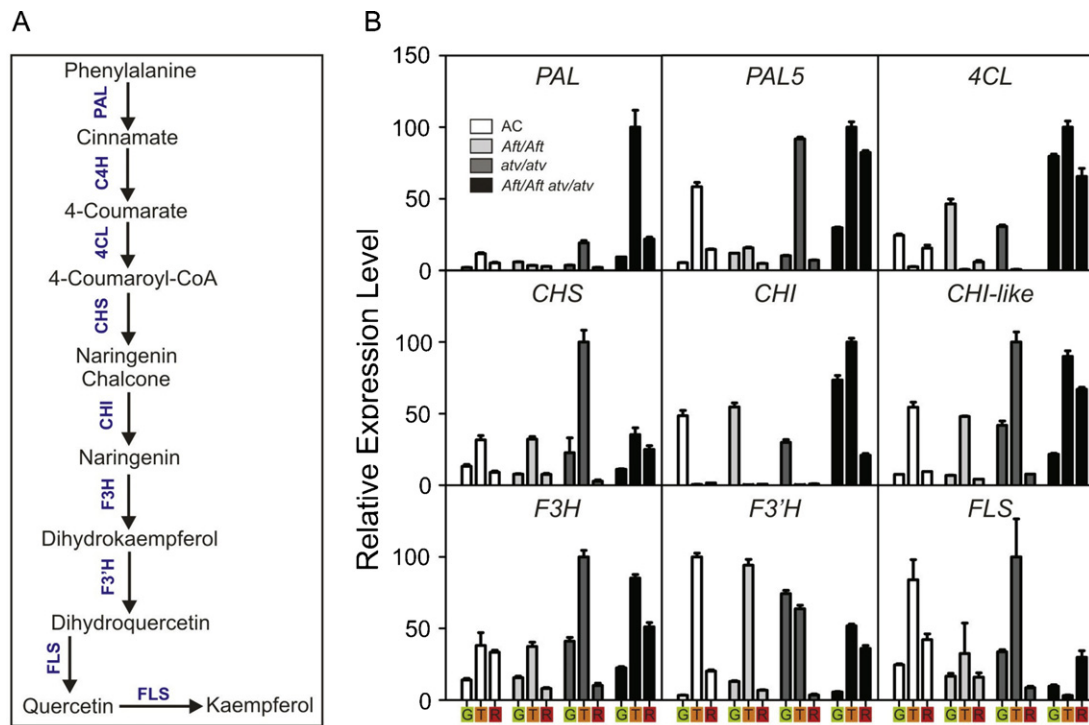


Fig. 2. Analysis of gene expression in the early steps of the anthocyanin biosynthetic pathway in the peel of tomato fruits. (A) Early steps in the anthocyanin biosynthetic pathway. Enzyme names were abbreviated as follows: phenyl alanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonols synthase (FLS). (B) Analysis of the expression pattern of early biosynthetic genes leading to dihydroflavonols and flavonols in the various tomato genotypes. Relative expression levels are shown, as measured by quantitative RT-PCR in mature green (G, green box), turning red (T, orange box) and red (R, red box) stages of ripening, assuming the highest level of expression as 100 in each dataset. Data are means of three replicates \pm SD.

fruits, whereas *atv/atv* expression patterns were not different from those observed in AC, where a negligible expression of these genes was found (Fig. 3B). Another gene encoding a glucosyltransferase (5GT) was highly expressed in the *Aft/Aft atv/atv* tomato in the T and R stages. However, this gene was also expressed in the other genotypes (Fig. 3B). Therefore, it could be involved also in the glycosylation of other classes of flavonoids. *F3'5'H* and *DFR* were strongly expressed in *Aft/Aft atv/atv*. These genes are both necessary to activate delphinidin-type anthocyanin production (Fig. 3A) and are considered as catalyzing limiting steps in the tomato anthocyanin biosynthetic pathway (Bovy et al., 2007). These data (Fig. 3B) revealed a set of genes that are likely to be responsible for the unusual accumulation of anthocyanin in the *Aft/Aft atv/atv* genotype.

With respect to the biosynthetic pathway as a whole, *atv* seems to affect mostly the expression of EBGs, while LBG transcription is in some way influenced by *Aft*. However, when the two mutations are combined together, a synergistic effect takes place, leading to the final strong production of anthocyanins in *Aft/Aft atv/atv* fruits. Many of the genes acting in the two parts of the anthocyanin pathway, particularly LBGs, indeed showed an up-regulation in the double mutant that was much higher than in the corresponding single mutant parental line (Figs. 2B and 3B).

Genes belonging to different families are differentially regulated in *Aft/Aft atv/atv*

Anthocyanin metabolism may be tightly interconnected with other important physiological processes. Accordingly, the *Aft/Aft atv/atv* genotype may directly or indirectly result in a more general transcriptional reprogramming of genes involved in different metabolic pathways. The transcriptional profile was thus analyzed in single and double mutant fruits compared to AC using the

GeneChip[®] Tomato Genome Array. Different aspects were considered before carrying out the transcriptome analysis. First, the two candidates for *Aft* mutation, *SIAN2* and *SIANT1*, were predominantly expressed in the peel of green fruits (Fig. 1D), suggesting an early effect of these genes on the activation of the anthocyanin pathway. For the *atv* mutation, there are not gene candidates at present. However, since qPCR analyses showed a more pronounced effect of this mutation on the early phase of flavonoid biosynthesis (Fig. 2B), it is likely that the *Atv* gene is also expressed early. Consequently, it is plausible that direct or indirect effects of both *Aft* and *atv* mutations on fruit peel transcriptome should be observed in early phases of anthocyanin production. Our previous analyses showed that anthocyanin pigments were already present in the fruit peel at the G stage, both in *Aft/Aft* and *Aft/Aft atv/atv* genotypes (Fig. 1C). For all these reasons, RNA from the green fruit peel was used for the transcriptome analysis.

We identified 214 differentially expressed genes (DEGs) (P -value ≤ 0.001 ; see Table S2) in *Aft/Aft atv/atv* fruit peel compared to AC (131 up-regulated and 83 down-regulated). We cannot exclude the possibility that some alterations in the expression pattern might be due to the lack of isogenicity between AC and the double mutant line (see "Materials and methods"). In addition, the genetic differences among the tomato lines made the choice of the stage of ripening more difficult. For this reason, we carefully collected samples considering specific criteria (see "Materials and methods"). However, the identical growth conditions and the high similarity in fruit size and maturation time between the two genotypes allowed us to consider the presence/absence of anthocyanins in the peel as a major trigger in differential genetic expression.

As expected, a substantial portion (6%) of DEGs was represented by transcripts involved in the flavonoid and anthocyanin metabolism, which were all up-regulated in the double mutant (Fig. 4). All the transcripts of

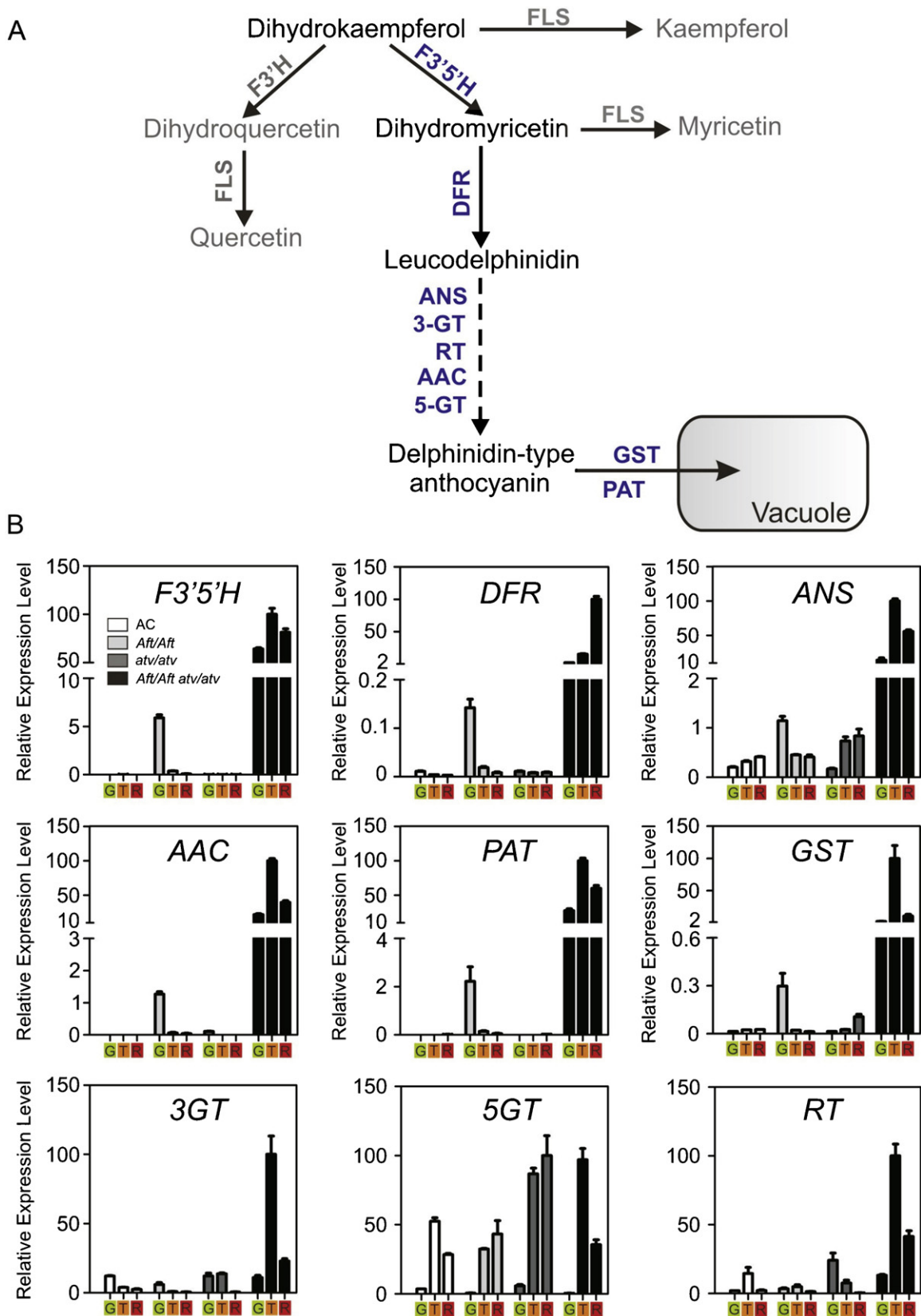


Fig. 3. Analysis of gene expression in the late steps of the anthocyanin biosynthetic pathway in the peel of tomato fruits. (A) Late steps in the anthocyanin biosynthetic pathway. Enzyme names were abbreviated as follows: flavonoid 3'-hydroxylase (F3'H), flavonols synthase (FLS), flavonoid 3'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (ANS), 3-O-glucosyltransferase (3-GT), rhamnosyl transferase (RT), anthocyanin acyltransferase (AAC), 5-O-glucosyltransferase (5-GT), glutathione S-transferase (GST), putative anthocyanin transporter (PAT). (B) Analysis of the expression pattern of late biosynthetic genes for anthocyanin production and accumulation. Relative expression levels, measured by quantitative RT-PCR in mature green (G, green box), turning red (T, orange box) and red (R, red box) stages of ripening, are shown, assuming the highest level of expression as 100 for each dataset. Data are means of three replicates \pm SD.

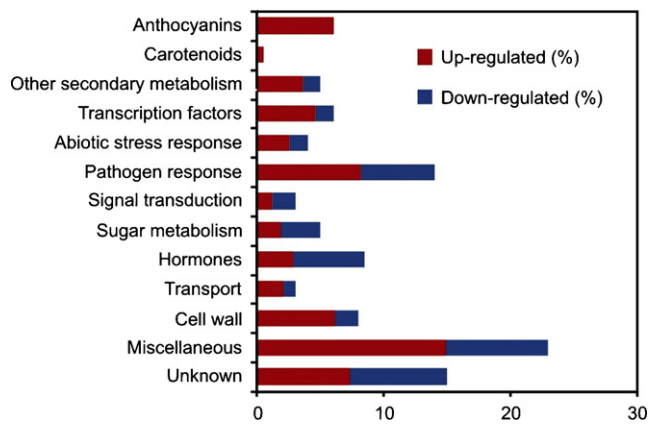


Fig. 4. Functional categories of the peel-associated transcripts displaying differential expressions when comparing AC with *Aft/Aft atv/atv* fruits. The distribution of categories is given as a percentage of the total 214 differentially expressed transcripts ($P \leq 0.001$).

the anthocyanin biosynthetic pathway that are included in the GeneChip, such as *PAL* (Les.4271.2.S1_at), *PAL5* (Les.4271.1.S1_at), *4CL* (Les.1097.1.A1_at), *CHS* (Les.3650.1.S1_at), *CHI-like* (LesAffx.68320.1.S1_at), *DFR* (Les.3659.1.S1_at), *ANS* (LesAffx.17064.1.A1_at), *RT* (Les.5842.1.S1_at), *PAT* (Les.4452.1.S1_at), and *GST* (LesAffx.57342.1.S1_at) (Table S2) were up-regulated. Additionally, several genes involved in biotic (14%) and abiotic stress (4%) responses displayed an altered mRNA level in *Aft/Aft atv/atv*. For example, different genes encoding putative peroxidases (LesAffx.70492.1.S1_at, LesAffx.32359.1.S1_at, LesAffx.71388.1.S1_at), arginases (LesAffx.1.1.S1_at, Les.3299.2.A1_s.at), and proteinase-inhibitors (Les.1675.1.S1_at, Les.3940.2.A1_at, Les.3621.1.S1_at, Les.2971.2.A1_at, Les.4022.1.S1_at, Les.3034.1.S1.at), among others, were up-regulated in the double mutant (Table S2). This may be a consequence of the accumulation of anthocyanins,

since these compounds represent signaling molecules for the activation of defense processes (Gould, 2004) or for the scavenging of stress-related free radical species (Rice-Evans et al., 1997; Wang et al., 1997). A “high anthocyanin level” may thus signal a status of general stress in the plant, which leads to the activation of both biotic and abiotic defense programs. Alternatively, it is possible that stress-related genes can be regulated by the same transcription factors which trigger anthocyanin biosynthesis, as part of the same general defense program.

Nine percent of the DEGs in *Aft/Aft atv/atv* compared to AC were related to hormone metabolism. Remarkably, five out of the six genes involved in ethylene biosynthesis (Les.2560.1.S1_at, Les.132.1.S1_at, Les.3225.1.A1_at, Les.3225.2.S1_at, Les.3225.3.S1_at) showed a clear down-regulation in *Aft/Aft atv/atv* (Table S2). This suggests a possible inhibition, or at least a delay, in ethylene production associated with the strong accumulation of anthocyanins. The expression of some genes encoding ripening-regulated cell wall proteins is modulated by ethylene (Bennett and Labavitch, 2008). Interestingly, several transcripts involved in cell wall metabolism and composition were strongly up-regulated in *Aft/Aft atv/atv* compared to AC (Fig. 4). This may suggest that anthocyanin accumulation could induce some effects on the composition or the structure of the cell wall in tomato fruit peel. Further analyses, also carried out in successive stages of ripening, could contribute to elucidate this important aspect.

About 6% of DEGs in *Aft/Aft atv/atv* were represented by mRNAs encoding for transcription factors. Transcriptional regulators of anthocyanin biosynthesis are well known in many plant species (Quattrocchio et al., 2006), and only some of them have been identified in tomato to date (Mathews et al., 2003; Boches et al., 2009). Therefore, the identification of transcription factors differentially expressed in AC and high anthocyanin tomatoes in early phases of pigment accumulation is of great interest. Indeed, MYB (Les.5091.1.S1_at) and bHLH (LesAffx.17051.1.S1_at) transcription factors, which are similar to *Petunia hybrida* PH2 (PhPH2) (Avila

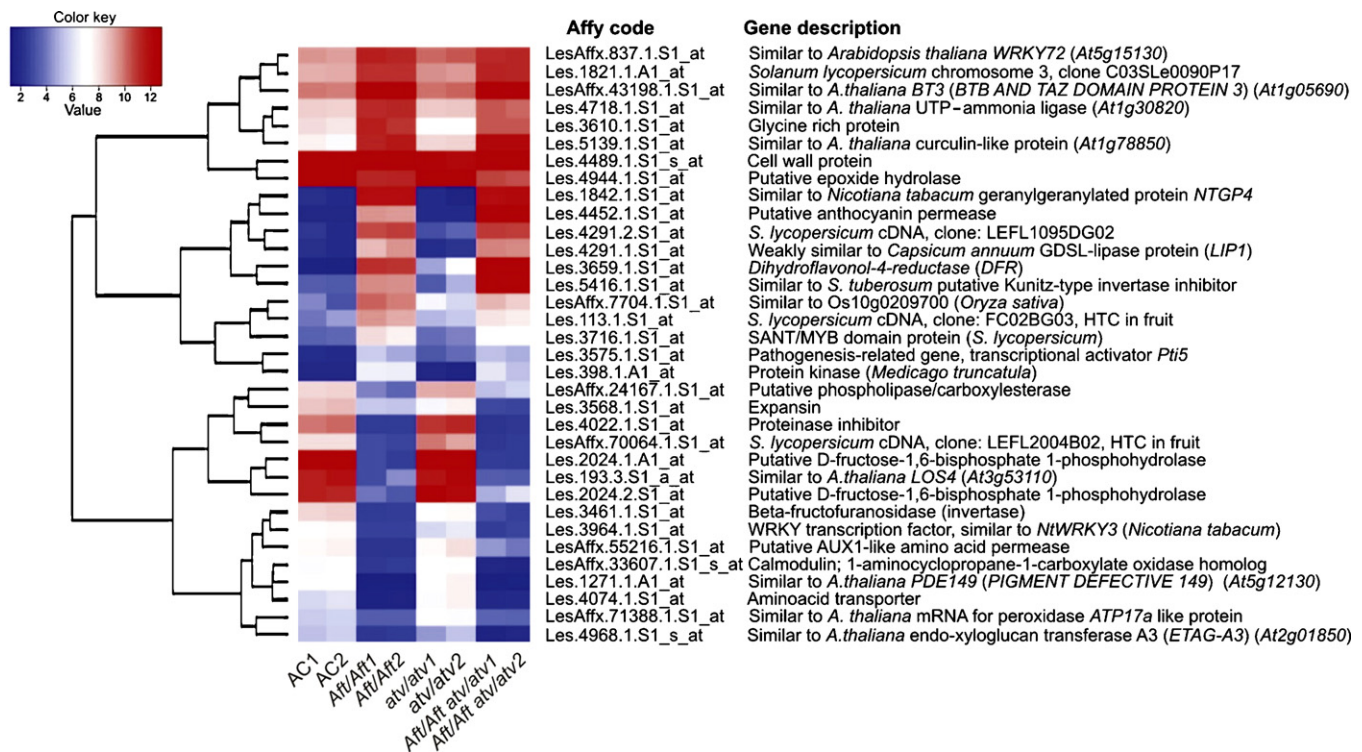


Fig. 5. Heatmap of differentially expressed genes showing a common expression pattern in *Aft/Aft atv/atv* and *Aft/Aft* fruit peel. Two different biological replicates for each genotype (indicated as 1 and 2) were used for this experiment.

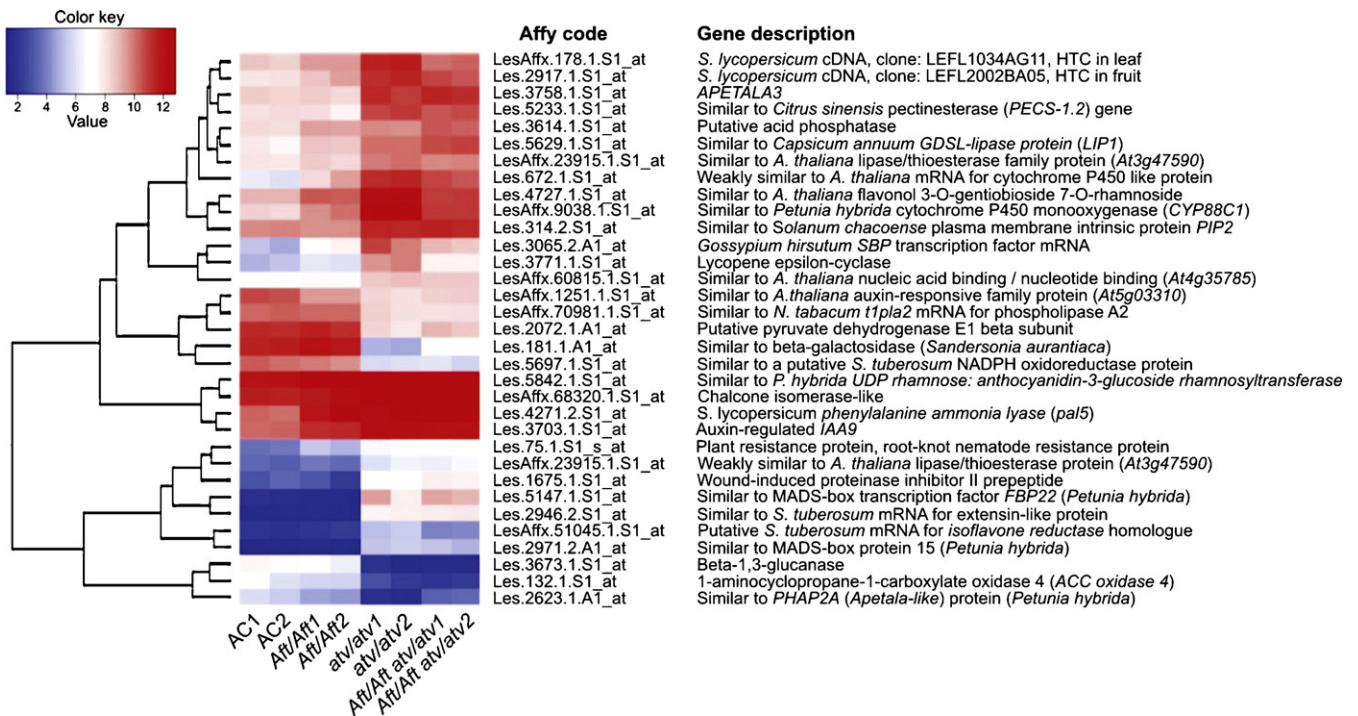


Fig. 6. Heatmap of differentially expressed genes showing a common expression pattern in *Aft/Aft atv/atv* and *atv/atv* fruit peel. Two different biological replicates for each genotype (indicated as 1 and 2) were used for this experiment.

et al., 1993) and a *Pisum sativum* bHLH (*PsGBF*) (Qian et al., 2007), respectively, involved in anthocyanin biosynthesis and *CHS* promoter activation, were up-regulated in the double mutant fruit and could be related to its high anthocyanin phenotype (Table S2).

Finally, several genes involved in sugar (5%) and secondary metabolisms (5%) were also differentially modulated in the double *Aft/Aft atv/atv* mutant when compared to AC (Fig. 4; Table S2).

Common gene expression patterns in *Aft/Aft atv/atv* and *Aft/Aft* fruit peel

Several DEGs displayed a shared expression pattern in the double mutant and one of the two parental mutant lines, different from both AC and the other mutant genotype. This suggests that their differential expression in *Aft/Aft atv/atv* fruit peel could be a consequence of the inheritance of either *atv* or *Aft*. Indeed, potential transcriptomic differences due to the genotype, to the effects of the environment or to the ripening stage should be abolished or strongly reduced in this kind of comparison.

As expected from qPCR analyses (Fig. 3B), *Aft*-associated DEGs (Fig. 5; Tables S3 and S4) include transcripts involved in anthocyanin biosynthesis, such as *PAT* and *DFR*. Both of these genes were expressed in *Aft/Aft* and *Aft/Aft atv/atv* at a much higher level than in AC and *atv/atv* (Fig. 5). However, their expression in the double mutant was considerably higher than in *Aft/Aft* (Fig. 3B). This is in line with the scarce presence of anthocyanins in *Aft/Aft* fruits. These results confirm the previous ones obtained by qPCR, suggesting that *Aft* might affect the expression of key LBGs, such as *DFR*, thus influencing the downstream steps of the anthocyanin pathway. This supports the hypothesis that *SIAN2* or *SIANT1* may be candidates for *Aft* mutation, since these two transcription factors, as well as their orthologs in other plant species, modulate the expression of *DFR* and other downstream structural genes involved in the anthocyanin biosynthesis (Quattrocchio et al., 1999; Mathews et al., 2003; Teng et al., 2005; Boches et al., 2009).

Other transcripts co-regulated in *Aft/Aft* and *Aft/Aft atv/atv* include genes coding for cell wall proteins, such as a glycine

rich protein (Les.3610.1.S1.at), a lysine and tyrosine enriched cell wall protein (Les.4489.1.S1_s.at), hypothesized as having a specialized structural function in tomato (Domingo et al., 1994), and an expansin (Les.3568.1.S1.at) (Fig. 5; Table S3, S4). Furthermore, a considerable number of genes putatively involved in defense pathways and in sugar metabolism show a similar pattern of expression in both *Aft/Aft* and *Aft/Aft atv/atv*. A Kunitz-type protease/invertase inhibitor precursor (Les.5416.1.S1.at) was up-regulated in both of these genotypes (Fig. 5; Table S3). Besides their metabolic role, plant protease or invertase inhibitors have been found to be involved in plant defense against insects and pathogens (Glaczinski et al., 2002). Invertases may also play a role in responses to wounds and pathogen attack (Tymowska-Lalanne and Kreis, 1998), and the inhibition of fungal invertases can restrict pathogen growth, therefore increasing plant resistance (Heisteruber et al., 1994). A gene (LesAffx.837.1.S1.at) similar to *Arabidopsis thaliana* *WRKY22*, involved in plant defense and elicitation by chitin and pathogens (Libault et al., 2007), was also commonly up-regulated in *Aft/Aft* and *Aft/Aft atv/atv* (Fig. 5; Table S3). A GDSL lipase transcript (Les.4291.1.S1.at) encoding a protein similar to *Capsicum annuum* *GLIP1*, which modulates disease susceptibility and abiotic stress tolerance (Hong et al., 2008) and the pathogenesis-related transcriptional activator *Pti5* (Les.3575.1.S1.at) (Zhou et al., 1997) were also up-regulated in *Aft/Aft* and *Aft/Aft atv/atv* (Fig. 5; Table S3).

Overall, these results suggest that *Aft* mutation mostly affects the gene expression involved in late steps of anthocyanin biosynthesis, in cell wall structure, and in plant defense.

Common gene expression patterns in *Aft/Aft atv/atv* and *atv/atv* fruit peel

Genes sharing the expression pattern in *Aft/Aft atv/atv* and *atv/atv* when both genotypes were compared to AC and *Aft/Aft* (Fig. 6; Tables S5 and S6) were identified. The transcripts for *PAL5*, *CHS* and *CHI*-like, encoding enzymes involved in the early steps of the flavonoid pathway, were strongly accumulated in both *atv/atv* and *Aft/Aft atv/atv* (Fig. 6; Table S5), in agreement with the

qPCR data (Fig. 2B). Genes involved in the flavonol metabolism and in other branches of the flavonoid pathway were also commonly up-regulated in *atv/atv* and *Aft/Aft atv/atv*. They include Les.4727.1.S1.at (similar to an *A. thaliana* locus affecting the accumulation of flavonol 3-O-gentiobioside 7-O-rhamnoside), and a gene (LesAffx.51045.1.S1.at) similar to a *S. tuberosum* isoflavone reductase homologue (Fig. 6, Table S5). Moreover, one of the commonly up-regulated genes in *atv* and the double mutant was a putative UDP rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase (Les.5842.1.S1.at), possibly involved in anthocyanin glycosylation (Fig. 6; Table S5). These data mirror the qPCR results, showing that *RT* mRNA was high in *atv/atv* and, to a lesser extent, in *Aft/Aft atv/atv* during the G phase (Fig. 3B). However, since this gene was also expressed in AC tomatoes in the T stage (Fig. 3B), it is possible that it can be related to flavonol synthesis as well. In *P. hybrida*, *PhRT* expression acting in the final part of the anthocyanin pathway generally coincides with pigment accumulation in the flower, and follows a similar induction pattern to *PhPAL*, *PhCHS*, *PhCHI* and *PhDFR* genes in terms of sugar and high-light activation (Brugliera et al., 1994). The higher activation of *RT* in *Aft/Aft atv/atv* fruits, possibly inherited from *atv* (Figs. 3B and 6; Table S5), might significantly contribute to the final glycosylation pattern of the anthocyanins produced.

Several other genes that were involved in metabolic pathways not directly related to flavonoid metabolism resulted co-expressed in *atv/atv* and *Aft/Aft atv/atv*. Genes coding for a cytochrome P450 monooxygenase (LesAffx.9038.1.S1.at), similar to *P. hybrida* *CYP88C1*, and a cytochrome P450-like protein (Les.672.1.S1.at), possibly involved in the biosynthetic pathway of other secondary metabolites, were co-modulated (Fig. 6; Table S5). Genes coding for a wound-induced proteinase inhibitor (Les.1675.1.S1.at) and a putative plant resistance protein (Les.75.1.S1.s.at), both involved in plant defense, and genes similar to pectinesterases (Les.5233.1.S1.at) and extensin-like proteins (Les.2946.2.S1.at), both associated with cell wall metabolism, were all up-regulated in both *atv/atv* and *Aft/Aft atv/atv* (Fig. 6; Table S5). For the initiation of environmental stress responses, and for protecting against UV and pathogens, the activation of *PAL* and *PAL5* genes, common to the two genotypes (Fig. 2B), could be also crucial (Lee et al., 1992).

In summary, genes involved in important steps of flavonoid biosynthesis, cell wall metabolism and defense pathways, different from the ones found in the previous paragraph, were commonly regulated in *atv/atv* and *Aft/Aft atv/atv* fruits.

Conclusions

Transcriptome analysis showed that anthocyanin production in *Aft/Aft atv/atv* tomatoes coincides with the strong activation of specific genes involved in this pathway. In *Aft* and *atv* fruits a similar process could not be observed; we observed instead only a partial activation of some steps of the pathway. Our data indicate that *Aft* mainly affects the expression of genes directly involved in anthocyanin production, such as *DFR* and other LBGs. This supports the hypothesis that *Aft* may represent an allele of *SIAN2* or *SIANT1*, which encode MYB transcription factors highly expressed in anthocyanin-accumulating fruits (Fig. 1D). In other plant species, such as *Arabidopsis* or petunia, MYB transcription factors specifically affect the expression of LBGs of the anthocyanin pathway (Borevitz et al., 2000; Quattrocchio et al., 2006). Similarly, the high expression of *SIAN2* and/or *SIANT1* in *Aft* green fruits could be responsible for the activation of the late steps of the biosynthetic pathway (Figs. 1D and 3B), leading to the final limited anthocyanin production (Fig. 1A–C). On the other hand, qPCR and microarray data indicate that the *atv* mutation might influence the expression of some of the EBGs involved in the biosynthesis of flavonoid pre-

cursors in the fruit (Figs. 2B and 6). This would be in accordance with the hypothesis of a role of this gene in the photomorphogenesis and in the phytochrome signaling pathway, as suggested in earlier studies (Kendrick et al., 1997). Indeed, other photomorphogenic mutants show a sustained activation of flavonoid production, such as *hp* mutants (Torres et al., 2005; van Tuinen et al., 2006). However, when *atv* is introduced in an *Aft* background, not only some important EBGs, such as *PAL*, *PAL5*, *4CL*, *CHI* and *F3H* were up-regulated (Fig. 2B), but also, and even more strikingly, most of the LBGs (Fig. 3B). It is therefore likely that *atv* gene synergistically acts with *Aft* on LBG transcription. For example, a combined effect of transcriptional activation, sustained by the high expression of *SIAN2* and/or *SIANT1*, and transcriptional de-repression, exerted by the mutation in the gene *Atv*, could take place. Negative regulators of anthocyanin biosynthesis, belonging to the R3-MYB class, have been recently identified in *A. thaliana* (*AtMYBL2*) (Dubos et al., 2008; Matsui et al., 2008) and in petunia (*PhMYBX*) (Quattrocchio et al., 2006). *Atv* could carry out a similar function in tomato.

Transcriptome analysis also revealed that, in *Aft/Aft atv/atv* fruit peel, not only anthocyanin production but also other important metabolic processes are considerably altered. We cannot exclude the possibility that some differentially expressed transcripts in the double mutant can be due either to the lack of isogenicity of the selected lines, or to small differences in the stage of ripening among them. However, it is likely that many of these transcripts were inherited from one of the two parental mutant lines, being coherently present in both genotypes and absent in both AC and the other mutant (Figs. 5 and 6). In particular, some of the genes involved in the cell wall metabolism and in stress responses were up-regulated. These alterations could be due to the specific effects of either *Aft* or *atv* mutations (Figs. 5 and 6). They could be also the consequence of changes in anthocyanin metabolism, since this is tightly interconnected with other important physiological processes, particularly with the pathway of biotic/abiotic stress responses. Research is ongoing to verify whether gene expression changes in *Aft/Aft atv/atv* can determine the acquisition of physiological and nutritional properties. This could be very important for tomato production and marketing, for example in terms of resistance to biotic and abiotic stresses and antioxidant content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.07.022.

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